Metabolic and developmental consequences of continuous *vs.* refreshed culture of bovine embryos in BBH7 media.

Jennifer Rose Wills

B.Sc. (Hons), University of Newcastle upon Tyne

Thesis submitted for the Degree of Master of Medical Science

December 2013

School of Clinical Science

D Floor, East Block

Queen's Medical Centre

Nottingham, NG7 2UH

Declaration

I hereby declare that this thesis has been composed by myself and has not been submitted for any other degree, in Nottingham or elsewhere. The work presented herein is my own, and all work of other authors is duly acknowledged. I also acknowledge the assistance given to me during the design and execution of the experiments contained in this thesis and during its preparation.

Jennifer Rose Wills

Abstract

In vitro production of bovine embryos is an effective tool for quickly and efficiently improving herd genetics. Previously, detrimental effects of continuous culture (to day 7) on embryonic development have been reported due to a toxic build-up of ammonia. BBH7 is a serum-free *in vitro* culture media that has been specifically developed to improve the yield and cryo-tolerance of bovine embryos. Currently, bovine embryos are cultured continuously in BBH7 and it is the aim of this thesis to determine whether this culture system is detrimental to embryo development.

Oocytes were aspirated from follicles (4-9 mm) of abattoir-derived ovaries, matured overnight and fertilised with frozen-thawed semen. Putative zygotes were randomly assigned to two treatments; 1) continuous culture in BBH7 for 144 h, and 2) refreshed media at 48 h intervals over 144 h. Spent media was collected and snap-frozen (-196 °C) at each 48 h interval and final 144 h time points. Cleaved of inseminated (48 h post-fertilisation), blastocysts of cleaved (by 144 h), and embryo grade (IETS) were assessed. Furthermore, ammonia concentrations were determined in spent media by an enzymatic ultraviolet method, and sex ratio was determined by polymerase chain reaction.

Zygote and blastocyst yields did not differ between treatments. There was also no effect of treatment on final stage of embryonic development. Ammonia concentrations 1) increased significantly over time in basal media (P=0.014); 2) increased significantly from 0 h (P=0.005) after each 48 h interval, but not between each interval; and 3) were significantly greater in the 'no change' group following 144 h (P=0.048) than in the 'change' group. Furthermore, sex ratio did not differ between treatments.

Although there is reported evidence of the detrimental effects of ammonia accumulation on preimplantation embryo development during continuous culture, these data suggest there is currently no benefit in refreshing BBH7 at 48 h intervals. Equally, there was no effect of refreshing BBH7 on sex ratio skew. However, the spontaneous breakdown of amino acids in serum-free media may compromise embryo development by increasing circumjacent ammonia concentrations. Finally, effects on amino acid metabolism await analysis, and post-transfer pregnancy rates await experimentation.

Acknowledgements

Firstly, I would like to thank Prof Kevin Sinclair for his support and guidance throughout this project. Also, I would like to acknowledge and thank Mrs Pat Fisher for both her technical assistance and friendship over the last three months. It has been a great pleasure to work with you both.

To Annie, you have been a great lab partner and I have enjoyed the time we spent together out at Sutton Bonington, despite everything seemingly conspiring against us at times! You are an inspirational young woman and I wish you every success in the future, I hope we stay in touch.

Huge thanks go to my family for your love and support as I complete this degree. To Hamish, thank you (again) for supporting me emotionally and financially this year. It's been a difficult year for us all, so thank you all for everything.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AA	Amino acid
AAP	Amino acid profiling
AAT	Amino acid turnover
ADP	Adenosine diphosphate
AI	Artificial insemination
AMH	Anti-Müllerian hormone
ANOVA	Analysis of variance
ART	Assisted Reproduction Technology
ATP	Adenosine triphosphate
B	Blastocyst
BBH	Block Bonila Hansen
BCB	Brilliant cresyl blue
BHB	Beta-hydroxybutyrate
bp	Base pairs
BSE	Bovine spongiform encephalitis
BSP	Bovine specific primers
C	Centigrade
C	Carbon
Ca	Calcium
CJD	Creutzfeldt-Jakob disease
Cl	Chloride
CoA	Coenzmye A
COC	Cumulus-oocyte complex
CoQ	Coenzyme Q
DNA	Deoxyribonucleic acid
DO	Denuded oocyte
EB	Expanded blastocyst
EGA	Embryonic genome activation
ET	Embryo transfer
ETC	Electron transport chain
F6P	Fructose-6-phosphate
FP	Forward primer
FSH	Follicle stimulating hormone
g	Gravity
G3P	Glyceradehyde-3-phosphate
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GCMS	Gas chromatography-mass spectrometry
GLDH	Glutamate dehydrogenase
GLM	Generalised linear mixed model
GLUT	Glucose transporter

GTP	Guanosine triphosphate
H	Hatched blastocyst
h	Hours
H	Hydrogen
HFEA	Human Fertilisation & Embryology Authority
Hing	Hatching blastocyst
HSA	Human serum albumin
HPLC	High-performance liquid chromatogrpahy
ICM	Inner cell mass
IETS	International Embryo Transfer Society
ITS	Insuin-transferrin-sodium selenite
IVC	*In vitro* culture
IVF	*In vitro* fertilisation
IVM	*In vitro maturation*
IVP	*In vitro* production
K	Potassium
LH	Luteinising hormone
LN_2	Liquid nitrogen
M	Morula
Mg	Magnesium
ml	Millilitre
mins	Minutes
MOET	Multiple ovulation and embryo transfer
mOsm	Milliosmole
mRNA	Messenger ribonucleic scid
MSC	Migration sedimentation chamber
Na	Sodium
NADH	Nicotinamide adenine dinuclotide
NADP	Nicotinamide adenine dinuclotide phosphate
NEB	Negative energy balance
O	Oxygen
OPU	Ovum pick-up
P	Phosphorus
pb	Polar body
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPP	Pentose phosphate pathway
PZ	Putative zygotes
r	Range
REML	Restricted Maximum Likelihood Model
rHSA	Recombinant human serum albumin
ROS	Reactive oxygen species
RP	Reverse primer
S	Sulphur
SEM	Standard error of the mean

SRY	Sex-determining region Y
TCA	Tricarboxylic acid cycle
TE	Trophectoderm
UV	Ultraviolet
ZP	Zona pellucida
µl	microlitre

Chapter 1: Literature Review

1.1 Introduction

The global decline in dairy cow fertility has been the focus of much research in recent years. Causal factors include poor nutritional management (Roche, 2006) and a negative correlation between improved milk performance and fecundity (Nebel & McGillard, 1993). Lucy (2007) identified four "primary mechanisms" affecting fertility; 1) anovulation and anoestrus, 2) irregular oestrous activity, 3) preimplantation failure, and 4) uterine incompetence.

Initially, controlled breeding programs using both natural and artificial insemination (AI), were employed in an attempt to reverse this decline, however, little progress was made. Next, the 1980s saw the advent of multiple ovulation and embryo transfer (MOET) technology which effectively increased the number of calves of high genetic merit from a single animal (Merton *et al.,* 2003). A decade later saw the introduction of controlled ovarian stimulation and oocyte retrieval for *in vitro* fertilisation (IVF) and culture (IVC) of embryos, allowing greater control over the manipulation of reproductive cycles (Hassler *et al.,* 1995), and overcoming infertility caused primarily by ovulation and fertilisation failure (Hansen, 2006). This technology is now well on its way to becoming a routine part of domestic animal reproduction, and breeding companies are prepared to invest in this technology in order to generate animals of the highest genetic merit, whose gametes and/or embryos may be retailed globally.

Advantages of *in vitro* production (IVP) and embryo transfer (ET) include transfer of embryos known to be fertilised (as opposed to natural or artificial conception, during which confirmation of preimplantation fertilisation is not established), embryos known to have originated from top quality donors, increased selection intensity and a reduced generation interval (Merton *et al.,* 2003), as well as sex selection (Wheeler, 2006). However, disadvantages include the increased costs of veterinary intervention (Hansen, 2006), reports of abnormal foetuses and large offspring syndrome (Young, Sinclair & Wilmut, 1998), as well as a negative public perception (Ouédraogo, 2004).

As in vitro production of embryos for both commercial and research purposes has increased in popularity, a number of developments in commercial culture media have been reported. The inclusion of heparin into culture media was demonstrated to improve IVF success rates (Brackett & Zuelke, 1993); following which, embryo culture systems including heparin with additional penicillamine, hypotaurine and epinephrine also improved oocyte cleavage and enhanced developmental competence of preimplantation embryos (Miller *et al.,* 1994).

Supplementation of embryo culture media with serum is a much-debated issue; the benefits of inclusion include additional hormones, growth factors and attachment factors, amongst numerous smaller nutrients (Francis, 2010). However, serum also carries the risk of infection, and can vary significantly between samples (Francis, 2010). As it has been discovered that amino acids (AAs) in the secretions of the oviduct can be used as an energy substrate for the embryo (Gardner & Lane, 1993; Lane & Gardner, 1995), AAs are now routinely added to embryo culture media. This may be of particular importance to serum-free embryo culture systems as serum proteins are an important source of AAs. The catabolism of AAs produces ammonia, known to exert toxic effects on the embryo, thereby affecting developmental competence (Lane & Gardner, 2003). As such, the study of the appearance and disappearance of AAs in culture media (termed 'amino acid turnover'; AAT) is an emerging science, and potential tool in the early differentiation of developmentally competent embryos (Leary *et al.,* 2012). Sequential media systems that cater to the changing metabolic requirements of the developing embryo and remove toxic compounds (such as ammonia) are also popular in bovine embryo culture systems (Lane *et al.,* 2003).

Bovine IVP has the potential for a significant economic contribution to British (and global) agriculture, however there is still little standardisation of IVP protocols. There is no doubt that this field of reproductive biology is still expanding and there is much to learn about optimising bovine IVP protocols.

BBH7 (BoviPro™, Minitube of America, Verona WI, USA) is a serum-free embryo culture media that claims to increase the yield and cyrotolerance of bovine blastocysts *in vitro* (Block, Bonilla, & Hansen, 2010). Currently, continuous culture

in BBH7 is used in the production of commercial bovine embryos; however, there is reason to believe that this may lead to a toxic build-up of ammonia. Thus, the aim of this thesis is to elucidate if continuous culture of bovine embryos in serum-free BBH7 is detrimental to embryo development, by examining post-fertilisation development, build-up of toxic ammonia concentrations, and sex ratio of embryos cultured both continuously, and following a transfer into fresh BBH7 at 48 h intervals.

1.2 Gross anatomy of the oviduct

The first name given to the oviduct, in the sixteenth century by the Italian anatomist Gabriele Fallopius, was "tuba uteri", owing to its resemblance of the Tuba (a brass musical instrument). More recently, the terms 'oviduct', 'uterine tube' and 'Fallopian tube' are used inter-changeably. The oviduct is constructed from two muscular layers (an exterior longitudinal layer and a complimentary interior circular layer) as well as a mucous lining (Leese, 1988). The surface of the oviduct is predominantly made-up of ciliated cells, designed for tubal transfer, that undergo cyclical changes in response to elevated oestradiol concentrations in the primate (Hess, Nayak & Giudice, 2006) and the bovine (Nayak & Ellington, 1977); these are interspersed with secretory 'peg cells' that synthesise and secrete tubal fluid. Furthermore, oviducts consist of four distinct regions; the infundibulum is located at the cranial ovarian end, opening into the peritoneal cavity via the tubal ostium, and is fringed by fimbriae. The ampulla is the widest and largest region of the oviduct, distinguished by a high number of mucosal folds (known as 'rugae' or 'plicae'; Leese, 1988); ciliated cells are most numerous in the infundibulum and ampulla. The isthmus is the narrowest part of the oviduct and can act as a functional reservoir in which sperm can be stored prior to fertilisation; it is joined to the ampulla by the ampullary-isthmus junction. Finally, the utero-tubal junction joins the oviduct to the uterus. In the bovine, it has been reported that there is an observable change in tubal structure (Fig. 1.1), notably there is a decrease in the proportion of mucosal cells, the degree of mucosal folding and surface area of the tubal lumen on approaching the uterus. Conversely, there is a corresponding increase in muscle tissue towards the utero-tubal junction (El-Banna & Hafez, 1970; Leese, 1988). The reason for this anatomical change most likely relates to tubal transfer requirements of the fertilised zygote; following fertilisation, requirement for mucosal folds to harbour sperm

diminishes whereas muscular tissue is needed to facilitate the zygote's passage into the uterus.

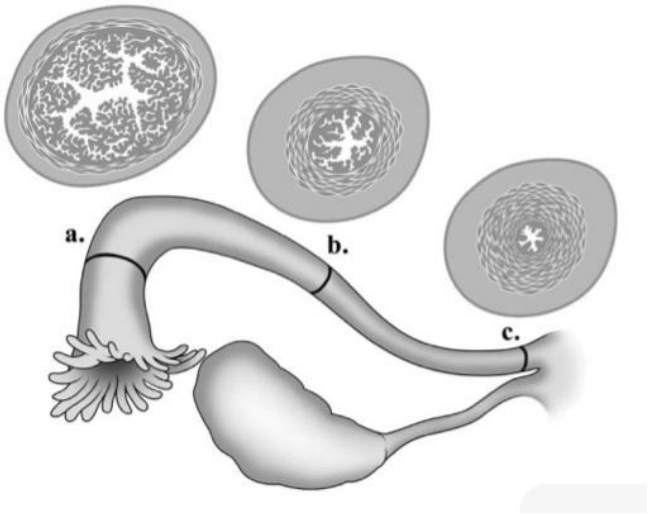


Figure 1.1: Illustration of the gradual change in longitudinal folds of mucosa along cross-sections of the oviduct from a) the infundibulum, b) the ampulla, and c) the isthmus (adapted from Lyons, Saridogan & Djahanbakhch, 2006).

1.3 Fertilisation and preimplantation embryo development

In mammals, a mature oocyte (previously arrested in the diplotene stage of meiosis I) with enclosing cumulus cells is released from the ovary into the peritoneal cavity in response to the pre-ovulatory surge of luteinising hormone (LH) from the anterior pituitary. The oocyte is collected by the fimbriated ostium of the oviduct and swept towards the uterus by cilia, having now resumed meiosis and extruded the first polar body. Tubal transfer of oocytes and embryos relies on a combination of muscular contractions, ciliary activity and the flow of oviduct fluid (Lyons, Saridogan & Djahanbakhch, 2006). Spermatazoa deposited in the vagina during coitus must undergo the process of capacitation during which they gain the ability to fertilise the oocyte. Travel to the oocyte is predominantly achieved by self-propulsion but is, in part, aided by cilia in the oviduct. Thermo- (Bahat & Eisenbach, 2006) and chemo- (Kaupp, Kashikar & Weyand, 2008) tactic gradients have also been identified as aiding sperm in the location of the cumulus-oocyte complex (COC). Fertilisation occurs in the ampulla of the oviduct; a single spermatozoon binds to the zona pellucida (ZP) of the oocyte and initiates the acrosome reaction, whereby the membranes of both gametes fuse and the contents of the acrosome are released into the oocyte. The fertilised oocyte (now known as a 'zygote') must establish only one

set of chromosomes, thus it undergoes the second meiotic division and expels surplus chromosomes as the second polar body. The zygote then continues its journey along the oviduct.

A series of independent cell-cleavage stages now face the fertilised zygote, during which it will exhibit distinct changes in metabolism, protein synthesis, requirement for energy, and AA uptake (Johnson, 2007). Early cellular cleavage results from oocyte derived mRNA (Brevini *et al.,* 2006); each cleavage event doubles the number of cells (known as blastomeres) within the ZP without increasing the volume of the zygote. Furthermore, as the size of the blastomeres is reduced with each cleavage, the high cytoplasmic:nuclear ratio is restored to adult levels (Johnson, 2007). In primates (e.g. humans) and ruminants (e.g. cows), embryonic genome activation (characterised by a major burst of transcription) occurs at the 4-8 cell (Braude, Bolton & Moore, 1988) or 8-16 cell stage (Frei, Schultz & Church, 1989), respectively.

After the 8-cell stage, the embryo undergoes the process of compaction to form a 'morula' whereby morphogenic and cellular differentiation occurs. Previously spherical cells become wedge-shaped to ensure maximal surface contact with opposing and adjacent cells (Johnson, 2007). As a result, by the 8-16 cell and 16-32 cell stage, individual blastomeres will no longer be observable as gap junctions begin to form between the cells, and the cell phenotype changes from radially symmetrical to highly polarised or epithelioid (Johnson, 2007), giving rise to two distinct populations of polar and apolar cells. This polarisation will cause the aggregation of apolar cells within the centre of the embryo, enveloped by the now polarised outer cells (Johnson & Ziomek, 1981) (Fig. 1.2).

Division of polarised cells results in the clear formation of the polarised trophectoderm (TE) (which will give rise to extra-embryonic tissues, such as the placenta or chorion), and the apolar inner cell mass (ICM) (that will give rise to the foetus) which sits against, or embedded within, the TE (Fig. 1.2). TE cells pump fluid into the embryo resulting in the formation of an impermeable fluid filled cavity

known as the 'blastocoele' (Gardner & Papaioannou, 1975; Watson, Dampsy & Kidder, 1990; Pfaf *et al.,* 1998). The embryo is now known as a 'blastocyst' and during transition from a morula to a blastocyst, has left the oviduct and entered the uterus.

