Development of Ovum Pickup and In Vitro Embryo Production to Assess Fertility Responses for Mineral Intervention Studies.

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Dedication

I would like to dedicate this thesis to;

My parents; Hugh and Doreen

&

‘The Girls’; Sue, Katie, Becca and Isla

Abstract

As nutrition is of central importance to cattle fertility, this study sought to assess how veterinarians and nutritional advisers manage trace element imbalance in the UK; diagnosis and treatment. The study also sought to develop a robust system for oocyte recovery (ovum pick-up (OPU)) and *in vitro* embryo production (IVP) for commercial use, and to identify key factors influencing success, including oestrus synchrony and ovarian stimulation prior to OPU. The intention originally was to use OPU/IVP to investigate the impact of mineral imbalances on bovine oocyte quality, early embryo development and pregnancy establishment following embryo transfer (ET).

In the first survey of its kind in the UK, the understanding and approach of advisers to mineral nutrition on farms was investigated. Of the 173 respondents, 78% were vets in practice. The overall importance of minerals was recorded by vets as low 33%, medium 37%, and high 30%, while non-vets scored importance as low 17%, medium 48%, and high 35%. There was little consensus amongst the advisers, or within the vet and non-vet subgroups about mechanisms and interactions associated with deficiency, and particularly of copper responsive conditions. The most frequently identified deficiencies were selenium, copper and iodine, while the most commonly identified toxicity was molybdenum. For copper responsive conditions, all of the listed treatments were used at least “occasionally”; the most frequently being glass boluses, in-feed supplementation, matrix boluses, and then copper injections. While there was a diverse choice of treatments, altering the ration was relatively rarely selected.

This thesis also provides the first large-scale retrospective analysis of factors influencing the establishment of a commercially robust ovum pick up (OPU) and *in vitro* embryo production (IVP) platform in the UK. Over a 5-year period, a system was developed and validated for use in the UK with 2,138 cycles of OPU. These cycles were analysed as four sets of data and included two IVP laboratories and 6 OPU teams. Factors in these analyses included OPU team, IVP laboratory, ovarian stimulation protocol and semen type (unsorted vs sex-sorted).

The mean number of follicles aspirated by the OPU teams ranged from 6.5 to 14.9 ($P<0.001$), while the number of oocytes collected was between 4.0 and 12.4 ($P<0.001$). There was an

indication (P=0.055) that the blastocyst per oocyte rate varied between teams. The proportion of blastocysts from oocytes that cleaved was higher (P=0.01) for unsorted than sexed semen. Two commercial products containing different ratios of follicle stimulating hormone (FSH) to luteinising hormone (LH) (Folltropin® and Pluset®) were compared in ovarian stimulation programs. The addition of 'coasting' (short-term (typically 48h) hormonal withdrawal after FSH stimulation), prior to OPU was also investigated. Pluset® resulted in a greater (P<0.001) mean number of follicles aspirated, more (P=0.003) blastocysts per oocyte matured and more (P<0.001) embryos per cycle (2.45), compared with Folltropin® (1.17) or with no stimulation (1.24). Throughout the study there was a steady improvement in blastocyst production per OPU cycle.

In a separate analysis, Grade 1 cumulus oocyte complexes (COCs) as a proportion of COCs recovered, oocytes that cleaved as a proportion of total COCs, and blastocysts as a proportion of total COCs, were all greater (P<0.05) for stimulated than non-stimulated cycles, irrespective of FSH/LH product. A composite score of oocyte quality and quantity was proposed (sCOC); Log Total Mean sCOC was correlated (P<0.001) with both the proportion of blastocysts per oocyte collected, and the total number of embryos produced per cycle.
Finally, twelve peri-pubertal heifers (approximately 10 months old) participated in a crossover trial which compared PRID® (Delta®) vs CIDR® progesterone releasing intravaginal devices for use in OPU/IVP cycles. Vaginoscopic examination found higher vaginal inflammation grades for PRID® than CIDR® (P<0.001). There was evidence of vaginal inflammation continuing for at least 2 weeks after device withdrawal. The proportion cleaved of oocytes inseminated was higher for PRID® than CIDR® (P<0.05). Numerically but not significantly there was a higher proportion of blastocysts per cycle and a higher Log Total Mean sCOC score per cycle with PRID® than CIDR® treatments, but blastocyst yield was low throughout, suggesting a need to repeat the trial.