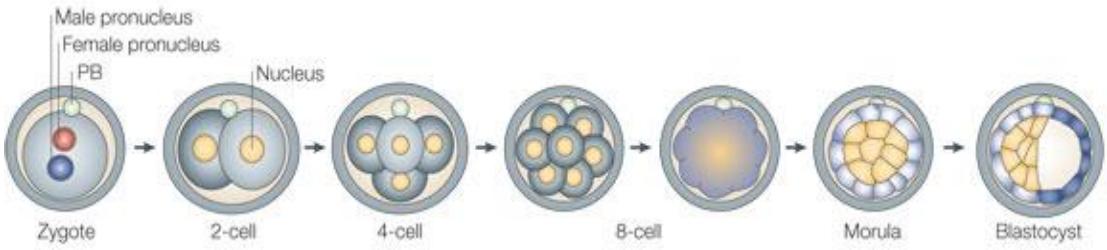


Figure 1.2: Cell cleavage events following fertilisation. Cleavage from 1-2 cells, 2-4 cells, 4-8 cells followed by compaction to morula stage, differentiation into two distinct cell lineages, and formation of the blastocoelic cavity (Zernica-Goetz, 2005).

PB = polar body

As the embryo continues to acquire blastocoelic fluid it becomes an 'expanded blastocyst' and eventually the ZP cracks under the pressure from increased embryonic volume such that the embryos 'hatches'. This process is essential to allow the hatched blastocyst to adhere to the uterine wall. Implantation can be either invasive (e.g. primates) or non-invasive (e.g. ruminants) and subsequent embryo survival is dependent on both progesterone secretion from the corpora lutea, and how well the uterus responds to this progesterone (Senger, 2005). A summary of these events can be found in Table 1.1.

Table 1.1: Time (in days) following ovulation of embryonic events in the oviduct and uterus in invasive (e.g. human) and non-invasive species (e.g. cow). Adapted from Johnson (2007). [a]Johnson, 2007; [b]Braude, Bolton & Moore, 1988; [c]Frei, Schultz & Church, 1989.

EGA = Embryonic genome activation.

	2-4 cell	EGA	Conceptus enters uterus	Blastocyst formation	Time of attachment
Human	2[a]	4-8 cells[b]	3.5 [a]	4.5 [a]	7-9 [a]
Cow	2-3[a]	8-16 cells[c]	3-4 [a]	7-8 [a]	30-45 [a]

Tubal fluid

Following fertilisation, the developing embryo is dependent on tubal and uterine fluids for survival prior to implantation and placental development (Ellington, 1991; Leese, 1995; Bavister, 2000). The surface of the oviduct is fundamentally a secretory one (despite bidirectional diffusion of molecules across the epithelium), and tubal fluid is selectively derived from plasma, supplemented by additional proteins produced by the tubal epithelium (Leese & Gray, 1985; Leese, 1988; Gandolfi *et al.,* 1993). The ampulla produces two thirds of tubal fluid whilst the isthmus produces the rest (Aguilar & Reyley, 2005); furthermore, oestrogen stimulates, and progesterone inhibits tubal fluid production; thus, a cyclic variation in the volume of secretion, and composition of tubal fluid is observed (Aguilar & Reyley, 2005).

Electrolytes are primarily responsible for maintaining the pH and osmolarity of tubal secretions. Ion concentrations in tubal fluid are similar to those of serum across most species, with few exceptions (Aquilar & Reyley, 2005). For example, during the bovine oestrous cycle, sodium (Na) and chloride (Cl) ions have been reported to be present in high concentrations in tubal fluid (Hugentobler *et al.,* 2007). Potassium (K) ion concentrations are consistently higher in human tubal fluid (Lippes *et al.,* 1972; Borland *et al.,* 1980) as well as increasing in concentration in oestrous cattle (Olds & Van Denmark, 1957).

Energy substrates are required by the oocyte, spermatozoa, and preimplantation embryo for nourishment (Leese & Gray, 1985; Varghese *et al.,* 2011). The principle energy substrates used by human and bovine gametes and early embryos are glucose and pyruvate (Guyader-Joly *et al.*, 1996; Hugentobler *et al.,* 2008), however lactate has also been identified in human tubal fluid (Dickens *et al.,* 1995). As reviewed by Leese (1988), the presence of energy substrates within tubal fluid is facilitated by three means: 1) diffusion across the epithelium from plasma, 2) synthesis and secretion by tubal epithelial cells, and/or 3) synthesis by cumulus cells (namely pyruvate and lactate).

Albumin and immunoglobulin G are the predominant proteins present in tubal fluid, constituting 95% of tubal proteins (Oliphant *et al*., 1978); the function of tubal proteins has yet to be fully elucidated. Tubal proteins may be derived from the blood or synthesised by tubal epithelium under ovarian steroidal regulation (Buhi, Alvarez & Kouba, 2000). Cyclic fluctuations in the synthesis of oviductal glycoproteins has been reported (Boice *et al.,* 1990), as well as some specificity in the site of production (Ampulla > Isthmus) (Hernández, 1996). Systemically, albumin serves two important roles within the body. Firstly, it is essential for maintaining the osmotic pressure and pH of blood by binding water and cations. Secondly, albumin can bind and transport physiologically important ligands such as hydrophobic steroid hormones, lipids and AAs. Albumin in tubal fluid is derived from plasma. Within the ampullary region of the oviduct, albumin has been reported to act as a sterol acceptor molecule, facilitating sperm capacitation (Ravnik, Albers & Muller, 1993). Furthermore, Gardner (2008) demonstrated that albumin alters the surface tension of gametes and embryos, such that they become lubricated, avoiding adhesion to culture vessels (including IVC apparatus).

1.4 Embryo metabolism

Glucose metabolism in the COC

Glucose, derived from plasma, is taken into the cumulus cells (mediated by insulin; Purcell, Chi & Moley, 2012) via the glucose transporters (GLUT) GLUT-1 and GLUT-4 (Lequarre *et al.,* 1997; Dan-Goor *et al.,* 1997), where it is anaerobically metabolised via glycolysis within the cytosol. The products of glycolysis are two molecules of pyruvate, two molecules of adenosine triphosphate (ATP) (i.e. energy) and two molecules of nicotinamide adenine dinucleotide plus hydrogen (NADH) (a reducing agent), per one molecule of glucose (Fig. 1.3).

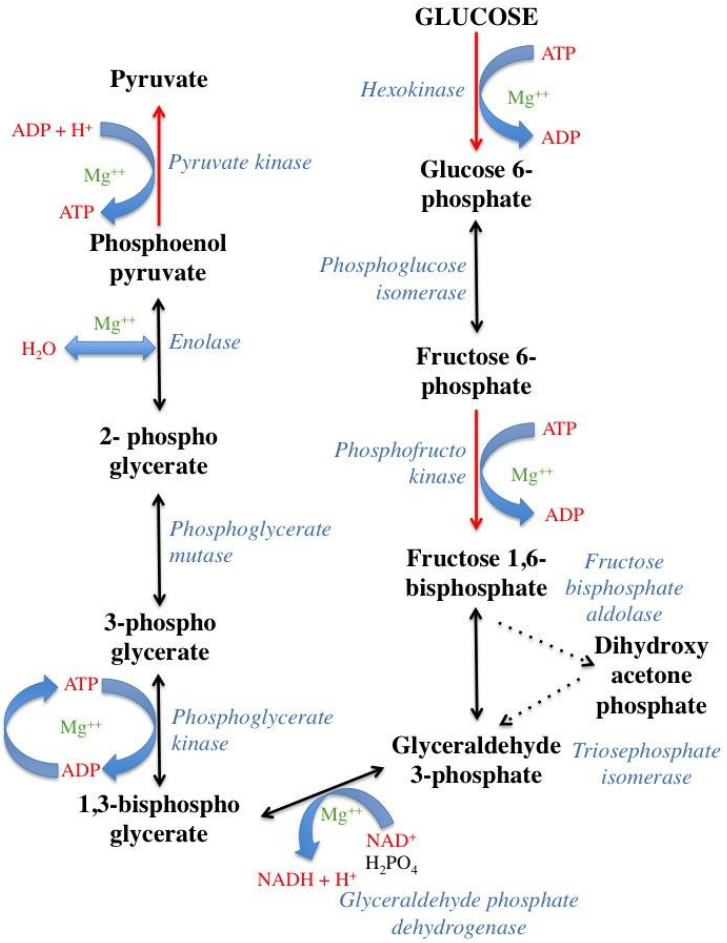


Figure 1.3: Glycolysis; two molecules of pyruvate, two molecules of nicotinaminde adenine dinucleotide plus hydrogen (NADH), and two molecules of adenine triphosphate (ATP) are produced per molecule of glucose.

H_2PO_4 = Inorganic phosphate; Mg^{++} = magnesium ion (cofactor); ADP = adenine diphosphate; one-sided arrow = irreversible reaction; double headed arrow = reversible reaction; enzymes are depicted in blue text.

The Pentose Phosphate Pathway (PPP) is an alternative to glycolysis (i.e. the PPP is anabolic rather than catabolic); glyceraldehyde-3-phosphate (G3P), fructose-6-phosphate (F6P) and NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate ($NADP^+$)) are generated from glucose-6-phosphate (G6P) (Fig. 1.4). G3P and F6P can both be utilised in glycolysis to produce pyruvate. Furthermore, NADPH is a coenzyme that is used as a reducing agent in nucleic acid and lipid synthesis (important in the production of cell membranes as the embryo expands), as well as controlling the toxicity of reactive oxygen species (ROS) produced as a result of the Tricarboxylic Acid (TCA) cycle.

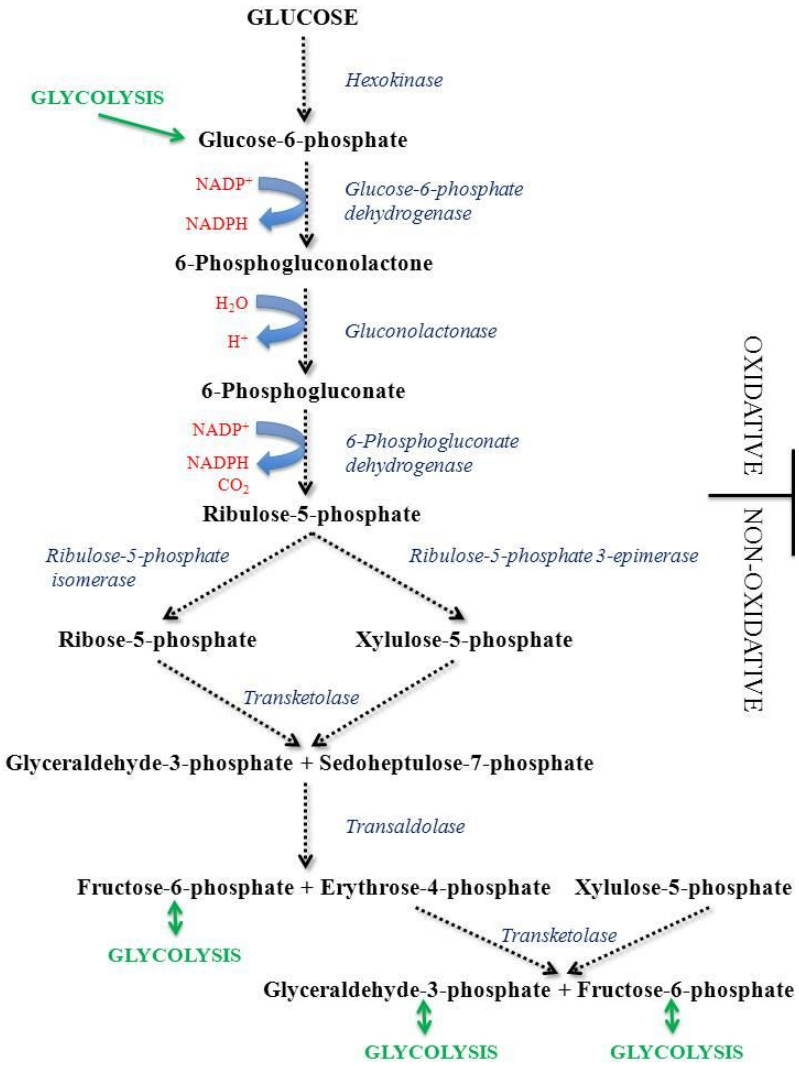


Figure 1.4: The Pentose Phosphate Pathway (PPP). An alternative to glycolysis, the PPP produces glyceraldehyde-3-phosphate and fructose-6-phosphate as well as the NADPH for use in other metabolic cycles.

Enzymes are depicted in blue and points of entry into glycolysis are depicted in green.

Cumulus cells are connected to the oocyte by heterologous gap junctions that penetrate the ZP and terminate at the oolemma (Tanghe *et al.,* 2002), permitting passage of nutritional and regulatory factors. Glycolysis in the oocyte is not very active and glucose is, instead, metabolised through the PPP; pyruvate (and lactate; produced from pyruvate by lactate dehydrogenase) moves across these gap junctions from the cumulus cells to the oocyte where it is converted into acetyl-CoA by decarboxylation within the mitochondria. Acetyl-CoA next enters into the TCA

cycle (Fig. 1.5) producing NADH (x3) and guanosine triphosphate (GTP) (x1) per molecule of pyruvate, as well as precursors for other metabolic processes.

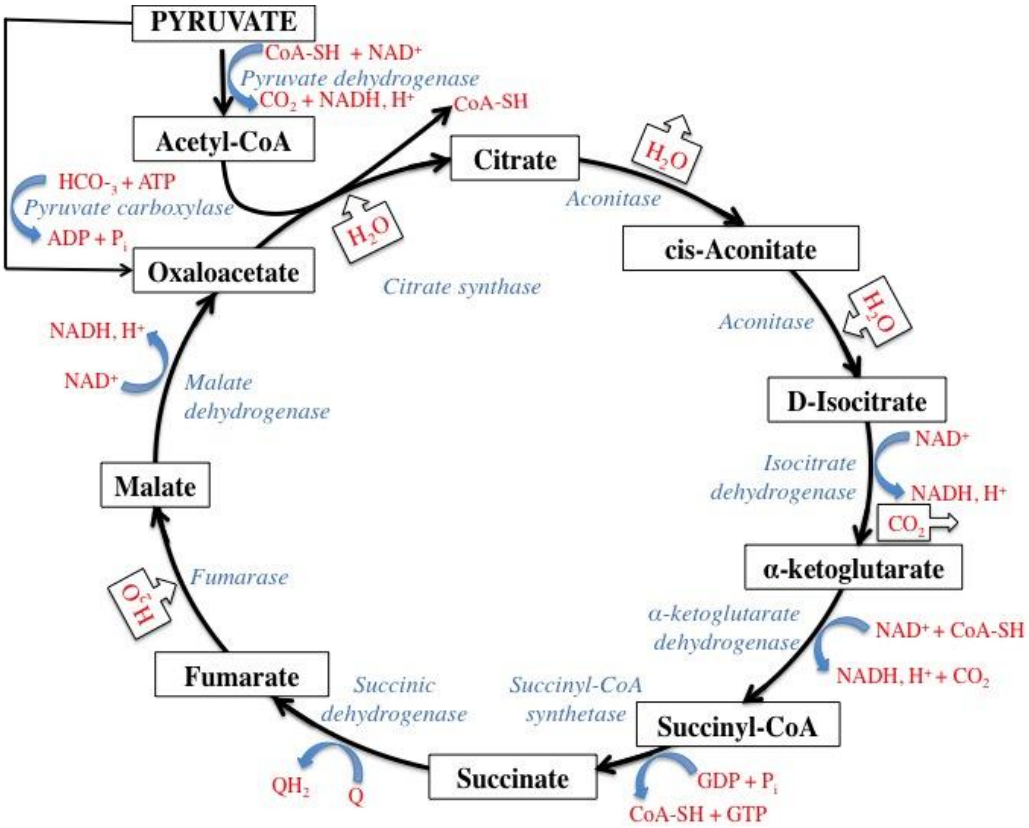


Figure 1.5: The Tricarboxylic Acid (TCA) cycle. Acetyl-CoA enters the cycle producing three molecules of nicotinamide adenine diphosphate plus hydrogen (NADH) and one molecule of guanosine triphosphate (GTP), as well as additional precursors of further metabolic processes.

H = hydrogen; H_2O = water; CO_2 = carbon dioxide; CoA-SH = Coenzyme A; Q = Coenzyme Q; enzymes are depicted in blue text

Subsequently, NADH and succinate (produced by the TCA cycle; Fig. 1.5) are oxidised through the Electron Transport Chain (ETC), at the site of the inner cell membrane. Electrons are passed from a donor to an electronegative acceptor, releasing energy and prompting a series of electron transfers until electrons are passed either to oxygen, or to the terminal electron acceptor in the chain. As a result, a proton gradient is created across the membrane, whereby protons are 'pumped' into the inter-membrane space. In summary, the ETC produces ATP (through phosphorylation of adenosine diphosphate (ADP)) and water.

Direct uptake of glucose by the oocyte itself is also possible; however it is unlikely that this occurs via GLUTs (Biggers, Whittingham & Donahue, 1967; Downs & Utecht, 1999). Furthermore the comparative study by Downs & Utecht (1999) demonstrated preferential uptake of glucose by COCs *vs.* denuded oocytes (DO).

Fatty acids

Fatty acids consist of a carboxylic acid and a long aliphatic tail that is either unsaturated (carbon atoms joined by double bonds; e.g. Linoleic acid) or saturated (no double bonds; e.g. Palmitic acid), derived nutritionally from phospholipids and triglycerides. Fatty acids are broken down by the liver to produce ketone bodies including acetone, acetoacetate and beta-hydroxybutyrate (BHB), in a process known as ketogenesis. As a by-product of ketogenesis, acetyl-CoA is produced, which subsequently enters the TCA cycle and is oxidised as before (Fig. 1.5). Thus, fatty acids can be used as an alternate energy substrate (Sturmey *et al.,* 2009b; Collado-Fernandez, Picton & Dumollard, 2012). However, it is important to note that ketogenesis does not occur in the embryo, but occurs in the mother's liver in situations when glucose availability is low: ketogenesis is not preferential to glycolysis (Somogyi, 1942).

Amino acids

From plasma, AAs diffuse into luminal fluid and are taken-up by the preimplantaiton embryo (Leese 1988). AAs consist of a carboxylic acid group, an amino group and an additional side chain (specific to each AA), linked to a single carbon atom. Most AAs can be synthesised within the body (non-essential AA). However, other AAs cannot be synthesised and must, instead, be incorporated into the diet (essential AAs). AAs considered to be essential may also differ between species (e.g. primates *vs.* ruminants) due to the anatomy and physiology of respective gastrointestinal tracts. Furthermore, AAs considered to be essential in the diet differ from those in culture media (Elhassen *et al.,* 2001). Henceforth, AAs described to be essential or non-essential, are those considered so in cell culture media rather than in the diet.

It has been reported that there are 500 AAs occurring in nature, however, only 21 are used by eukaryotes for *de novo* protein and nucleotide synthesis (Epstein & Smith, 1973; Wagner & Musso, 1983; Alexiou & Leese, 1992). Furthermore, AAs serve a number of additional purposes within the preimplantation embryo (Table 1.2): AAs (particularly glycine) act as osmolytes, maintaining cell volume by replacing inorganic ions and providing intracellular osmotic support (Dawson *et al.,* 1998; Baltz, 2012). Cysteine, glycine and glutamic acid are precursors of the antioxidant glutathione, known to occur in oocytes. Supplementation with cysteine has demonstrated an increase in glutathione production, thus protecting the embryo from oxidative damage and ROS (Guerin *et al.,* 2001; Harvey *et al.,* 2002). As early embryos lack the mechanism whereby they are able to alleviate acid loads (caused by pyruvate and lactate from glycolysis; Baltz, Biggers & Lechenne, 1991), AAs act as buffers of intracellular pH; Edwards, Williams & Gardner (1998) demonstrated that non-essential AAs buffer intracellular pH up until the 4-cell stage. At compaction, the morula generates a permeability seal that may help it to regulate its own pH (Edwards, Williams & Gardner, 1998), thus, diminishing the role of AAs in this capacity. Finally, Van Winkle, Haghighat & Campione (1990) demonstrated a role for glycine in the protection of early embryos from the toxic effects of heavy metal ions (i.e. as a chelator).

Degradation of AAs for removal from the body requires deamination of one or more amino groups. Urea is routinely produced from the deamination of AAs in the liver, as a safe method by which to transport and excrete excess nitrogen. However, in the preimplantation embryo, AAs are aerobically metabolised to produce glucogenic or ketogenic substrates to enter the TCA cycle. Catabolism of AAs (particularly glutamine; Gardner & Lane, 1993; Lane & Gardner, 1995) produces ammonia that impairs an embryo's ability to regulate intracellular pH, reduces ICM development, decreases blastocyst cell number, alters gene expression, perturbs metabolism and subsequently increases apoptosis (Lane & Gardner, 2003). Thus, appearance/depletion of AAs (i.e. AAT) in spent and unspent media measured by high-performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GCMS) have become metabolomic markers of the developmental potential of an embryo (reviewed by Piction *et al.,* 2010). Furthermore, it has been proposed that viable embryos suffer less cellular and molecular damage than their

less viable equivalents, thus AA uptake to repair the aforementioned damage is diminished (Sturmey *et al.,* 2010). This has led to the development of the 'quiet embryo hypothesis' which states that embryos with the most developmental potential have a lower rate of AAT (Leese, 2002; Baumann *et al.,* 2007; Leese *et al.,* 2007; Sturmey *et al.,* 2009a; Sturmey *et al.,* 2010). Measuring AAT could, therefore, assist in the selection of embryos at the cleavage and early blastocyst stage for further culture and ET.

Table 1.2: Twenty-one amino acids predominantly used by eukaryotes, the group to which they belong (essential *vs.* non-essential) and an indication of their function.

AA = Amino acid

	Amino Acid	Abbreviation	Function	Reference
ESSENTIAL	Arginine	Arg	Embryo development to the 4-cell stage is inhibited by Cys, Ile, Leu, Phe, Thr, & Val, but supported subsequent to the 8-cell stage. His supports development up to the 4-cell stage. Cys is a precursor for the antioxidant Gln.	Gardner & Lane, 1993 Gardner & Lane, 1997 Van Winkle, 2001
	Cysteine	Cys		
	Histidine	His		
	Isoleucine	Ile		
	Leucine	Leu		
	Lysine	Lys		
	Methionine	Met		
	Phenylalanine	Phe		
	Threonine	Thr		
	Tryptophan	Trp		
	Tyrosine	Tyr		
	Valine	Val		
NON-ESSENTIAL	Alanine	Ala	Early embryo development (to the 4-cell stage) is supported by Asn, Asp, Gly, Ser and Gln. Non-essential AAs (particularly Gln) buffer intracellular pH to the 4-cell stage by alleviating acid loads of pyruvate and lactate. Glu and Gly are precursors for the antioxidant Gln. Gly acts as an osmolyte providing intracellular support, and also as a chelator to remove heavy metal ions.	Gardner & Lane, 1993 Gardner & Lane, 1997 Van Winkle, 2001 Tiboni *et al.*, 1997 Edwards, Williams & Gardner, 1998 Baltz, 2012
	Asparagine	Asn		
	Aspartic Acid	Asp		
	Glutamic Acid	Glu		
	Glutamine	Gln		
	Glycine	Gly		
	Proline	Pro		
	Serine	Ser		
	Selenocysteine	Sec		

Amino acid profiling (AAP) has also identified some sex-specific differences in AAT of bovine embryos cultured *in vivo vs. in vitro*. The AAP of female *in vitro* produced embryos demonstrated increased concentrations of glycine and increased depletion of arginine, glutamine and methionine (Sturmey *et al.,* 2010); by comparison, male embryos demonstrated increased depletion of phenylalanine, tyrosine and valine (Sturmey *et al.,* 2010). Although there is little supporting evidence of this theory at present, if further developed, AAP could provide a non-invasive means by which embryos could be sex-selected prior to transfer (as an alternative to embryo biopsy for polymerase chain reaction (PCR)).

In recent years, AAs have been implicated in the epigenetic regulation of gene expression by phosphorylation, acetylation and methylation of histone tails (also known as 'histone modification') as well as deoxyribonucleic acid (DNA) methylation (Oommen *et al.,* 2005; Kouzarides, 2007; Wu, 2010). Epigenetics can be defined as chemical changes to chromatin structure that result in, sometimes heritable, changes to gene expression without alterations in the nucleotide sequence. DNA is packaged around proteins known as histones; AA tails extend from the histones and are a site of post-translational modification during protein biosynthesis, thus affecting gene regulation (Mersfelder & Parthun, 2006).

1.5 Embryo culture systems

Historically, embryo culture systems were adapted from those used in the culture of somatic cells. However, in the late 19th Century it was proposed that an environment that closely mimics that *in vivo*, and a medium that supports the metabolic function of the embryo, should be provided in order to maximise growth and developmental potential (Freshney & Freshney, 2002). Ringer (1884) subsequently isolated the beating hearts of amphibians in a salt solution and thus went on to investigate how different salts affected cardiac performance *in vitro* (Ringer, 1884). Henceforth, a number of advances were made in the development of culture medium until modern-day culture systems were established, i.e. a simple salt solution ($Na^{=}$, K^{+}, Cl^{-}, Ca^{2+} Mg^{2+}, SO_4^{2-} and sometimes PO_4^{2-}; Baltz, 2012) in ultra-filtered water, supplemented with: an energy substrate (e.g. glucose), macromolecules (e.g. albumin), essential/non-essential AAs, vitamins, antibiotics and chelators.

Currently there are two methods by which embryos are cultured *in vitro*: continuous and sequential culture. Traditionally, continuous culture was used in human clinical embryology as embryos were cultured and transferred at day 2/3 post fertilisation (cleavage stage) (Gardner, Lane & Schoolcraft, 2000). Unfortunately, this technique resulted in low implantation rates *in utero* (Gardner *et al.,* 1998) and subsequently multiple ET became common practice to compensate, increasing the risk of multiple births. In recent years, the Human Fertilisation & Embryology Association (HFEA) has advised participating members that they must adopt a strategy whereby a reduction in the number of multiple births occurring annually is seen (HFEA, 2010). Thus, the need to select one viable embryo for transfer has driven developments in embryo culture systems. In particular, IVC to the blastocyst stage (day 5) boasts a number of advantages; transferring a single blastocyst stage embryo has been demonstrated to maintain a high level of pregnancy (Bolton, Wren & Parson, 1991). Furthermore, allowing more time for development *in vitro* facilitates the identification of developmentally incompetent embryos to be discarded that may otherwise have been selected for ET (Jones *et al.,* 1998; Jones & Trounson, 1999). Most importantly, however, extended culture to the blastocyst stage allows better synchronicity between the embryo transferred and the uterine environment of the recipient, as the embryo would naturally enter the uterus five days following fertilisation, potentially resulting in increased implantation rates.

Gardner (1998) described the changing requirements (i.e. shift from pyruvate to glucose and non-essential to essential AAs) of the preimplantation embryo, and thus a need for a sequential media system that catered to evolving metabolic requirements. The author also reported that AAs are essential for embryogenesis both pre- and post- activation of the embryonic genome, and that use of this system resulted in the late-stage viable blastocysts for transfer (Gardner, 1998). The initial sequential media system comprised of two solutions, G1 and G2; these were later developed into the commercially available S1 and S2, followed by G1.2 and G2.2, and finally by the G*III* series. Embryos are cultured in the first solution for 24 hours and then transferred into the second, containing all of the components an embryo would need to successfully develop to blastocyst stage.

Co-culture systems (i.e. the culture of somatic cells alongside embryos) were developed in the 1980s, as a means by which to alleviate the developmental block associated with EGA (Allen & Wright 1984; Gandolfi & Moor 1987). During early cleavage, the maternal-to-embryonic genome transition is not yet complete, thus assessment of embryos at this stage may fail to measure their true viability (Braude *et al.*, 1988; Taylor *et al.*, 1997). The first reported practical application for bovine co-culture technology in assisted reproduction was the extended culture of embryos to blastocyst stage for transfer (reviewed by Ménézo *et al.,* 2012), as transfer of cleavage stage embryos resulted in poor implantation. The mechanism by which co-culture with somatic cells alleviates the developmental block has yet to be elucidated (Orsi & Reischl, 2007); however the technique has successfully been translated into human embryo co-culture resulting in good quality blastocysts for single ET (Ménézo, Sakkas & Janny, 1995; Mercader *et al.,* 2003). There has been very little published in the last 20 years regarding co-culture and IVP of bovine embryos. This is most likely owing to pressure from commercial sponsors to focus on defined culture media, which has demonstrated the ability to support early embryonic development more so than co-culture (Catt, 1994). However, there is no denying that co-culture has yielded promising results and may again, in the future, become the focus of further research.

"Block-Bonilla-Hansen 7 (BBH7; BoviPro™, Minitube of America, Verona WI, USA) is a defined, serum-free culture medium that was developed to increase the yield and cryotolerance of bovine blastocysts produced in vitro" (Block, Bonilla & Hansen, 2010). Serum has historically been included into embryo culture medium as it provides a source of hormones, growth factors and attachment factors, amongst numerous smaller nutrients (Francis, 2010). However, serum may: contaminate culture media with mycoplasmas and viruses; is potentially variable from sample to sample (thus preventing a confident sample-sample comparison); and may occasionally be limited in supply (Francis, 2010). Furthermore, since the bovine spongiform encephalopathy (BSE) epidemic and resultant threat of Creutzfeldt-Jakob disease (CJD), a need to replace animal derived products (such as serum) now exists.

The effects of the inclusion of serum into embryo culture media are well documented in the literature. Serum appears to exhibit a biphasic effect on bovine embryos, inhibiting development of cleavage stage embryos but stimulating advanced morula compaction and blastocyst development up to, but not beyond, six days following fertilisation (Pinyopummintr & Bavister, 1991; Rizos *et al.,* 2002). This acceleration in embryo development is most likely the result of increased glycolytic activity as reported by Krisher, Lane & Bavister, (1999). However, a number of authors have reported poor cryotolerance of embryos cultured in serum, demonstrating superior survival and hatching rates post-thaw in embryos cultured in the absence of serum (Rizos *et al.,* 2002; Hiroyuki & Hiroyoshi, 2003 Mucci *et al.,* 2006; Gómez *et al.,* 2008). An abnormal accumulation of cytoplasmic lipid and lipid droplets in embryos cultured in the presence of serum has been reported (Abe *et al.,* 2002), it has been hypothesised that this accumulation of lipids may alter embryo sensitivity to cryopreservation (i.e. vitrification or slow-freezing) (Hiroyuki & Hiroyoshi, 2003). Lipids are a major component of cell membranes, the composition of which is prone to change at decreased temperatures (Zeron *et al.,* 2001). Upon thawing, these changes may affect the osmotic properties of an embryo (Rottem *et al.,* 1973), thus, impacting post-thaw viability.

Therefore, although embryo culture media supplemented with serum has been shown to accelerate the time to which blastocyst stage is reached, cryotolerance of the resultant blastocysts is low. Furthermore, the availability of serum-replacement products such as CPSR-3 (Sigma-Aldrich, St. Louis MO, USA) and Ultroser™ (Pall Corporation, Port Washington NY, USA) for inclusion into embryo culture media (Duque *et al.,* 2003), and evidence reporting blastocyst development in serum-free media excelling that of serum-inclusion culture systems (Abe & Hoshi, 2003), suggests little requirement for serum in future embryo culture protocols.

It is, however, important to remember that comparison of published literature regarding embryo culture systems may be difficult. Systems designed for human clinical IVF (but often initially designed and executed in animal models like the cow; Zander-Fox & Lane, 2012) aim to produce one or two top quality embryos for

transfer (Lane & Gardner, 2007). Conversely, culture systems designed for the IVP of domestic species are often designed to generate a cohort of top quality oocytes for transfer (fresh or frozen-thawed) into one or several recipients (Moore & Thatcher, 2006).

1.6 Clinical applications

Bovine applications

Following the Second World War there was a shift from the "productivist focus" to breed improvement through genetic technologies (Gibbs *et al.,* 2009). Conventional breeding is limited by availability of animals on-site or in close vicinity to each other. Furthermore, breeding animals that originate from the same geographic region may limit their resistance to disease (McEwan & Fedorka-Cray, 2002). Whilst this may be overcome using vaccines, antimicrobials and probiotics, these represent an additional cost to the producer. Cows are non-seasonally polyoestrous monovulatory animals producing, on average, only one calf annually (although twinning occurs in approximately 4% of births; Esslemont & Kossaibati, 2002). The commercial longevity of cows depends on a number of factors, including poor general health, udder health (position of teats and susceptibility to mastitis), and irregular cyclic activity (i.e. susceptibility to conditions that induce anoestrus, e.g. ovarian cysts), all of which require costly veterinary intervention. Thus, it is important that animals and the calves they produce justify the cost of production; with conventional breeding techniques, however, this is not always the case. Traditional genetic improvement systems, therefore, have been augmented using firstly AI (Salisbury, VanDenmark & Lodge, 1978), secondly MOET (involving controlled ovarian stimulation), and now by IVP of embryos (Carolan *et al.,* 1996).

Artificial Insemination

The advent of AI and long-term cold storage (-80 °C) of sperm revolutionised the breeding of agricultural stock globally. There are a number of advantages when using AI, both regarding the breeding animals and the related costs. The use of AI helps to minimise the spread of disease, protects animals from injuries potentially sustained during coitus, synchronises births, and minimises risk to stockpersons

associated with male sexual aggressiveness. Furthermore, AI allows for semen processing and quality control. AI also allows owners to maximise the economic potential of an animal (living or deceased) by increasing the number of reproductive events from a single ejaculate; whilst clients benefit from the reduced cost of globally transporting frozen semen straws *vs.* appropriating the animal in its entirety. However, ease of access to popular animals has seen an increase in inbreeding such that mating amongst farm animals is now closely monitored to avoid associated negative consequences (such as poor fertility; Walsh, Williams & Evans, 2011). Other disadvantages of AI include a requirement for otherwise unnecessary veterinary intervention, increased cost and a potential risk of laboratory/human error in semen analysis and sample selection.

Multiple ovulation & embryo transfer

Following AI, MOET was employed by dairy farmers as a means by which genetic improvement could be accelerated and a number of valuable offspring could be generated from one desirable animal. Multiple ovulation (also known as 'super-ovulation') is induced in female animals by administering gonadotrophins during follicle recruitment (Mapletoft, Steward & Adams 2002), following which the animal is inseminated (naturally or artificially) with genetically superior semen, and the resultant embryos are removed (via a uterine flush) prior to implantation. Recipient animals primed for ET (again, either by monitoring cyclic activity or artificially manipulating their oestrous cycle) are then used to gestate these embryos to term. Although, this technique is effective at producing a large number of superior animals, the use of super-ovulatory drugs is costly and undesirable at a consumer level.