Data collection and analyses are ongoing, to identify other key performance indicators within the OPU/IVP embryo transfer (ET) system, with a view to refining the sCOC composite score model. A robust OPU/IVP/ET system has been developed and this could be used to investigate further how mineral imbalances impact oocyte competence and blastocyst yield.

Declaration

The author was the project lead for all the studies undertaken and upon which this thesis is based, and would like to acknowledge fellow veterinarians and technicians who undertook some of the cow-side and laboratory procedures.

The preparation of this thesis is the unaided work of the author, except where acknowledgement is made by reference. The work described in this thesis has not been previously accepted for, or is currently being submitted for, another degree of qualification.

David H. Black

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I, first of all, must thank my supervisors, Dr Nigel Kendall and Professor Kevin Sinclair. It has not been an easy task for them to deal with an older, slightly stubborn student, readily distracted by his 'day job' and whose gradually aging grey matter no longer seems as effective as that of his younger counterparts. However, their patience, prompting, guidance and support has been invaluable and very gratefully received. Nigel's enthusiasm and knowledge of mineral nutrition, combined with Kevin's immense breadth of understating of reproductive biology have neatly complemented each other. I would like to thank Kevin for helping teach me the statistics and for continually encouraging me to write in a crisp scientific style. And it would be remiss of me not to mention and thank the late Professor Keith Campbell who was a significant influence and inspiration for me to undertake this in the first place.

I would like to thank all respondents who took the time to complete the mineral questionnaire and all the farmers and breeders whose cattle we have worked with. I am also grateful to the vets and technicians in the wider XLVets network who have contributed data and who continue to work with us as part of Activf-ET to deliver OPU/IVP to the wider cattle industry.

Without the support of colleagues at Paragon Veterinary Group, who have given me the time and support to not only undertake the projects, but latterly to write up this thesis, it could never have happened. Thank you to those vets, embryologists and support staff who have worked alongside me throughout this project undertaking cow-side, laboratory, logistic, client care and administrative tasks to deliver the TSB/Innovate UK projects. Specifically, Stuart Mullan, Gemma Dobson, Rob Simmons, Charlotte Smith and Jenny Turner. And a very special mention to Will Christie who back in the late 1990s set Paragon off on this journey – we are getting there Will!

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Publications

BLACK, D.H. & KENDALL, N.R. 2010. The Attitudes and Approach to Trace Element Diagnosis and Treatment in the UK. *Cattle Practice*, 18, 67-72. (Black and Kendall, 2010)

BLACK, D.H. & KENDALL, N.R. 2010. The Attitudes and Approach to Trace Element Diagnosis and Treatment in the UK. *Proceedings of World Association for Buiatrics – 26th Congress*, Poster Presentation, 216.

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BLACK, D.H. & SINCLAIR, K.D. 2017. Preliminary findings from an in vitro embryo production optimisation study in cattle in the UK. *Cattle Practice*, 25, 276-278.

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Associated work completed with academic partners in parallel with this study;

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Abbreviations

AI	Artificial Insemination
AFP	Antral Follicle Population
AMH	Anti-Mullerian Hormone
BCB	Brilliant cresyl blue
bFSH	Bovine Follicle Stimulating Hormone
CL	Corpus Lutuem
Cu	Copper
CHX	Cyclohexamide
DM	Dry Matter
EFSA	European Food Safety Authority
EG	Ethylene Glycol
EGF	Epidermal Growth Factor
ET	Embryo Transfer
Fe	Iron
FCS	Foetal Calf Serum
FSH	Follicle Stimulating Hormone
G6PDH	Glucose-6-phosphate dehydrogenase
GHG	Greenhouse Gas
GSHPx	Glutathione peroxidase
HM	Holding Medium
I	Iodine
ICSI	Intracytoplasmic sperm injection
IGF1	Insulin-like Growth Factor 1
ITS	Insulin-Transferrin-Selenium
INFT	interferon τ
IVF	*In-Vitro* Fertilisation
IVM	*In-Vitro* Maturation
IVP	*In-Vitro* Production
Lab	Laboratory

LH	Luteinising Hormone
LOS	Large offspring syndrome
Mo	Molybdenum
MOET	Multiple Ovulation Embryo Transfer
MSC	Migration Sedimentation Chamber
OPU	Ovum Pick-Up
P4	Progesterone
PAM	Peptidylglycine α-amidating monooxygenase
PBS	Phosphate Buffered Saline
PMSG	Pregnant mare serum gonadotrophin
ROS	Reactive Oxygen Species
RT-PCR	Real Time Polymerase Chain Reaction
S	Sulphur
Sig	Significance
Se	Selenium
SBV	Schmallenberg virus
SNP	SIngle nucleotide polymorphysm
SOD	Superoxide Dismutase
T3	Triiodothyronine
T4	Tetraiodothyronine (or Thyroxine)
TH	Thyroid Hormone(s)
TTM	Tetrathiomolybdate
VHM	Vitrification Holding Medium

Introduction and context statement

Development of Ovum Pickup and In Vitro Embryo Production to Assess Fertility Responses for Mineral Intervention Studies.

Introduction

It is recognised that there is an increasing global demand for animal products; the Food and Agriculture Organization of the United Nations (UN FAO), predicts that between 2015 and 2030, global demand for animal protein will double, driven by population growth to 9.8 billion (FAO 2015, UN 2017). Increasing affluence in developing countries, with a concurrent switch from largely vegetable based diets to meat and dairy products, may be more of a driver than simple population growth (Kastner et al., 2012). Further concerns arise from climate change, farming effects on greenhouse gas (GHG) emissions, the sustainability of importing (for example) soya protein and livestock farming's demand for fresh water.

Increasing production and sustainability of domestically supplied animal protein for food, through increasing the efficiency of both feed conversion and use of other resources within the livestock sector will improve both the financial performance of the industry, as well as reducing the quantity of GHGs emitted and resources consumed. There is an increasing recognition of the importance of optimal mineral and vitamin nutrition, particularly in regard to animal health, fertility and immune function. Some specific mechanisms are understood but there remains much to learn about the micronutrient status of animals and their optimal requirements depending on genetics and environmental conditions (Kegley et al., 2016). Healthier animals require fewer therapeutic interventions and antimicrobial resistance is a very real threat to the world population, with the World Health Organisation quoting that "a post-antibiotic era—in which common infections and minor injuries can kill—far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century" (WHO, 2014).

There is a wealth of ongoing recent and current research investigating new traits and phenotypes of cattle, so that higher reliability of breeding values can be achieved, with large phenotypic databases being correlated with genotyping (Egger-Danner et al., 2015). Genomics is being more widely used to identify health traits, and disease resistance, improvements in which would lead to reduced dependence on antimicrobials (Plastow, 2016). Although there is a recognition that bull fertility traits are important (Thundathil et al.,

2016), there is also a need to amplify and accelerate genetic gains through the female breeding lines (Faber et al., 2003).

Trials to evaluate the effects of dietary or pharmaceutical interventions may result in relatively small differences in the measurable fertility outcomes, and therefore tend to require a large number of animals over relatively long periods of time to reach significant conclusions. The initial basis of this project was the suggestion that ovum-pickup (OPU) and in-vitro production (IVP) of embryos may allow us to identify fertility markers as early as the actual collection of the cumulus-oocyte-complex (COC) or during the first few days of embryo development. These might give some insights prior to larger and lengthier cattle trials which have pregnancy or even calving as the outcome variable. At the same time as developing the platform and methodology for these studies, the opportunity was taken to investigate some of the key factors that affected successful and consistent OPU/IVP embryo production in the UK; whether these be donor effects, cow-side techniques and protocols, laboratory techniques and protocols or recipient traits.

Initial Hypothesis

That OPU/IVP can be used to assess fertility responses for mineral intervention studies

Initial Aims

i. Assess the attitudes and approach to trace element diagnosis and treatment in the UK
ii. Establish a robust OPU/IVP system under UK conditions and identify key markers in the system
iii. Assess a crossover trial utilising OPU/IVP to compare mineral supplementations that may affect fertility

Context Statement

Inevitably with a part-time study in a rapidly developing field, there have been advances that have influenced the final outcomes of this study.

The original emphasis was to address the fact that there is still much debate about the significance of micro-minerals in bovine fertility, and of how to measure, monitor and manage imbalances. It was hypothesised that ovum pickup (OPU) and *in-vitro* production (IVP) of bovine embryos may be used as a tool to help understand mineral supplementation or

manipulation in cattle better. To establish the need for this tool a survey was undertaken to establish the current attitudes and approach to trace element diagnosis and treatment in the UK and although it became unfeasible to undertake a cross-over trial utilising mineral supplementation, the concept was investigated utilising a pharmacological intervention instead.