In vitro fertilisation and embryo transfer

Further developments in technology have seen the application of IVF and ET in farm species (particularly equine, ovine and bovine) (Flores-Foxworth, 1995; Squires *et al.,* 2003; Hansen, 2006). Currently, there are at least eight veterinary practices offering advanced assisted reproduction technology (ART) to clients (sheep, beef and dairy) in the UK (Fig. 1.6). Following a period of controlled ovarian stimulation, immature oocytes are collected from the ovaries of genetically superior animals using a transvaginal ultrasound probe and aspiration needle (known as ovum pick-

up; OPU). These oocytes are matured overnight, fertilised *in vitro*, and cultured for seven days to blastocyst stage. Following which, embryos are transferred (fresh or frozen-thawed) into recipient animals after a period of uterine preparation.

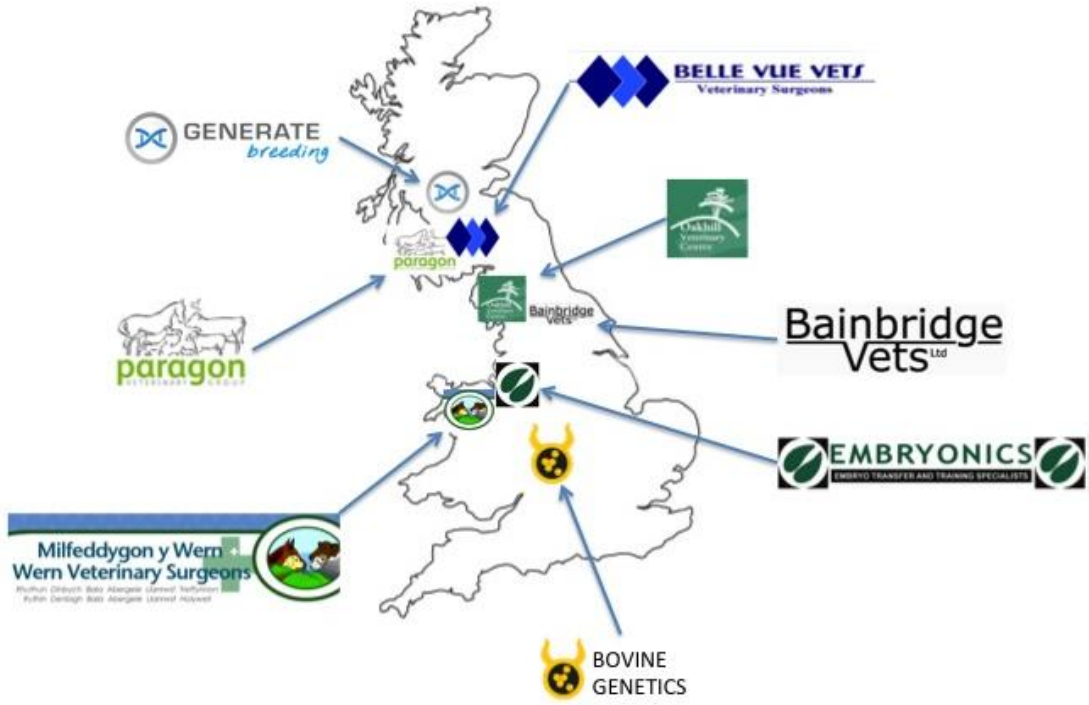


Figure 1.6: Veterinary practices in England, Scotland and Wales that offer embryo transfer technologies. Generate Breeding is based in the Scottish borders; Belle Vue and Paragon are located within Cumbria; Oakhill Veterinary Centre and Bainbridge Vets can be found in Lancashire and North Yorkshire, respectively; Embryonics Ltd. is operated within Cheshire; Wern Veterinary Surgeons was established in North Wales; most Southernly, Bovine Genetics is based in Warwickshire.

Recipients both nationally and internationally (subject to local regulation) can benefit from this service. Furthermore, if required, the sex of an embryo can be guaranteed through the use of sexed-semen (Hohenboken, 1999), embryo sexing technology (Macháty *et al.,* 1993) or amino acid profiling (Sturmey *et al.,* 2010). Previously, however, IVP systems have reportedly resulted in abnormally large offspring, caused not by genetic or chromosomal abnormalities, but by the environment in which the embryos were cultured (reviewed by Young, Sinclair & Wilmut, 1998). Large offspring syndrome is commonly associated with a number of pathological changes including increased birth-weight and extended gestation (Lazarri *et al.,* 2002). At the production level, large offspring syndrome often results

in dystocia, requiring veterinary intervention at calving (Kruip & den Daas, 1997). Thus, developments in bovine IVP have been hindered as the increased cost of complications associated with this technology are undesired by producers. Specifically, the inclusion of serum into embryo culture media has been reported to induce large offspring syndrome in subsequent embryos in bovine and ovine specimens (Behboodi *et al*., 1995; Farin & Farin, 1995; Thompson *et al*., 1995; Brown and Radziewic, 1996; Kruip & den Daas, 1997; Sinclair *et al*., 1998). Thus, optimising IVP of bovine embryos in a serum-free media has the potential to advance IVP technology into everyday dairy production.

1.7 Development of working hypotheses

Currently, IVP embryos are produced by continuous culture in BBH7 (Block, Bonilla & Hansen, 2010); although this method currently yields acceptable results, the biology of embryo metabolism suggests there may be some detrimental effects of toxic ammonia build-up on embryo development. Thus, this thesis seeks to explore whether continuous culture has a detrimental effect on embryo development, compared with a similar culture system that incorporates a change to fresh media at 48 hour intervals following fertilisation. It is hypothesised that this change in culture media will support development of significantly more embryos to the blastocyst stage by removing embryo-toxic ammonia, and replenishing amino acids in the culture environment.

This will be assessed by:

1. Culturing two groups of embryos in commercially available serum-free BBH7 media for a period of 7 days. One group will remain in their initial drop of BBH7 whilst the other will be moved at 48 h intervals into fresh drops.
2. Assessing cleavage rate 48 h following transfer into initial culture media in both groups and removing any uncleaved embryos.
3. Assessing final embryo development after 144 h of IVC by recording the number and stage of any resulting blastocysts according to the International Embryo Transfer Society (IETS) coding for embryonic stage.
4. Determination of ammonia and AA concentrations in spent culture media by enzymatic ultraviolet (UV) assay and GCMS, respectively.
5. Determination of sex ratio by PCR analysis to identify any consequential effect of treatment on sex ratios.

Chapter 2: Materials and Methods

2.1 Oocyte retrieval and maturation

COCs were aspirated from follicles 4-9mm in diameter of abattoir-derived ovaries washed thrice in sterile phosphate buffered saline (PBS). Aspirates were collected into 50 ml centrifuge tubes kept at 37 °C in a warming cabinet, and were sorted and washed in 'wash media' (Appendix A1 for full recipe), osmolarity to 295-325 mOsm (Advanced® Model 3300 Micro-Osmometer, Advanced Instruments Inc., Norword MA, USA). COCs with good even cytoplasm and in excess of 4 layers of cumulus cells (Grades 1 & 2) were preferentially selected for inclusion into this experiment, good quality grade 3 embryos were also selected if necessary; however, no grade 4 embryos were selected for this study (Table 2.1).

Table 2.1: Oocyte grading; system used in this study to identify best quality embryos for culture (criteria adapted from Goodhand *et al.,* 2000).

Grade	Definition
1	Good even cytoplasm with very good cumulus cells (>4-5 layers).
2	Good even cytoplasm with good cumulus cells (4-5 layers).
3	Patchy cytoplasm and a slightly expanded cumulus (<4-5 layers).
4	Expanded cumulus cells or denuded oocyte with or without cracked zona pellucida.

COCs were then incubated at 39 °C in 5 % CO_2 for 24 h in 'maturation media' (Appendix A2 for full recipe) to induce final maturation of previously arrested oocytes, that would otherwise naturally have occurred at the LH surge.

2.2 Sperm preparation and fertilisation

Preparation of fertilisation media

Micro-drops of G-IVF™ Plus (Vitrolife AB, Göteborg, Sweden) supplemented with human serum albumin (HSA) and a preparation of hypotaurine, heparin and penaciliamine (Appendix A3 for full recipe), were prepared for IVF under paraffin

oil (Ovoil; Vitrolife AB, Göteborg, Sweden). Once prepared, fertilisation dishes were allowed to equilibrate to 39 °C in 5 % CO_2 for at least 2 hours prior to fertilisation.

Swim-up protocol

Frozen-thawed semen (supplier no longer in business) was prepared for fertilisation using a Migration Sedimentation Chamber (MSC; Research Instruments, Cornwall, UK). A calcium-free media (See Appendix A4 for full recipe) (1.5 ml) was pipetted into the bottom of the MSC and the frozen-thawed semen was layered around the gallery (Fig. 2.1). The MSC was incubated at 39 °C and 5 % CO_2 for 1-2 h to allow vigorous motile sperm to swim-up out of the gallery and precipitate in the bottom of the MSC. Media was subsequently aspirated from the bottom of the MSC into a 1.5 ml eppendorf and centrifuged at 3000 g for 5 minutes.

Figure 2.1: Sperm preparation by the swim-up method using the Migration Sedimentation Chamber (MSC). 1) Calcium-free media (1.5 ml) is pipetted into the well of the MSC, thawed semen is layered around the gallery and the tube is incubated at 39 °C for 1-2 hours to allow vigorous motile sperm to swim-up and be separated from non-motile or abnormal sperm. 2) Media is aspirated from the bottom of the MSC and centrifuged to remove immotile sperm. Figure adapted from Research Instruments Ltd., (2010).

Oocyte preparation

Oocytes were retrieved from maturation media and washed twice through 0.5 ml of G-MOPS™ Plus (Vitrolife AB, Göteborg, Sweden). Following which, embryos were washed in G-IVF™ Plus (Vitrolife AB, Göteborg, Sweden) supplemented with HSA and a preparation of hypotaurine, heparin and penaciliamine, before transfer within

10 µl into equilibrated fertilisation drops and incubated at 39 °C and 5 % CO_2 until sperm preparation was complete.

Fertilisation

Following centrifugation, the supernatant was removed and discarded and 10 µl of G-IVF™ Plus (Vitrolife AB, Göteborg, Sweden) for each fertilisation drop prepared was added to the pellet. After mixing thoroughly, 5 µl of the sperm preparation was added to 95 µl of distilled water (creating a 1 in 20 dilution) inducing osmotic stress and allowing a sperm count to be conducted. The number of immobilised sperm was counted in 5 squares of a Neubauer Improved haemocytometer, and the sperm concentration was determined using the following equation:

$$Sperm\ concentration = \frac{total\ number\ of\ sperm\ counted}{5} \times 20 \times 10^4$$

Where '5' was the number of squares counted, '20' was the dilution of sperm in distilled water and '10^4' was the volume of the haemocytometer.

Once calculated, the volume of G-IVF™ Plus (Vitrolife AB, Göteborg, Sweden) required to dilute the sperm preparation to 5 x10^6 ml^{-1} was determined using the following equation:

$$\frac{sperm\ concentration\ (10^7)}{5 \times 10^6}$$

This produced a dilution factor that was then multiplied by the volume of G-IVF™ Plus (Vitrolife AB, Göteborg, Sweden) added earlier to the sperm preparation. Sperm preparation (10 µl) was next added to the equilibrated fertilisation drops producing a final sperm concentration of 1 x10^6 ml^{-1}, and the fertilisation dish was incubated for 24 h at 39 °C and 5 % CO_2. The final volume of fertilisation drops was 50 µl.

2.3 *In vitro* culture

Following a 24 h incubation period, putative zygotes (PZ) were removed from fertilisation drops and washed twice in G-MOPS™ Plus (Vitrolife AB, Göteborg,

Sweden) to remove remaining cumulus cells. From here, PZ were washed through a micro-drop of BBH7 (BoviPro™, Minitube of America, Verona WI, USA) and then transferred to equilibrated micro-drops of BBH7, maintaining a ratio of 1 PZ:2 µl of media. PZ were divided equally into two groups; the 'no change' group were to remain in the same micro-drops of BBH7 for the entirety of the 7 d culture, and the 'change' group were to be transferred into a fresh micro-drop of BBH7 at 48 hour intervals following transfer into initial culture drops. Simultaneously, a dish containing 4 micro-drops of BBH7 only (i.e. no PZ), was also incubated alongside both treatment groups to be sampled with the 'change' group at 0, 48, 96, and 144 h time points to provide a baseline for comparison.

Cleavage assessment and sampling after 48 h of culture

Embryo cleavage was assessed 48 h post fertilisation in both the 'change' and 'no change' groups. Un-cleaved embryos were removed in enough media to maintain the 2:1 ratio for remaining embryos. Embryos in the 'no change' groups were returned to the incubator (39 °C, 5 % CO_2, 5 % O_2) for a further 96 h. Embryos from the 'change' group were transferred through two equilibrated wash drops and into fresh equilibrated micro-drops of BBH7 (BoviPro™, Minitube of America, Verona WI, USA), the volume of which was subsequently altered to maintain the 2:1 ratio. Following which, the embryos were incubated (39 °C, 5 % CO_2, 5 % O_2) for a further 48 h, and the initial micro-drops of BBH7 were sampled and immediately preserved in liquid nitrogen (LN_2) (approximately -196 °C) (also known as 'snap-freezing'), and stored long-term at -80 °C.

Sampling of spent media following a further 48 h of culture

After a further 48 h incubation, the 'change' group of embryos were transferred through two wash drops into fresh micro-drops of BBH7 (BoviPro™, Minitube of America, Verona WI, USA) and incubated (39 °C, 5 % CO_2, 5 % O_2) for a further 48 h. The micro-drops of BBH7 from which the embryos were transferred were sampled, snap-frozen and transferred to long-term storage at -80 °C). The 'no change' group of embryos remained in the incubator and were untouched.

Final assessment and sampling

'Change group' - After a final incubation period of a further 48 h, the number of developed embryos and their stage of development (according to the International Embryo Transfer Society; Table 2.2) was recorded; spent media was sampled and snap-frozen along with the 4th control drop. 'No change group' - embryos were counted and the stage of development recorded (Table 2.2); spent media was sampled and snap-frozen.

Table 2.2: Embryo grading according to the International Embryo Transfer Society.

TE = trophectoderm; ICM = inner cell mass; ZP = zona pellucida

Code	Stage	Morphological description
3	Early morula	16-32 non-compacted blastomeres.
4	Morula	32-64 smaller compacted blastomeres.
5	Early blastocyst	Blastocoel < 50 %.
6	Blastocyst	Blastocoel > 50 %; TE and ICM discernible.
7	Expanding blastocyst	Diameter of embryo increases; ZP thinner.
8	Hatching blastocyst	Rupture of ZP; partially hatched embryo.
9	Hatched blastocyst	Fully hatched embryo; further increasing in sized.

Subsequently, the ZP was removed from all embryos using proteinases (2 mg ml^{-1} Pronase in PBS; Sigma-Aldrich, Dorset, UK) (Velásquez *et al.,* 2013) and embryos were stored individually at -20 °C until time of analysis.

2.4 Polymerase chain reaction

The sex of bovine blastocysts was determined using PCR; DNA from bovine embryos was amplified using two sets of bovine sexing primers and subsequently separated by gel electrophoresis. The PCR products were then detected by Safe-Red™ (ABM, Richmond BC, Canada) staining and imaging under UV light.

Digest

In order to isolate DNA for PCR, 10 µl of 0.2 mg ml^{-1} proteinase K buffer (Sigma-Aldrich, Dorset, UK) (pH 8.4) was added to each embryo (previously stored individually at -20 °C in 1 µl of sterile PBS). The embryos were then incubated overnight at 37 °C to allow for histone breakdown and release of DNA (Bondioli *et al.,* 1989). The following day residual proteinase K was inactivated by heating the embryos to 95 °C for 10 mins.

PCR reaction mix

ImmunoMix Red (Bioline, London, UK) is a prefabricated heat-activated reaction-mix requiring the addition of water, primers and DNA. Use of ImmunoMix Red (Bioline, London, UK) minimises both the risk of contamination and preparation time. Furthermore, as no additional loading buffer is required (due to the density of the inert red dye present within) greater reproducibility is ensured.

A PCR Master Mix was set-up as follows; per sample, to 10 µl of ImmunoMix Red (Bioline, London, UK) 7.8 µl of water, 0.1 µl of both the forward and reverse bovine specific primers (BSP) (chromosome X), and 0.5 µl of both forward and reverse sex-determining region Y (SRY) primers (chromosome Y) (Table 2.3 & 2.4) were added. Following a thorough mix, 19 µl of this Master Mix was added to 0.2 µl PCR tubes (Fisher Scientific, Loughborough, Leicestershire, UK), and 1 µl of digested DNA per embryo was added (i.e. DNA from one embryo per tube).

Table 2.3: Bespoke primers used for gender differentiation of bovine embryos (Sigma-Aldrich, Dorset, UK).

SA = size of amplicon; AN = accession number; bp = base pairs; BSP = bovine specific primer; FP = forward primer; RP = reverse primer; SRY = sex-determining region Y.

Primer	Primer sequence	SA	AN
BSP FP1	5'-TTTACCTTAGAACAAACCGAGGCAC-3'	538 bp	X00979
BSP RP1	5'-TACGGAAAGGAAAGATGACCTGACC-3'		
SRY FP6022	5'-TGAAACAAGACCAAAACCGGG-3'	339 bp	EU581861
SRY RP6360	5'-TCCATGGACTTGCTCTACTGT-3'		

Table 2.4: Amount of each component added per embryo to create a master mix for the polymerase chain reaction.