After having enrolled part-time for the Doctor of Veterinary Medicine (DVM) degree at The University of Nottingham, opportunities arose to bid for funding to develop and expand the OPU/IVP aspects of the project. The projects, which could be up to five years long, had to be business- led (either business-to-business or business-to-science collaborations) and aim to develop new products or processes. Funding was allocated to proposals in the applied R&D or experimental development categories. The competitions aimed to harness the potential held within technologies to derive commercial solutions that would benefit consortia members directly, as well as benefiting the wider community through commercial exploitation and uptake by industry and farm businesses alike.

Sustainable Protein Production Competition for Collaborative R&D Funding

On 4th April 2011, the Technology Strategy Board (TSB), in partnership with the Department for Environment, Food and Rural Affairs (DEFRA), the Biotechnology and Biological Sciences Research Council (BBSRC) and the Scottish Government launched a collaborative R&D competition with up to £15 million available to invest in projects focusing on the challenge of sustainable protein production.

An application was submitted which was successful and the project was titled;

"Applying Advanced Breeding Technologies to Amplify and Distribute Bovine Genetics to Increase Production Efficiency and Sustainability"

This project aimed to apply advanced breeding technologies to amplify and distribute bovine genetics to increase production efficiency and sustainability via 2 main strands;

i. Utilisation of "ovum pickup" (OPU) and "in-vitro embryo production" (IVP) to accelerate the speed of genetic gain in cattle. Need were identified to;
    - improve the reliability and consistency of embryo production
    - improve the efficacy of sexed semen in IVF
    - develop "quick thaw" systems

- develop a network of collection and transfer centres
- create a route to export for sexed, frozen embryos.

ii. Development of robust techniques to allow rapid, affordable, real-time assessment of bovine fertility interventions, and identify criteria within the OPU/IVP system that may act as fertility markers.

Measurement Technologies for Efficient Agri-food Systems

On 18th March 2013, the same funding partners launched a further collaborative R&D competition with up to £8.75 million available to help businesses develop innovative measurement technologies for efficient agri-food systems.

An application was submitted which was successful and the project was titled;

"Optimising the Delivery of Superior Genetics through Advanced Genomic Selection of Bovine Embryos"

This project built on the developments of the first to apply advanced breeding technologies to produce (both in-vivo and in-vitro) pre-implantation bovine embryos from which biopsies could be taken to interrogate their genomic makeup using single nucleotide polymorphism arrays (SNP chips). It was currently possible to screen dairy and beef cattle genomically, but responses to selection are impeded by waiting for the gestation of the calf on which genomic selection is performed. The screening of bovine embryos, however, would optimise the delivery and amplification of superior genetics by advancing the time of selection and reducing "wastage" of unwanted calves. Therefore combining advanced embryo breeding technologies with state-of-the-art genomic screening (so called pre-implantation genetic diagnosis), and karyomapping (combining parental DNA information with the offspring's genomic information to provide more genetic detail) allowed the development of strategies for optimal bovine embryo biopsy, cryopreservation and genomic screening of small cell numbers, and provided proof of principle that these new technologies can be used to deliver superior genetics more efficiently to the breeding herd.

Both projects were designed to create a greater amplification and distribution of outputs from other projects and research, both previous and current, so that maximum benefits could be achieved. Achieving these aims would result in significant economic, environmental and food security benefits to both the UK and the wider global economy.

The projects were both led by myself at Paragon Veterinary Group, with the collaboration of XLVet UK Ltd., and in the initial project Cogent Breeding Ltd., and Professor Keith Campbell's lab at The University of Nottingham. After the sad and untimely death of Professor Keith Campbell, Professor Kevin Sinclair and his lab became involved. The second project worked in collaboration with Semex UK substituting for Cogent Breeding Ltd., and with the addition of Professor Darren Griffin and his lab at the University of Kent.

Chapter 1: Literature review - minerals and fertility in the cow

1.1. General introduction

In line with the initial aims of this study, the current literature was reviewed to consider the minerals most associated with fertility in cattle; selenium and iodine, as well as copper and its interactions with molybdenum, sulphur and iron. The assessment of the status of these minerals was considered as well as the mechanisms and impact on bovine fertility. A parallel review was undertaken of the literature available on OPU and IVP in cattle, and in particular the areas of limitation of these techniques.