BSP = bovine specific primer; DNA = deoxyribonucleic acid; FP = forward primer; RP = reverse primer; SRY = sex-determining region Y.

Component	Single Reaction
	Volume (µl)
Immunomix Red	10
BSP FP1	0.1
BSP RP1	0.1
SRY FP6022	0.5
SRY RP6360	0.5
Water	7.8
DNA	1.0
Total volume	20

PCR

DNA was amplified using a thermocycler (Mastercycler EP Gradient S; Eppendorf, Hamburg, Germany). A 'hot start' (designed to initially reduce non-specific amplification) to activate the ImmunoMix Red (Bioline, London, UK), was followed by 40 cycles of denaturation, primer annealing and extension. Following which, a final extension step was included to ensure that any single stranded DNA remaining following the final PCR cycle was fully extended (summarised in Table 2.5).

Table 2.5: Conditions for the polymerase chain reaction used to amplify a bovine deoxyribonucleic acid (DNA) for the purpose of gender differentiation. Following a hot start, DNA was denatured and subjected to 40 cycles of exponential amplification, followed by a final protracted extension step.

Step	Temperature (°C)	Duration	
Hot start	95	10 minutes	x1
Denaturation	94	1 minute	x40
Primer annealing	55	1 minute	
Primer extension	72	1 minute	
Final extension	94	1 minute	x1
	55	1 minute	
	72	7 minutes	

Upon completion, the PCR products (10 µl) were loaded into a 1.6 % agarose + Safe Red™ (ABM, Richmond BC, Canada) gel, separated by gel electrophoresis (100 volts for approximately 25-30 mins), and imaged under UV light (Fig. 2.2).

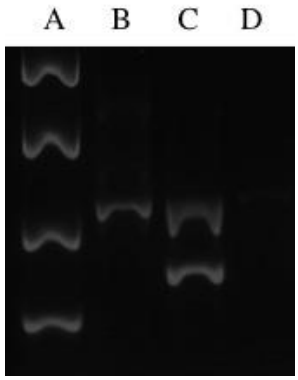


Figure 2.2: Example of a gender differentiation achieved using polymerase chain reaction followed by separation using gel electrophoresis and imaged under ultra violet light. (A) 100 base pair marker; (B) female positive control (538 base pairs); (C) male positive control (339 base pairs); (D) negative control (nuclease-free water).

2.5 Ammonia assay

The ammonia concentration of spent culture media was determined using an enzymatic UV method (Cat. No. AM 3979; Randox Laboratories Ltd., Belfast, UK), on a clinical chemistry auto-analyser (RX Imola; Randox Laboratories Ltd., Belfast, UK). Although this kit was designed for the determination of ammonia concentrations in plasma, it has previously been altered to measure ammonia concentrations in ruminal fluid (Vargas-Bello-Pérez, 2011). The principle of the assay is as follows; in the presence of glutamate dehydrogenase (GLDH), ammonia combines with α-ketoglutarate and NADPH producing glutamate and NADP+; measured proportionally as a corresponding decrease in absorbance at 340 nm. The assay was calibrated using a multi-calibrator supplied by Randox (MC1382; Randox Laboratories Ltd., Belfast, UK), and both ammonia supplied by Randox (Randox Laboratories Ltd., Belfast, UK) and unconsumed BBH7 spiked with ammonia were used as controls (Table 2.6). The assay was performed in singlicate to the manufacturer's specifications and the intra-assay % CV was <10 %.

Table 2.6: Ammonia controls and intra-assay % coefficient of variation (%CV).

r = range

Control	Target (µmol/l)	Manufacturer code	Intra-assay % CV
Ethanol Ammonia 1	55.0 r: 44-66	EA1366	2.8
Ethanol Ammonia 2	167.0 r: 134-200	EA1367	7.4
BBH7 + Ethanol Ammonia 2	76.7 r: 67-80	N/A	4.4

2.6 Statistical analysis

All statistical procedures were conducted using Genstat release 12 (Genstat 12, VSN International, Hemstead, UK). The proportions of oocytes that cleaved, and blastocysts of cleaved, were analysed using Generalised Linear Mixed (GLM) models, where experimental occasion and treatment (media change *vs.* no media

change) formed the fixed effect, and culture plate (two media drops per plate) formed the random effect. These models assumed binomial errors and used logit-link functions. Zygote development at 48 h post-insemination (i.e. number of 2, 4, >4 cells), and embryo development on Day 7 (i.e. number of morulae, expanding, expanded, hatching and hatched blastocysts), were also analysed using this Restricted Maximum Likelihood Model (REML) but, on these occasions, the models assumed Poisson errors and used log-link functions. Data are presented as back-transformed predicted means ± SEMs.

Media ammonia concentrations were analysed by analysis of variance (ANOVA). For the comparison of media changes at 48 h intervals to that of basal media, the variation associated with the 3 degrees of freedom for 0 *vs.* 48 *vs.* 96 *vs.* 144 h were partitioned into the following orthogonal contrast (i.e. 0 h *vs.* media change). Data are presented as predicted means ± SEMs.

Finally, the proportion male and female blastocysts were analysed using the Chi-Square test.

Chapter 3: Results

3.1 Post-fertilisation embryo development

Follicles (4-9 mm in diameter) were aspirated from approximately 240 ovaries of Holstein, Holstein-Friesian, and Dexter cows. In total, 566 oocytes were fertilised and cultured for this experiment. These were allocated to the 'change' (n= 288) and 'no-change' (n= 278) treatment groups and cultured for up to 7 days. In the 'no-change' group, a total of 133 embryos (mean 33.3; 49.1 %) had cleaved by 48 h post-fertilisation. Of those cleaved a total of 29 (mean 7.3; 24.3 %) developed to the blastocyst stage. Within the 'change' group, a total of 119 embryos (mean 29.8; 44.1 %) had cleaved by 48 h post-fertilisation. Of those cleaved a total of 31 (mean 7.8; 24.5 %) developed to the blastocyst stage. There was no significant effect of treatment on cleavage or blastocyst rate (Fig. 3.1).

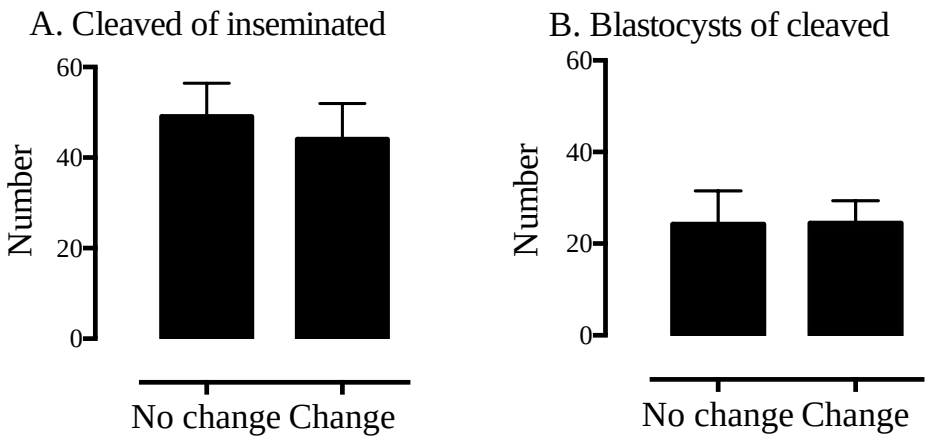
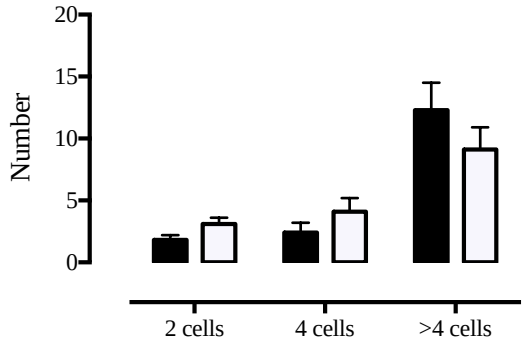


Figure 3.1: Effects of 48 h media change on post-fertilisation embryo development. (A) Cleaved of inseminated; (B) Blastocysts of cleaved. Data presented here were calculated as back transformed means and plotted ± SEM.

There was no significant difference in the number of cells of cleaved embryos between groups by 48 h (Fig. 3.2 A); nor was there a significant difference in the stage of embryo development at final assessment (Day 7) (Fig. 3.2 B).

A. Number of 2, 4 and >4 embryos at 48 h



B. Stage of blastocyst development

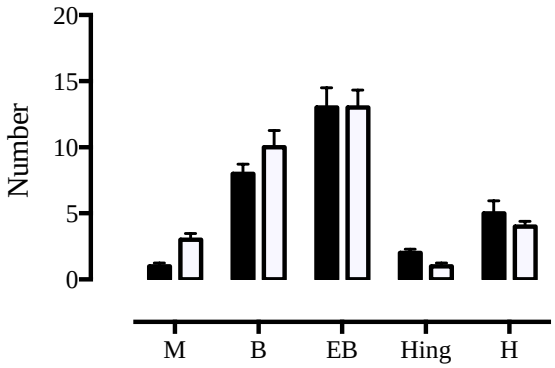


Figure 3.2: Average number of cells (± SEM) at cleavage and final stage of blastocyst development in the 'no change' (black) and 'change' (white) groups. (A) Number of 2, 4 and >4 cell embryos after 48 hours of culture; (B) Stage of blastocyst development at final assessment.

M = morula; B = blastocyst; EB = expanded blastocyst; Hing = hatching; H = hatched.

3.2 Ammonia

Ammonia concentrations measured in basal media significantly increased (P=0.014) over time (Fig. 3.3 A) in the absence of embryos. Ammonia concentrations in spent culture media after each 48 h change differed significantly from 0 h but not from each other (P=0.005) (Fig. 3.3 B). After 144 h culture, ammonia concentrations were significantly higher in the 'no change' group compared to the 'change' group (P=0.048) (Fig. 3.3 C).

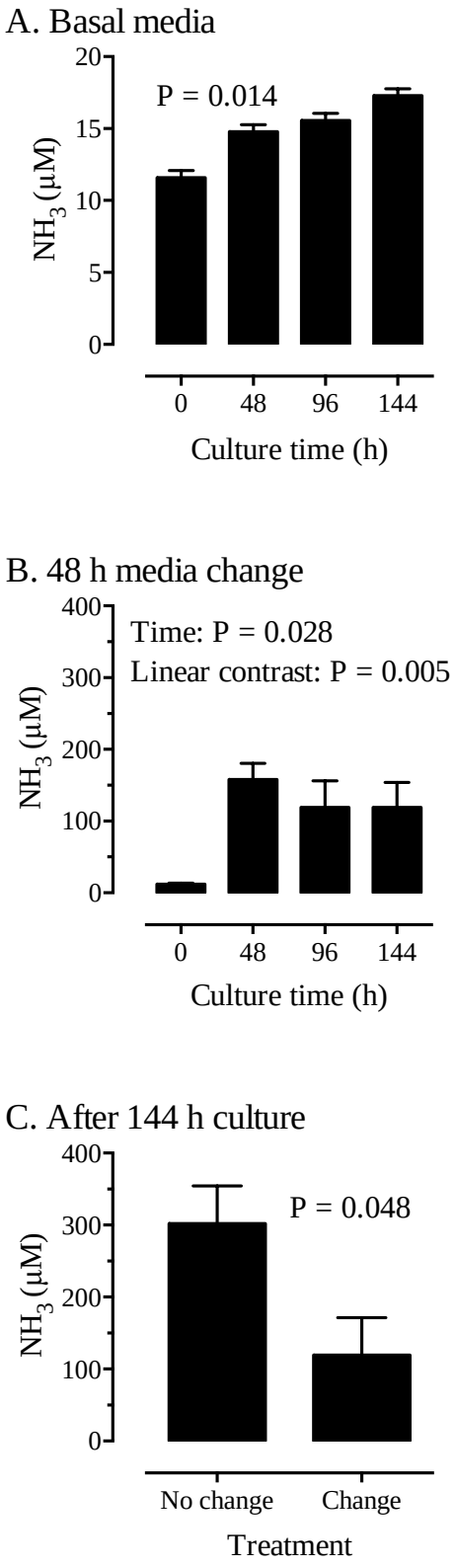


Figure 3.3: Ammonia concentrations (± SEM) in spent culture media. (A) Concentrations in basal BBH7 with no embryos after various periods of culture; (B) Concentrations after various periods of culture with embryos and with media change every 48 h; (C) Concentrations after 144 h culture with embryos and without media change at 48 h intervals.

3.3 Sex ratio

Approximately two thirds of all embryos, irrespective of treatment, were male. There was no significant difference in sex ratio between groups (Fig 3.4).

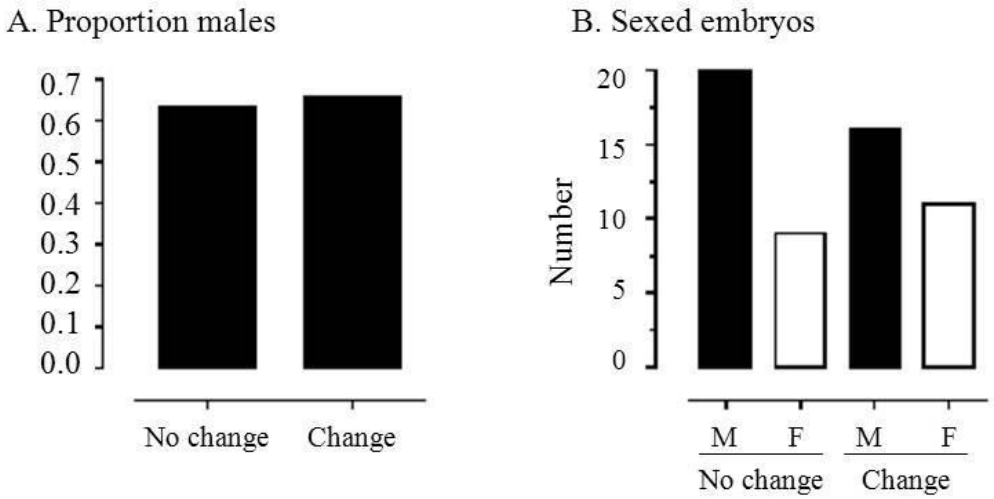


Figure 3.4: Effects of 48 h media on change sex ratio. (A) Proportion male embryos; (B) Absolute numbers of male and female embryos.

Chapter 4: Discussion

Catabolism of amino acids by the embryo produces ammonia, known to impair embryo development by disrupting intracellular pH, ICM development, blastocyst cell number, gene expression and embryo metabolism (Lane & Gardner, 2003). Developments in embryo culture systems have recognised the need to transfer embryos in extended culture from a primary, to a secondary media better designed to cater to changing metabolic requirements (Gardner, 1998). Currently, commercial IVP of bovine embryos is performed using a continuous culture system, and it was the aim of this thesis to elucidate whether a change of culture media would enhance production.

The main findings of this thesis are summarised as follows:

- There was a significant increase in the ammonia concentration of basal media over time.
- In the 'change' group, there was a significant increase in the ammonia concentrations between 0 h and each 48 h media change, but not between 48 h media changes.
- At final assessment, the average ammonia concentration of the 'no change' group was significantly higher than the 'change' group.
- There was no significant effect on the number of blastocysts developed, or on final stage of development reached, in the 'no change' *vs.* 'change' group.
- There was no significant difference in proportion of male embryos or absolute number of males and females in the 'no change' *vs.* 'change' group.

4.1 Post-fertilisation embryo development

Cleavage rates ranged from an average of 41.1% (change group) to 49.1% (no change group); noticeably lower than those previously reported in both a similar culture system (BBH7; 80.1%) and modified synthetic oviduct fluid (80.0%) (Block *et al.,* 2010). There are a number of potential factors (both physiological and methodological) that may have resulted in low cleavage rates.

As the oocytes were collected from abattoir-derived ovaries, there was great variability in the age and breed of cows (Holstein, Holstein-Friesian, and Dexter). It is also likely that oocytes were collected from animals at different stages of the oestrous cycle. It has been reported that the late luteal and early follicular phase of the oestrous cycle is the optimal point for oocyte recovery, yielding a greater number of morphologically normal and meiotically competent oocytes (Hagemann *et al.,* 1999; Machatkova *et al.,* 2008).

Furthermore, follicle size has been shown to have an effect on oocyte competence. Although meiotic maturation of bovine oocytes occurs spontaneously following oocyte retrieval, developmental competence of oocytes has been shown to increase with increasing follicle size, and only one-third of oocytes go on to produce viable embryos (Lonergan *et al.*, 1994). Importantly, oocytes collected from small follicles (<3 mm) are known to be cytoplasmically deficient (Marchal *et al.,* 2008), and unable to accumulate the glutathione needed for final cytoplasmic and nuclear maturation (Ali, 2007).

Moreover, there is a documented effect of dominant follicle presence on the developmental competence of oocytes within subordinate follicles (Hagemannn, 1999). A significant increase in the proportion of apoptotic cells within subordinate follicles in the dominant *vs.* the growing phase has been identified (Hagemann, 1999, Hagemann *et al.,* 1999). This may be the result of decreasing follicle stimulating hormone (FSH) concentrations sustained by elevated oestradiol and inhibin concentrations from the newly selected dominant follicle, causing subordinate follicles to enter atresia and regress before the next wave of follicle growth. Thus, if

oocytes were collected from cows during the mid to late follicular phase and the early luteal phase, it may have adversely affected fertilisation and cleavage rates.

Elevated temperatures during the summer months are a well-documented cause of decreased fertility in cattle (Al-Katanani, Paula-Lopes & Hansen, 2002; West, 2003; Collier, Dahl & VanBaale, 2006). A comprehensive review by De Rensis & Scaramuzzi (2003) identifies the two major pathways by which heat-stress leads to infertility; 1) Increased body temperature is accompanied by increased lethargy and a compromised uterine environment, consequentially oestrus is harder to detect, fewer cows are inseminated, and mistimed inseminations result in reduced pregnancy rates. 2) Increased body temperature results in a reduction in appetite causing cattle to enter a state of negative energy balance (NEB), leading to a hormone and growth factor imbalance, which in turn compromises oestrus detection, results in fewer inseminations, more mistimed inseminations, and an increase in poor quality oocytes. Thus, if this experiment was to be repeated in cooler months (i.e. October-March), a higher cleavage rate may have been observed.

In this study, oocytes selected for *in vitro* maturation (IVM) were chosen based on their morphology. Bovine oocytes have dark cytoplasm (due to a high lipid content; Nagano, Katagiri & Takahashi, 2006) that, when examined by light microscopy, may impair assessment (i.e. identification of granularity and vacuoles). Furthermore, bovine oocytes are often shrouded in a mass of equally dark cumulus cells, again causing complications in the identification and assessment of viable COCs. Brilliant cresyl blue (BCB) is a supravital stain that may be used to assess the developmental competence of oocytes (Wu *et al.,* 2007). Glucose-6-phosphate dehydrogenase (G6PDH) is a cytosolic enzyme involved in the PPP (Fig. 1.4; page 10), G6PDH catalyses the following reaction:

$$G6P + NADP^{+} \leftrightharpoons 6\text{-phospho-D-glucono-1,5-lactone} + NADPH + H^{+}$$

Consequently, BCB is reduced to a colourless substance and can, therefore, be used as a non-invasive indicator of intracellular activity (Goovaerts *et al.,* 2010). G6PDH activity in competent oocytes is low (Torner *et al.,* 2008) and as such BCB would retain its blue colour (and facilitate selection for IVM); *vice versa,* G6PDH is high in

underdeveloped oocytes (Torner *et al.,* 2008), thus incompetent oocytes would reduce the BCB to colourless. However, selection using BCB requires a 90 minute incubation period, which may in itself affect developmental competence (Vandaele, 2008), and not all authors agree that BCB facilitates selection of developmentally competent oocytes to a greater extent than morphological assessment (For: Alm *et al.,* 2005; Heleil *et al.,* 2010; Su *et al.,* 2012; Castaneda *et al.,* 2013 - Against: Ishizaki *et al,* 2009).

4.2 Effects of increasing ammonia concentrations on embryo development

The effect of ammonia on embryo development during IVC varies according to the developmental stage of the embryo at exposure, the duration of exposure, and ammonia concentration (Hammon, Wang & Holyoak, 2000). Relatively little is known about the metabolism of ammonia by embryos. As previously mentioned, in adults ammonia is metabolised to urea via the urea cycle in the liver (predominantly) and kidneys (to a lesser extent) then excreted; however, no such system has been identified in bovine embryos (Orsi & Leese, 2004). Instead it has been hypothesised that pyruvate (following transamination to alanine) is used as an "ammonia sink" (Partridge & Leese, 1996; Donnay, Partridge & Leese, 1999; Orsi & Leese, 2004), thereby preventing the accumulation of intracellular ammonia, particularly in serum-free media (Thompson, 2000). This theory is consistent with a reported and sustained rise in alanine concentrations during embryo culture (Partridge & Leese, 1996; Houghton *et al.,* 2001), as well as data that indicate a synergistic effect on blastocyst development in embryos cultured in a chemically defined media supplemented with alanine (Moore & Bondioli, 1993; Lee & Fukui, 1996; Thompson, 1996).

The pathways by which ammonia effects embryo development (positively and negatively) remain, as yet, unclear; however, a number of theories have been hypothesised; Gardner & Lane, (1993) suggested that ammonia facilitated the conversion of α-ketoglutarate to glutamate rather than succinyl co-A (Fig. 1.5, page 11), decreasing TCA cycle activity and consequentially reducing ATP production. Building on this theory, Hammon, Wang & Holyoak, (2000) suggested that this decrease in ATP post-compaction inhibits blastocyst development and increases the

proportion of degenerate ova. Accumulation of ammonia is also hypothesised to occur in the blastocoel fluid through the activity of Na^+/K^+ ATPase and $Na^+/K^+/2Cl^-$ co-transporters (Gardner & Lane, 1993; Hammon, Wang & Holyoak, 2000; Orsi & Leese, 2004).

In this study, ammonia concentrations in basal media increased significantly over time, most likely due to the spontaneous deamination of AAs present in BBH7. Although the exact formulation of BBH7 remains undisclosed by manufacturer BoviPro™, these data are consistent with a study by Berg *et al.,* (2002), who also reported a spontaneous increase in ammonia in protein-free (i.e. serum-free) culture in the absence of embryos. Following 144 h of continuous culture, in this experiment, ammonia concentrations were consistent with those reported by Gardner (1994), and significantly greater than refreshed culture. Although exposure to ammonia during the preimplantation period is known to affect embryo development, there was no significant difference in embryo quantity, quality or final developmental stage in this study; inconsistent with findings of Hammon, Wang & Holyoak (2000).

Furthermore, high yielding dairy cows require greater protein supplementation in the diet in order to sustain milk production. Ruminants require dietary protein to both satisfy rumen microorganisms and for breakdown to AA nitrogen at the tissue level (McDonald *et al.,* 2002). High-protein diets in dairy cows have been associated with a reduction in fertility (Dobson *et al.,* 2007) largely due to increased ammonia concentrations (Sinclair, Sinclair & Robinson, 2000; Boland, Lonergan & O'Callaghan, 2001). As well as altering the uterine, oviductal and intra-follicular environment, this increased dietary derived ammonia is also responsible for perturbed maturation, fertilisation and cleavage of bovine oocytes (Sinclair *et al.,* 2000; Leroy *et al.,* 2008). Therefore, the metabolic status of an animal is an important consideration when retrieving oocytes for commercial bovine IVP.

4.3 Sex ratio

Bovine IVP is increasingly employed to maximise on-farm production, specifically within the dairy sector. As most farms operate a 'closed-herd' policy in order to minimise the risk of disease-entry (i.e. a herd in which no outside breeding stock is introduced), female offspring are desired to serve as replacement animals for those reaching the end of their working life. Natural mating yields only a 50 % chance of female offspring, hampered further by the birth of freemartins (i.e. female-like offspring born with masculinised behaviour and non-functioning ovaries, due usually to exposure to anti-Müllerian hormone (AMH) from a male twin *in utero*). Thus, IVP (with or without sexed-semen) is an efficient way to safely guarantee offspring of a desired sex.

Importantly, a skew in male:female sex ratio under certain bovine embryo culture conditions has been reported (Iwasaki *et al.,* 1988; Bondioli *et al.,* 1989; Gutiérrez-Adán *et al.,* 2001a); culture media supplemented with serum has been demonstrated to skew the sex ratio in favour of males (Gutiérrez-Adán *et al.,* 2001b). The results from this study indicate that, despite two thirds of all embryos identified as male, there was no significant difference between continuous or refreshed serum-free culture on sex ratio, consistent with a study by Grisart *et al.,* (1999). As the majority of clients employing IVP technology do so in order to produce genetically preferential female replacements, it is vital that laboratory protocols are optimised to avoid an unfavourable skew in sex ratio.

4.4 Limitations of this thesis

Due to the time constraints of this project it was not possible to analyse the concentrations of AAs in spent culture media. As ammonia concentrations differed significantly between groups 144 h after initial culture was established, it is likely that similar differences in the concentration of certain AAs would also have been observed. Two AAs of particular interest would have been glutamine and alanine, as both have previously been hypothesised to neutralise the toxic effects of ammonia in the absence of a urea cycle (Partridge & Leese, 1996; Donnay, Partridge & Leese, 1999; Orsi & Leese, 2004). If elevated concentrations of alanine and glutamine were to be identified in the 'no change' *vs.* the 'change' group, this data may provide

some support to this theory. Currently, spent media (stored at -80 °C) is awaiting analysis by GCMS to determine AA concentrations.

Only semen from one bull was used to fertilise all of the oocytes cultured in this study, thus it is impossible to rule out any bull-effect on the results. If time had allowed, the experiment would have been repeated at least twice using semen from at least two different bulls in order to block for bull-effect during the statistical analysis of the data, thereby reducing variability within the data and increasing overall precision.

4.5 Final conclusions

Although there is reported evidence of the detrimental effects of ammonia accumulation on preimplantation embryo development during continuous bovine blastocyst culture, these data suggest there is currently no benefit in refreshing serum-free culture media at 48 h intervals. Equally, there was no consequential benefit to refreshing culture media on sex ratio skew (in favour of females). However, the spontaneous breakdown of AAs in serum-free media may compromise embryo development by increasing circumjacent ammonia concentrations.

Bibliography

ABE, H., HOSHI, H. 2003. Evaluation of bovine embryos produced in high performance serum-free media. *Journal of Reproduction & Development.* 49 (3): 193-202.

ABE, H., YAMASHITA, S., SATOH, T., HOSHI, H. 2002. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free of serum-containing media. *Molecular Reproduction & Development.* 61 (1): 57-66.

AGUILAR, J., REYLEY, M. 2005. The uterine tubal fluid: secretion, composition and biological effects. *Animal Reproduction.* 2 (2): 91-105.

AL-KATANANI, Y.M., PAULA-LOPES, F.F., HANSEN, P.J. 2002. Effect of season and exposure to heat stress on oocyte competence in Holstein cows. *Journal of Dairy Science.* 85 (2): 390-396.

ALEXIOU, M, LEESE, H.J. 1992. Purine utilisation, de novo synthesis d degradation in mouse preimplantation embryos. *Development.* 114: 185-192.

ALI, K.M., ABDALLA, S.M., KANDEIL, M.A., AMIN, K.A. 2007. *Impact of exogenous glutathione during in vitro maturation, fertilisation and development of buffalo oocytes*. Ras El Bar 25-28 July 2007. Society of physiological sciences & their applications.

ALM, H., TORNER, H., LÖHRKE, B., VIERGUTZ, T., GHONEIM, I.M., KANITZ, W. 2005. Bovine blastocyst development rate in vitro is influence by selection of oocytes by brilliant cresyl blue staining before IVM as indicator for glucose-6-phosphate dehydrogenase activity. *Theriogenology.* 63 (8): 2194-2205.

ALLEN, R.L., WRIGHT, R.W., Jr. 1984. In vitro development of porcine embryos in co-culture with endometrial cell monolayers or culture supernatants. *Journal of Animal Science.* 59: 1657-1661.

BAHAT, A., EISENBACH, M. 2006. Sperm thermotaxis. *Molecular and Cellular*

Endocrinology. 252 (1-2): 115-119.

BALTZ, J.M. 2012. Media composition: salts and osmolarity. *Methods in Molecular Biology*. 912: 61-80.

BALTZ, J.M., BIGGERS, J.D., LECHENE, C. 1991. Two-cell stage mouse embryos appear to lack mechanism for alleviating intracellular acid loads. *Journal of*

BAUMANN, C.G., MORRIS, D.G., SREENAN, J.M., LEESE, H.J. 2007. The quiet embryo hypothesis: Molecular characteristics favoring viability *Molecular Reproduction & Development* 74:1345-1353.

BAVISTER, B.D. 2000. Interactions between embryos and the culture milieu. *Theriogenology*. 53: 619–626.

BEHBOODI, E., ANDERSON, G.B., BONDURANT, R.H., CARGILL, S.L., KREUSCHER, B.R., MEDRANO, J.F., MURRAY, J.D. 1995. Birth of large calves that developed from *in vitro* derived bovine embryos. *Theriogenology.* 44: 227-232.

BERG, D.K., BEAUMONT, S.E., THOMPSON, J.G., PHUNG, H.T., DUNLOP, J. 2002. Exogenous protein reduces ammonium influx from in vitro-produced bovine blastocysts. *Reproduction.* 66 (Suppl 1): 152-160.

BIGGERS, J.D., WHITTINGHAM, D.G., DONAHUE, R.P. 1967. The pattern of energy metabolism in the mouse oocyte and zygote. *Proceedings of the National Academy of Sciences.* 58: 560–567.

BLOCK, J., BONILLA, L., HANSEN, P.J. 2010. Efficacy of in vitro embryo transfer in lactating dairy cows using fresh or vitrified embryos produced in a novel embryo culture medium. *Journal of Dairy Science.* 93: 5234-5242.

BOICE, M.L., GEISERT, R.D., BLAIR, R.M., VERGHAGE, H.G. 1990. Identification and characterization of bovine oviductal glycoproteins synthesised at oestrus. *Biology of Reproduction.* 43: 457-465.

BOLAND, M.P., LONERGAN, P., O'CALLAGHAN, D. 2001. Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. *Theriogenology.* 55: 1323-1340.

BOLTON, V.N., WREN, M.E., PARSON, J.H. 1991. Pregnancies after in vitro fertilization and transfer of human blastocysts. *Fertility & Sterility.* 55: 830-832.

BONDIOLI, K.R., ELLIS, S.B., PRYOR, J.H., WILLIAMS, M.W., HARPOLD, M.M. 1989. The use of male-specific chromosomal DNA fragments to determine the sex of bovine preimplantation embryos. *Theriogenology.* 31 (1): 95-104.

BORLAND, R.M., BIGGERS, J.D., LECHENE, C.P., TAYMOR, M.L. 1980. Elemental composition of fluid in the human Fallopian tube. *Journal of Reproduction & Fertility***.** 58: 479-482.

BRACKETT, B.G., ZUELKE, K.A. 1993. Analysis of factors involved in the *in vitro* production of bovine embryos. *Theriogenology.* 39: 43-64.

BRAUDE, P., BOLTON, V., MOORE, S. 1988. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature.* 332: 459-461.

BREVINI, T.A.L., CILLO, F., ANTONINI, S., TOSETTI, V., GANDOLFI, F. 2006. Temporal and spatial control of gene expression in early embryos of farm animals. *Reproduction, Fertility & Development.* 19 (1): 35-42.

BROWN, B.W. RADZIEWIC, T. 1996. *In vitro* production of sheep embryos and growth rates of resultant lambs *Proceedings of 13th International Congress of Animal Reproduction* **3** P22–16.

BUHI, W.C., ALVAREZ, I.M., KOUBA, A.J. 2000. Secreted proteins of the oviduct. *Cells Tissues Organs.* 166: 165-179.

CAROLAN, C., LONERGAN, P., KHATIR, H., MERMILLOD, P. 1996. In vitro production of bovine embryos using individual oocytes. *Molecular Reproduction & Development.* 45 (2): 145-150.

CASTANEDA, C.A., KAYE, P., PANTALEON, M., PHILLIPS, N., NORMAN, S., FRY, R., D'OCCHIO, M.J. 2012. Lipid content, active mitochondria and brilliant cresyl blue staining in bovine oocytes. *Theriogenology.* 79 (3): 417-422.

CATT, J.W. 1994. Bovine embryo co-culture. *Cell Biology International.* 18 (12): 1155-1162.

COLLADO-FERNANDEZ, E., PICTON, H.M., DUMOLLARD, R. 2012. Metabolism through follicle and oocyte development in mammals. *International Journal of Developmental Biology.* 56: 799-808.

COLLIER, R.J., DAHL, G.E., VANBAALE, M.J. 2006. Major advances associated with environmental effects on dairy cattle. *Journal of Dairy Science.* 89 (4): 1244-1253.

DAN-GOOR, M., SASSON, S., DAVARASHVILI, A., ALMAGOR, M. 1997 Expression of glucose transporter and glucose uptake in human oocytes and preimplantation embryos. *Human Reproduction.* 12: 2508-2510.

DAWSON, K.M., COLLINS, J.L., BALTZ, J.M. 1998. Osmolarity dependent glycine accumulation indicates a role for glycine as an organic osmolyte in early preimplantation mouse embryos. *Biology of Reproduction.* 59: 225-232.

DE RENSIS, F., SCARAMUZZI, R.J. 2003. Heat stress and seasonal effects on reproduction in the dairy cow – a review. *Theriogenology.* 60 (6): 1139-1151.

DICKENS, C.J., MAGUINESS, S.D., COMER, M.T., PALMER, A., RUTHERFORD, A.J., LEESE, H.J. 1995. Human tubal fluid: formation and composition during vascular perfusion of the fallopian tube. *Human Reproduction.* 10 (3): 505-508.

DOBSON, H., SMITH, R.F., ROYAL, M.D., KNIGHT, C.H., SHELDON, I.M. 2007. The high producing dairy cow and its reproductive performance. *Reproduction in Domestic Animals.* 42 (Suppl. 2): 17-23.

DONNAY, I., PARTRIDGE, R.J., LEESE, H.J. 1999. Can embryo metabolism be used for selecting bovine embryos before transfer? *Reproduction, Nutrition and Development* 39: 523–533.

DOWNS, S.M., UTECHT, A.M. 1999. Metabolism of radiolabeled glucose by mouse oocytes and oocyte-cumulus cell complexes. *Biology of Reproduction.* 60 (6): 1446-1452.

DUFFY, K.P., ROCHE, J.F., BOLAND, M.P. 1997. Superovulation in cattle: effect of FSG type and method if administration on follicular growth, ovulatory response and endocrine patterns. *Animal Reproduction Science.* 46 (1): 1-14.

DUQUE, P., GÓMEZ, E., DÍAZ, E., FACAL, N., HIDALGO, C., DÍEZ, C. 2003. Use of two replacements of serum during bovine embryo culture in vitro. *Theriogenology.* 59 (3-4): 889-899.

EDWARDS, L.J., WILLIAMS, D.A., GARDNER, D.K. 1998. Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Human Reproduction.* 13 (12): 3441-3448.

EL-BANNA, A.A., HAFEZ, E.S.E. 1970. Profile analysis of the oviductal wall in rabbits and cattle. *The Anatomical Record.* 166: 469-478.

ELLINGTON, J.E. 1991. The bovine oviduct and its role in reproduction: a review of the literature. *Cornell Vet*. 81:313–328.

EPSTEIN, C.J., SMITH, S.A. 1973. Amino acid uptake and protein synthesis in preimplantation mouse embryos. *Developmental Biology.* 33: 171-184.

ESSLEMONT, R.J., KOSSAIBATI, M.A. 2002. The cost of poor fertility and disease in UK dairy herds, trends in DAISY herds over 10 seasons. Daisy Research Report No. 5. Milton Keynes: Intervet UK Ltd.

FARIN, P.W., FARRIN, C.E. 1995. Transfer of bovine embryos and produced *in vivo* or *in vitro*: survival and fetal development. *Biology of Reproduction.* 52: 676-682.

FLORES-FOXWORTH, G., COONROD, S.A., MORENO, J.F., BYRD, S.R., KRAEMER, D.C., WESTHUSIN, M. 1995. Interspecific transfer of IVM IVF-derived red sheep (Ovis orientalis gmelini) embryos to domestic sheep (Ovis aries). *Theriogenology.* 44 (5): 681-690.

FRANCIS, G.L. 2010. Albumin and mammalian cell culture. *Cryotechnology.* 62 (1): 1-16.

FREI, R.E., SCHULTZ, G.A., CHURCH, R.B. 1989. Qualitative and quantitative changes in protein synthesis occur at the 8-16 cell stage of embryogenesis in the cow. *Journal of Reproduction & Fertility.* 86: 637-641.

FRESHNEY, R.I., FRESHNEY, M.G. 2002. *Culture of Specialised Cells: Culture of Epithelial Cells* (Second Edition). New York NY, USA. Wiley-Liss Inc.

GANDOLFI, F., PASSONI, L., MODINA, S., BREVINI, T.A.L., VARGA, Z., LAURIA, A. 1993. Similarity of an oviduct- specific glycoprotein between different

species. *Reproduction Fertility & Development*. 5: 433-443.

GARDNER, D.K. 1994. Mammalian cell culture in the absence of serum or somatic cell support. *Cell Biology International*. 18 (12): 1163-1179.

GARDNER, D.K. 1998. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology* 49: 83-102.

GARDNER, R. L., PAPAIOANNOU, V. E. 1975. Differentiation in the trophectoderm and inner cell mass In: Balls, M., Wild, A.E. (Eds.) 1975. *The Early Development of Mammals* (edit. by Balls, M., and Wild, A. E.). Cambridge, UK. Cambridge University Press pp.107–132.

GARDNER, D.K., LANE, M. 1993. Amino acids and ammonium regulate mouse embryo development in culture. *Biology of Reproduction*. 48: 377-485.

GARDNER, D.K., LANE, M. 1997. Culture and selection of viable blastocysts: a feasible proposition for human IVF. *Human Reproduction Update.* 3: 367-382.

GARDNER, D.K., LANE, M., SCHOOLCRAFT, W.B. Culture systems and blastocyst development. In: Serono Symposia USA, International Symposium on ART and Human Blastocyst; 2000 March 30th – April 2nd: 14.

GIBBS, D., HOLLOWAY, L., GILNA, B., MORRIS, C. 2009. Genetic technologies for livestock breeding: Reconstructing institutional relationships in agriculture. *Geoforum.* 40 (6): 1041-1049.

GÓMEZ, E., RODRÍGUEZ, A., MUÑOZ, M., CAAMAÑO, J.N., HIDALGO, C.O., MORÁN, E., FACAL, N., DÍEZ, C. 2008. Serum free embryo culture medium improves in vitro survival of bovine blastocysts to vitrification. *Theriogenology.* 69 (8): 1013-1021.

GOODHAND, K.L., STAINES, M.E., HUTCHINSON, J.S.M., BROADBENT, P.J. 2000. In vivo oocyte recovery and in vitro embryo production from bovine oocyte donors treated with progestagen, oestradiol and FSH. *Animal Reproduction Science.* 63: 145-158.

GOOVAERTS, I.G.F., LEROY, J.L.M.R., JORSSEN, E.P.A., BOLS, P.E.J. 2010. Non-invasive bovine oocyte quality assessment: possibilities of a single oocyte culture. *Theriogenology.* 74: 1509-1520.

GRISART, B., MASSIP, A., COLLETTE, L., DESSY, F. 1995. The sex ratio of bovine embryos produced in vitro in serum-free oviduct cell-conditioned medium is not altered. *Theriogenology*. 43 (6):1097-1106.

GUERIN, P., EL MOUATASSIM, S., MENEZO, Y. 2001. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Human Reproduction Update.* 7(2): 175-189.

GUTIÉRREZ-ADÁN, A., GRANADOS, J., PINTADO, B., DE LA FUENTE, J. 2001a. Influence of glucose on the sex ratio of bovine IVM/IVF embryos cultured in vitro. *Reproduction, Fertility & Development.* 13 (5-6): 361-365.

GUTIÉRREZ-ADÁN, A., LONERGAN, P., RIZOS, D., WARD, F.A., BOLAND, M.P., PINTADO, B., DE LA FUENTE, J. 2001b. Effect of the in vitro culture system on the kinetics of blastocyst development and sex ratio of bovine embryos. *Theriogenology.* 55 (5): 1117-1126.

GUYADER-JOLY, C., KHATCHADOURIAN, C., MENEZO, Y. 1996. Comparative glucose and fructose incorporation and conversion by in vitro produced bovine embryos. *Zygote.* 4: 85-91.

HAGEMANN, L.J. 1999. Influence of the dominant follicle on oocytes from subordinate follicles. *Theriogenology.* 51: 449-459.

HAGEMANN, L.J., BEAUMONT, S.E., BERG, M., DONNISON, M.J., LEDGARD, A., PETERSON, A.J., SCHURMANN, A., TERVIT, H.R. 1988. Development during single IVP of bovine oocytes from dissected follicles: Interactive effects of oestrous cycle stage, follicle size and atresia. *Molecular Reproduction & Development.* 53: 451-458.

HAMMON, D.S., WANG, S., HOLYOAK, G.R. 2000. Effects of ammonia during different stage of culture on development of in vitro produced bovine embryos. *Animal Reproduction Science.* 59: 23-30.

HANSEN, P.J. 2006. Realising the promise of IVF in cattle – an overview. *Theriogenology.* 65 (1): 119-125.

HARVEY, A. J., KIND, K.L., THOMPSON, J.G. 2002. REDOX regulation of early embryo development. *Reproduction*. 123(4): 479-486.

HASLER, J.F., HENDERSON, W.B., HURTGEN, P.J., JIN, Z.Q., McCAULEY, A.D., MOWER, S.A., NEELY, B., SHUEY, L.S., STOKES, J.E., TRIMMER, S.A. 1995. Production, freezing, and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology.* 42 (1): 141-152.

HELEIL, B., KUZMINA, T., NOVIKOVA, N., TORNER, H., ALH. H. 2010. Effect of prolactin on developmental competence of bovine oocytes selected by brilliant cresyl blue staining. *Journal of Reproduction & Infertility.* 1 (1): 1-7.

HERNÁNDEZ, M.F. 1996. The mammalian oviductal epithelium: regional variations in cytological and functional aspects of the oviductal secretory cells. *Histology & Histopathology.* 11 (3): 743-768.

HESS, A.P., NAYAK, N.R., GIUDICE, L.C. 2006. Oviduct and endometrium; Cyclic changes in the primate oviduct and endometrium. In: Neill, J.D. 2006. *Knobil & Neill's Physiology of Reproduction*. St Louis MO, USA. Elsevier Academic Press.

HOHENBOKEN, W.D. 1999. Applications of sexed semen in cattle production. *Theriogenology.* 52 (8): 1421-1433.

HOUGHTON, F.D., HAWKHEAD, J.A., HUMPHERSON, P.G., HOGG, J.E., BALEN, A.H., RUTHERFORF, A.J., LEESE, H.J. 2001. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Human Reproduction.* 17 (4): 999-1005.

HUGENTOBLER, S.A., MORRIS, D.G., SREENAN, J.M., DISKIN, M.G. 2007. Ion concentrations in oviduct and uterine fluid and blood serum during the oestrous cycle in the bovine. *Theriogenology.* 68 (4): 538-548.

HUGENTOBLER, S.A., HUMPHERSON, P.G., LEESE, H.J., SREENAN, J.M., MORRIS, D.G. 2008. Energy substrates in bovine oviduct and uterine fluid and blood plasma during the estrous cycle. *Molecular Reproduction & Development.* 75: 496-503.

Human Fertilisation and Embryology Authority. 2010. Multiple Births Minimisation Strategy: achievements and compliance [online] available at: http://www.hfea.gov.uk/6195.html (accessed 28th June 2013).

ISHIZAKI, C., WATANABE, H., BHUIYAN, M.M., FUKUI, Y. 2009. Developmental competence of porcine oocytes selected by brilliant cresyl blue and matured individually in a chemically define culture media. *Theriogenology.* 72 (1): 72-80.

IWASAKI, S., SHIOYA, Y., HANADA, A., NAKAHARA, T. 1988. Chromosome preparation for two-cell bovine embryos derived from follicular oocytes fertilised in vivo. *Japanese Journal of Reproduction.* 34: 79-83.

JOHNSON, M.H. 2006. *Essential Reproduction*. Sixth Ed. Oxford, UK. Blackwell Publishing.

JOHNSON, M.H., ZIOMEK, C.A. 1981. Induction of polarity in mouse 8-cell blastomeres: specificity, geometry, and stability. *Journal of Cell Biology.* 91:303-308.

JONES, G.M., TROUNSON, A.O., LOLATIGAS, N., WOOD, C. 1998. Factors affecting the success of human blastocyst development and pregnancy following in vitro fertilization and embryo transfer. *Fertility & Sterility.* 70: 1022-1029.

JONES, G.M., TROUNSON, A.O. 1999. Blastocyst stage transfer: pitfalls and benefits (the benefits of extended culture). *Human Reproduction.* 14: 1405-1408.

KAUPP, U.B., KASHIKAR, N.D., WEYAND, I. 2008. Mechanisms of sperm chemotaxis. *Annual Review of Physiology.* 70: 93-117.

KOUZARIDES, T. 2007. Chromatin modifications and their function. *Cell*. 128: 693-705.

KRISHER, R.L., LANE, M., BAVISTER, B.D. 1999. Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined culture media. *Biology of Reproduction.* 60 (6): 1345-1352.

KRUIP, T.A.M., DEN DAAS, J.H.G. 1997. *In vitro* produced and cloned embryos effects on pregnancy, parturition and offspring. *Theriogenology.* 47: 43-52.

LANE, M., GARDNER, D.K. 1995. Removal of embryo-toxic ammonium from the culture medium by in situ enzymatic conversion to glutamate. *Journal Experimental Zoology.* 271: 356-363.

LANE, M., GARDNER, D.K. 2003. Ammonium induces aberrant blastocyst differentiation, metabolism, pH regulation, gene expression and subsequently alters fetal development in the mouse. *Biology of Reproduction.* 69 (4): 1109-1117.

LANE, M., GARDNER, D.K., HASLER, M.J., HASLER, J.F. 2003. Use of G1.2/G2.2 media for commercial bovine embryo culture: equivalent development and pregnancy rates compared to co-culture. *Theriogenology*. 60 (3): 407-419.

LANE, M., GARDNER, D.K. 2007. Embryo culture medium: which is best? *Best Practice & Research - Clinical Obstetrics & Gynaecology.* 21 (1): 83-100.

LAZARRI, G., WRENZYCKI, C., HERRMANN, D., DUCHI, R., KRUIP, T., NIEMANN, H., GALLI, C. 2002. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. *Biology of Reproduction.* 67 (3): 767-775.

LEARY, C.L., SMITH, D.G., LEESE, H.J., STURMEY, R.G. 2012. Amino acid turnover as a biomarker of embryo viability. IN: Nagy, Z.P., Varghese, A.C., Agarwal, a. (Eds.) 2012. Practice Manual of In Vitro Fertilisation. London. Springer Science+Business Media. pp. 431-438.

LEE, E.S., FUKUI, Y. 1996. Synergistic effect of alanine and glycine on bovine embryos cultured in a chemically defined medium and amino acid uptake by vitro-produced bovine morulae and blastocysts. *Biology of Reproduction.* 55 (6): 1383-1389.

LEESE, H.J. 1988. The formation and function of oviduct fluid. *Journal of Reproduction & Fertility.* 82: 843-856.

LEESE, H.J. 1995.Metabolic control during preimplantation mammalian development. *Human Reproduction Update.* 1:63–72.

LEESE H.J., GRAY, S.M. 1985. Vascular perfusion: a novel means of studying oviduct function. *American Journal of Physiology – Endocrinology & Metabolism.* 248:624-631.

LEESE, H.J. 2002. Quiet please, do not disturb: A hypothesis of embryo metabolism and viability. *Bioessays* 24:845-849.

LEESE, H.J., STURMEY, R.G., BAUMANN, C.G., MCEVOY, T.G. 2007. Embryo viability and metabolism: Obeying the quiet rules. *Human Reproduction* 22:3047-3050.

LEROY, J.L., VAN SOOM, A., OPSOMER, G., GOOVAERTS, I.G., BOLS, P.E. 2008. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in anger? Part II. Mechanisms linking nutrition and reduced oocyte and embryo quality in high-yielding dairy cows. *Reproduction in Domestic Ruminants.* 43 (5): 623-632.

LEQUARRE, A.S., GRISANT, B., MOREAU, B., SCHUURBIERS, N., MASSIP, A., DESSY, F. 1997. Glucose metabolism during bovine preimplantation development: Analysis of gene expression in single oocytes and embryos. *Molecular Reproduction & Development.* 48: 216-226.

LIPPES, J., ENDERS, R.G., PRAGAY, D.A., BARTHOLOMEW, W.R. 1972. The collection and analysis of human fallopian tubal fluid. *Contraception.* 5 (2): 85-103.

LONERGAN, P., MONAGHAN, P., RIZOS, D., BOLAND, M.P., GORDON, I. 1994. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilisation, and culture in vitro. *Molecular Reproduction & Development.* 37: 48-53.

LUCY, M.C. 2007. Fertility in high-producing dairy cows: reasons for decline and corrective strategies for sustainable improvement. *Society of Reproduction & Fertility Supplement.* 64: 237-254.

LYONS, R.A., SARIDOGAN, E., DJAHANBAKHCH, O. 2006. The reproductive significance of human Fallopian tube cilia. *Human Reproduction Update.* 12 (4): 363-372.

McDONALD, P., EDWARDS, R.A., GREENHALGH, J.F.D., MORGAN, C.A. 2002. *Animal Nutrition.* Sixth Edition. Essex, UK. Pearson Education Limited.

McEWEN, S.A., FEDORKA-CRAY, P.J. 2002. Antimicrobial use and resistance in animals. *Clinical Infection & Disease.* 34 (Suppl. 3): S93-S106.

MACHATKOVA, M., HULINSKA, P., HORAKOVA, J., RECKOVA, Z., HANZALOVA, K. 2008. Oestrous cycle stage influences the morphology and maturation of porcine oocytes in vitro. *Veterinarni Medicina.* 53 (2): 70-76.

MACHÁTY, Z., PÁLDI, A., CSÁKI, T., VARGA, Z., KISS, I., BÁRÁNDI, Z., VAJTA G. 1993. Biopsy and sex determination by PCR of IVF bovine embryos. *Journal of Reproduction & Fertility*. 98 (2): 467-470.

MAPLETOFT, R.J., STEWARD, K.B., ADAMS, G.P. 2002. Recent advances in the superovulation in cattle. *Reproduction, Nutrition, Development* 42 (6): 601-611.

MARCHAL, R., VIGNERON, C., PERREAU, C., BALI-PAPP, A., MERMILLD, P. 2002. Effect of follicular size on meiotic and developmental competence of porcine oocytes. *Theriogenology.* 57 (5): 1523-1532.

MÉNÉZO, Y.J., SAKKAS, D., JANNY, L. 1995. Co-culture of the early human embryo: Factors affecting human blastocyst formation in vitro. *Microscopy Research & Technique*. 32: 50-56.

MÉNÉZO, Y.J., SERVY, E., VEIGA, A., HAZOUT, A., ELDER, K. 2012. Culture systems: embryo co-culture. In: Smith, G.D., Swain, J.E., Pool, T.B. (Eds.) 2012. *Embryo Culture: Methods and Protocols*. London, UK. Humana Press, pp. 231-247.

MERCADER, A., GARCIA-VELASCO, J.A., ESCUDERO, E., REMOHÍ, J., PELLICER, A., SIMÓN, C. 2003. Clinical experience and perinatal outcome of blastocyst transfer after co-culture of human embryos with human endometrial epithelial cells: a 5-year follow-up study. *Fertility & Sterility*. 80 (5): 1162-1168.

MERSFELDER, E.L., PARTHUN, M.R. 2006. The tale beyond the tail: histone core domain modifications and the regulation of chromatin structure. *Nucleic Acids Research.* 34 (9): 2653-2662.

MERTON, J.S., DE ROOS, A.P.W., MULLAART, E., DE RUIGH, L., KAAL, L., VOS, P.L.A.M., DIELEMAN, S.J. 2003. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. *Theriogenology*. 59 (2): 651-674.

MILLER, G.F., GLIEDT, D.W., RAKES, J.M., RORIE, R.W. 1994. Addition of penicillamine, hypotaurine and epinephrine (PHE) or bovine oviductal epithelial cells (BOEC) alone or in combination to bovine in vitro fertilization medium increases the subsequent embryo cleavage rate. *Theriogenology*. 41: 689-696.

MOORE, K., BONDIOLI, K.R. 1993. Glycine and alanine supplementation of culture medium enhances development of in vitro matured and fertilized cattle embryos. *Biology of Reproduction.* 48 (4): 833-840.

MOORE, K., THATCHER, W.W. 2006. Major advances associated with reproduction in dairy cattle. *Journal of Dairy Science.* 89: 1254-1266.

MUCCI, N., ALLER, J., KAISER, G.G., HOZBOR, F., CABODEVILA, J., ALBERIO, R.H. 2006. Effect of oestrous cow serum during bovine embryo culture on blastocyst development and cryotolerance after slow freezing or vitrification. *Theriogenology*. 65 (8): 1551-1562.

NAGANO, M., KATAGIRI, S., TAKAHASHI, Y. 2006. Relationship between bovine oocyte morphology and in vitro developmental potential. *Zygote*. 14 (1): 53-61.

NAYAK, N.R., ELLINGTON E.F. 1977. Ultra-structural and ultra-cytochemical cyclic changes in the bovine uterine tube (oviduct) epithelium. *American Journal of Veterinary Research.* 38 (2): 157-168.

NEBEL, R.L., McGILLIARD, M.L. 1993. Interactions of high milk yield and reproductive performance in dairy cows *Journal of Dairy Science.* 76 (10): 3257-3268.

OLDS, D., VANDEMARK, N.L. 1957. Composition of luminal fluids in bovine female genitalia. *Fertility & Sterility*. 8: 345-354.

OLIPHANT, G., BOWLING, A., ENG, L.A., KEEN, S., RANDALL, P.A. 1978. The permeability of rabbit oviduct to proteins present in the serum. *Biology of Reproduction*. 18: 516-520.

OOMMEN, A.M., GRIFFIN, J.B., SARATH, G., ZEMPLENI, J. 2005. Roles for nutrients in epigenetic events. *Journal of Nutritional Biochemistry.* 16: 74–77.

ORSI, N.M., LEESE, H.J. 2004. Ammonium exposure and pyruvate affect the amino acid metabolism of bovine blastocysts in vitro. *Reproduction.* 127: 131-140.

ORSI, N.M., REISCHL, J.B. 2007. Mammalian embryo co-culture: Trials and tribulations of a misunderstood method. *Theriogenology.* 67 (3): 441-458.

OUÉDRAOGO, A.P. 2004. Public perceptions of reproductive biotechnologies: The case of farm animal breeding and reproduction in France and the United Kingdom. *Cloning & Stem Cells.* 6 (2): 182-189.

PARTRIDGE, R.J., LEESE, H.J. 1996. Consumption of amino acids by bovine preimplantation embryos. *Reproduction, Fertility & Development.* 8: 945-950.

PFAFF, R.T., LIU, J., GAO, D., PETER, A.T., LI, T.K., CRITSER, J.K. 1998. Water and DMSO membrane permeability characteristics of in-vivo and in-vitro derived and cultured murine oocytes and embryos. *Molecular Human Reproduction.* 4 (1): 51-59.

PICTON, H.M., ELDER, K., HOUGHTON, F.D., HAWKHEAD, J.A., RUTHERFORD, A.J., HOGG, J.E., LEESE, H.J., HARRIS, S.E. 2010. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development *in vitro. Molecular Human Reproduction.* 16 (8): 557-569.

PINYOPUMMINTR, T., BAVISTER, B.D. 1991. In vitro-matured/in vitro-fertilised bovine oocytes can develop into morulae/blastocysts in chemically define, protein-free culture media. *Biology of Reproduction.* 45 (5): 736-742.

PURCELL, S.H., CHI, M.M., MOLEY, K.H. 2012. Insulin-stimulated glucose uptake occurs in specialised cells within the cumulus oocyte complex. *Endocrinology.* 153 (5): 2444-2454.

RAVNIK, S.E., ALBERS, J.J., MULLER, C.H. 1993. A novel view of albumin-supported sperm capacitation: role of lipid transfer protein-I. *Fertility & Sterility.* 59 (3): 629-638.

Research Instruments Limited. 2010. RI MSC® [online] available at: http://www.research-instruments.com/static/products/consumables/msc (accessed 28th May 2013).

RINGER, S. 1884. An investigation regarding the action of rubidium and caesium salts compared with the action of potassium salts on the ventricle of the frog's heart. *The Journal of Physiology.* 4: 370-386.

RIZOS, D., GUTIÉRREZ-ADÁN, A., PÉREZ-GARNELO, S., DE LA FUENTE, J., BOLAND, M.P., LONERGAN, P. 2002. Bovine embryo culture in the presence or absence of serum: Implications for blastocyst development, cryotolerance, and messenger RNA expression. *Biology of Reproduction.* 68 (1): 236-243.

ROCHE, J.F. 2006. The effect of nutritional management of the dairy cow on reproductive efficiency. *Animal Reproduction Science.* 96 (3-4): 282-296.

ROTTEM, S., YASHOUV, J., NE'EMAN, A., RAZIN, A. 1973. Cholesterol in mycoplasma membranes. Composition, ultrastructure and biological properties of membranes from mycoplasma mycoides var. capri cells adapted to grow with low cholesterol concentrations. *Biochimica et Biophysica Acta.* 323: 495–508.

SALISBURY, G.W., VANDENMARK, N.L., LODGE, J.R. 1978. *Physiology of Reproduction and Artificial Insemination of Cattle.* San Francisco CA, USA. W.H. Freeman & Co.

SENGER.P.L. 2005. *Pathways to Pregnancy and Parturition* (Second revised Edition). Washington DC, USA. Current Conceptions Inc.

SINCLAIR, K.D., KURAN, M., GEBBIE, F.E., WEBB, R., McEVOY, T.G. 2000. Nitrogen metabolism and fertility in cattle: II. Development of oocytes recovered from heifers offered diets differing in their rate of nitrogen release in the rumen. *Journal of Animal Science.* 78: 2670-2680.

SINCLAIR, K.D., MCEVOY, T.G., CAROLAN, C., MAXFIELD, E.K., MALTIN, C.A., YOUNG, L.E., WILMUT, I., ROBINSON, J.J., BROADBENT, P.J. 1998. Conceptus growth and development following *in vitro* culture of ovine embryos in media supplemented with bovine sera. *Theriogenology.* 49: 218.

SINCLAIR, K.D., SINCLAIR, L.A., ROBINSON, J.J. 2000. Nitrogen metabolism and fertility in cattle: I. Adaptive changes in intake and metabolism to diets differing in their rate of energy and nitrogen release in the rumen. *Journal of Animal Science.* 78: 2659-2669.

SOMOGYI, M. 1942. Carbohydrate metabolism. *Annual Review of Biochemistry.* 11: 217-234.

SQUIRES, E.L., CARNEVALE, E.M., McCUE, P.M., BRUEMMER, J.E. 2003. Embryo technologies in the horse. *Theriogenology*. 59 (1): 151-170.

STURMEY, R.G., HAWKHEAD, J.A., BARKER, E.A., LEESE, H.J. 2009a. DNA damage and metabolic activity in the preimplantation embryo. *Human Reproduction* 24:81-91.

STURMEY, R.G., REIS, A., LEESE, H.J., MCEVOY, T.G. 2009b. Role of fatty acids in energy provision during oocyte maturation and early embryo development. *Reproduction in Domestic Animals.* 44 (Suppl. 3): 50-58.

STURMEY, R.G., BERMEJO-ALVAREZ, P., GUTIERREZ-ADAN, A., RIZOS, D., LEESE, H.J., LONERGAN, P. 2010. Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. *Molecular Reproduction & Development.* 77 (3): 285-296.

SU J, WANG Y, LI R, PENG H, HUA S, LI, Q., QUAN, F., GUO, Z., ZHANG, Y. 2012. Oocytes selected using BCB staining enhance nuclear reprogramming and the in vivo development of SCNT embryos in cattle. PLoS ONE 7(4): e36181. doi:10.1371/journal.pone.0036181

TANGHE, S., VAN SOOM, A., NAUWYNCK, H., CORYN, M., DE KRUIF, A. 2002. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation and fertilisation. *Molecular Reproduction & Development.* 61: 414-424.

TAYLOR, D.M., RAY, P.F., AO, A., WINSTON, R.M., HANDYSIDE, A.H. 1997. Paternal transcripts for glucose-6-phosphate dehydrogenase and adenosine deaminase are first detectable in the human preimplantation embryo at the three- to four-cell stage. *Molecular Reproduction & Development.* 48: 442-448.

THOMPSON, J.G., GARDNER, D.K., PUGH, P.A., MCMILLAN, W.H. TERVIT, H.R. 1995. Lamb birth weight is affected by culture system utilized during *in vitro* pre-elongation development of ovine embryos *Biology of Reproduction.* 53: 1385–1391.

THOMPSON, J.G. 2000. In vitro culture and embryo metabolism of cattle and sheep embryos – a decade of achievement. *Animal Reproduction Science.* 60-61: 263-275.

TIBONI, G.M., BUCCIARELLI, T., AMICARELLI, F., ANGELUCCI, S., IAMMARRONE, E., BELLATI, U., SACCHETTA, P., DI ILIO, C. 1997. Spatial distribution of glutathione, glutathione-related and antioxidant enzymes in cultured mouse embryos. *Archives of Toxicology.* 72 (1): 38-44.

TORNER, H., GHANEM, N., AMBROS, C., HÖLKER, M., TOMEK, W., PHATSARA, C., ALM, H., SIRARD, M.A., KANITZ, W., SCHELLANDER, K., TESFAYE, D. 2008. Molecular and subcellular characterization of oocytes screened for their developmental competence based on glucose-6-phosphate dehydrogenase activity. *Reproduction.* 135 (2): 197-212.

VAN WINKLE, L.J. 2001. Amino acid transport regulation and early embryo development. *Biology of Reproduction.* 64: 1-12.

VAN WINKLE, L.J., HAGHIGHAT, N., CAMPIONE, A.L. 1990. Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic ions of oviductal fluid. *Journal of Experimental Zoology.* 253: 215-219.

VANDAELE, L. 2008. *Intrinsic factors affecting apoptosis in bovine in vitro produced embryo*s. 2008. Ph.D. University of Ghent, Belgium. Chapter 5.2: pp. 109 –23.

VARGAS-BELLO-PÉREZ, E. 2011. Plasma transport and mammary uptake of *trans* fatty acids in dairy cows. Ph.D. University of Nottingham.

VARGHESE, A.C., LY, K.D., CORBIN, C., MENDIOLA, J., AGARWAL, A. 2011. Oocyte developmental competence and embryo development: impact of lifestyle and environmental risk factors. *Reproductive BioMedicine Online.* 22: 410-420.

VELÁSQUEZ, A.E., MANRÍQUEZ, J.R., CASTRO, F.O., RODRÍGUEZ-ALVAREZ, L.L. 2013. Effect of zona pellucida removal on early development of *in vitro* produced bovine embryos. *Archivos de Medicina Veterinaria* 45: 7-15.

VIGNEAULT, C., McGRAW, S., SIRARD, M. 2009. Spatiotemporal expression of transcriptional regulators in concert with the maternal-to-embryonic transition during bovine *in vitro* embryogenesis. *Reproduction.* 137: 13-21.

WAGNER, I., MUSSO, H. 1983. New naturally occurring amino acids. *Angewandte Chemie-International Edition in English.* 22 (22): 816-828.

WALSH, S.W., WILLIAMS, E.J., EVANS, A.C.O. 2011. A review of the causes of poor fertility in high milk producing dairy cows. *Animal Reproduction Science.* 123 (3-4): 127-138.

WATSON, A.J., DAMSKY, C.H., KIDDER, G.M. 1990. Differentiation of an epithelium: Factors affecting the polarised distribution of Na^+, K^+-ATPase in mouse trophectoderm. *Developmental Biology.* 141 (1): 104-114.

WEST, J.W. 2003. Effects of heat-stress on production in dairy cattle. *Theriogenology.* 59 (2): 651-674.

WHEELER, M.B., RUTLEDGE, J.J., FISCHER-BROWN, A., VANETTEN, T., MALUSKY, S., BEEBE, D.J. 2006. Application of sexed semen technology to in vitro embryo production in cattle. *Theriogenology.* 65 (1): 219-227.

WU, Y.G., LIU, Y., ZHOU, P., LAN, G.C., HAN, D., MIAO, D.Q., TAN, J.H. 2007. Selection of oocytes for in vitro maturation by brilliant cresyl blue staining: a study using the mouse model. *Cell Research.* 17 (8): 722-731.

WU, G. 2010. Functional amino acids in growth, reproduction, and health. *Advances in Nutrition.* 1: 31-37.

YOUNG, L.E., SINCLAIR, K.D., WILMUT, I. 1998. Large offspring syndrome in cattle and sheep. *Reviews in Reproduction.* 3: 155-163.

ZANDER-FOX, D., LANE, M. 2012. The future of human embryo culture media - or have we reached the ceiling? In: Yamada, S. (Ed.) 2012. *The Human Embryo*

[online] Available at: http://cdn.intechopen.com/pdfs/30621/InTech-The_future_of_human_embryo_culture_media_or_have_we_reached_the_ceiling_.pdf (accessed 20th June 2013).

ZERON, Y., OCHERETNY, A., KEDAR, O., BOROCHOV, A., SKLAN, D., ARAV, A. 2001. Seasonal changes in bovine fertility: relation to developmental competence of oocytes, membrane properties and fatty acid composition of follicles. *Reproduction.* 121: 447–454.

ZERNICA-GOETZ, M. 2005. Cleavage pattern and emerging asymmetry of the mouse embryo. *Nature Reviews Molecular Cell Biology*. 6: 919-928.

Appendices

Appendix A1: Wash Media Recipe

Working within a laminar flow hood, to a 25ml universal container add:

Component	Volume	Supplier
Medium M199	9 ml	Gibco®, Carlsberg CA, USA.
Foetal bovine serum (heat inactivated)	1 ml	
Gentamycin	10 µl	Sigma-Aldrich, Dorset UK

Vortex to mix thoroughly and check osmolarity (range: 295 – 325 mOsm) and filter using a 0.2 µ syringe filter.

Appendix A2: Maturation Media Recipe

Working within a laminar flow hood, to a 25 ml universal container add:

Component	Volume	Supplier
Medium M199	9 ml	Gibco®, Carlsberg CA, USA.
Foetal bovine serum (heat inactivated)	1 ml	
Glutamax (200 mM)	50 µl	
Epidermal growth factor (10 µg ml^{-1})	10 µl	Sigma-Aldrich, Dorset UK
Sodium pyruvate (11 mg ml^{-1})	42 µl	
Cysteamine (1.1 mg ml^{-1})	100 µl	
Gentamycin	10 µl	
Insuin-transferrin-sodium selenite (ITS)	100 µl	
Pluset (5 IU ml^{-1})	20 µl	Minitube of America, Verona WI, USA

Vortex to mix thoroughly and check osmolarity (range: 295 – 325 mOsm) and filter using a 0.2 µ syringe filter.

Appendix A3: Fertilisation media recipe

1. Heparin stock	Volume	Supplier
DPBS	12 ml	Sigma-Aldrich, Dorset UK
Heparin	30 mg	
Gentamycin	12 µl	
2. Hypotaurine stock		
DPBS	30 ml	Sigma-Aldrich, Dorset UK
Hypotaurine	10 mg	
Gentamycin	30 µl	
3. Penicillamine stock		
DPBS	20 ml	Sigma-Aldrich, Dorset UK
Penicillamine	10 mg	
Gentamycin	20 µl	
PH/HEP		
1. Heparin stock	67 µl	
2. Hypotaurine stock	50 µl	
3. Penicillamine stock	67 µl	
G-IVF	545 µl	Vitrolife AB, Göteborg, Sweden

Appendix A4: Calcium-free sperm preparation media recipe

Component	100ml	Supplier
NaCl	530mg	Sigma-Aldrich, Dorset UK
KCl	23mg	
$Na_2HPO_4.2H_2O$	0.425ml stock	
$MgCl_2.6H_2O$	1.5ml stock	
$NaHCO_3$	200mg	
Caffeine	10mg	
Phenol red (5mg/ml)	0.2ml	
Kanamycin	7.5mg	
Sodium pyruvate	100mg	
BSA	600mg	
Sodium lactate (60% syrup)	0.37ml	
pH	7.7	
Osmolarity	280-290 mOsmol	