1.2. Minerals

1.2.1. Introduction

There is much debate about the relevance of minerals in the nutrition of modern dairy with some authors virtually dismissing their importance (Whitaker 2005), while farmers as a rule tend to place great weight on mineral effects – often perhaps as they look for a simple one-stop answer to health problems. The answer is likely to be between these extremes, with a need to consider minerals as part of a holistic herd health approach.

When initially developed the recommended mineral intakes for cattle were based on the requirements for growth, maintenance, foetal growth and lactation (Agricultural Research Council, 1980, National Research Council, 2001). But the situation is more complex, with higher producing animals and the suggestion that there may be different requirements for optimum production in modern ruminants, and more focus on the effects on health and performance. Nutritional interactions, different formulations of mineral (for example chelates), and genomic differences further complicate our current understanding (Sinclair et al., 2015). In a survey of levels of minerals being fed during winter in the UK, Sinclair and Atkins (2015) found that when all feed, water and supplementation were taken into account, several minerals were being overfed as compared with NRC (2001) recommendations, with copper being fed at 1.5 times requirements in early lactation. The justification that this was to combat

the dietary antagonism of molybdenum was not supported by comparable high molybdenum levels on those farms. Overfeeding of minerals has cost implications but more importantly an adverse effect on animal health, consumers and on the environment and so the mineral tolerances of food producing animals must be better understood (NRC 2005).

This review considers selenium (Se) and iodine (I), as well as copper (Cu) and its interactions with molybdenum (Mo), sulphur (S) and iron (Fe), and their relevance in bovine reproduction.

1.2.2. Selenium

Selenium and vitamin E are often considered together as they have a similar function in the animal as antioxidants, preventing oxidative damage to cell membranes, and their deficiency has effects on growth, health and fertility (Suttle 2010). Selenium has now been identified in over 30 selenoproteins, virtually all containing selenocysteine, some of the most important being the iodothyronine deiodinases, which are involved in thyroid function (Arthur and Beckett, 1994, Beckett and Arthur, 1994, Beckett and Arthur, 2005). There are four known peroxidase selenoproteins, essential for the prevention of a build-up of free radicals and subsequent tissue damage. There are other antioxidant enzyme systems within the body (associated with copper, zinc, manganese and sulphur) as well as vitamin E which will also help protect, and these will complement each other. Therefore, there is an interbalance between the efficiency of these systems and the rate of production of free radicals. It has been shown that vitamin E and selenium act synergistically (Awad et al., 1994, Levander et al., 1995) but that each nutrient cannot fully compensate for the other.

In cattle, the contribution of erythrocyte selenium to whole-blood selenium is roughly 60%, while the half-life of selenium in the plasma compartment is around 6.6 hours in humans and is likely to be similar in other mammals. Therefore, the rapidly changing serum pool and the more slowly changing erythrocyte pool both affect whole-blood selenium concentration. This combination of short-term and long-term effects suggests that whole-blood selenium may be preferable as an assay than serum selenium for the determination of current selenium nutrition, although either can be

used (Herdt and Hoff, 2011). The selenium in the erythrocytes of most domestic animals is found primarily as the blood antioxidant glutathione peroxidase (GSHPx), the concentration of which is affected by dietary selenium uptakes (Koller et al., 1984). Caple et al. (1978), Koller et al. (1984) and Thompson et al. (1981) demonstrated a good correlation between blood selenium levels and GSHPx activity which has historically been simpler to assay and is still widely used. However, although GSHPx is found in various blood components, it is mainly found in red blood cells so the interpretation of "normal" GSHPx activity levels can be problematical. Slow turnover of erythrocytes reflects historical selenium status, which although usually taken to indicate selenium status some 4-6 weeks previously, Hidiroglou et al. (1985) suggest that this delay may be up to 4 months. More recently, with the development of inductively coupled plasma–mass spectrometry (ICP-MS) as a technique for performing trace metal analysis, selenium itself can be more economically assayed. Taken together with GSHPx this can further aid interpretation; a high plasma selenium and low GSHPx suggests that selenium status is increasing, while high GSHPx and low plasma selenium indicates a falling selenium status.

Hartley (1967) described an "ill-thrift" and increased perinatal mortality in sheep in New Zealand and the response to selenium, which was cumulative, and very significant economically for the farmer. However, the role of selenium itself in ruminant fertility is still a subject of some debate. In cows, sodium selenite-vitamin E injections have been shown to reduce losses from the birth of premature, weak or dead calves (Mace *et al* 1963) and to reduce the incidence of retained placenta (Trinder et al., 1973, Julien et al., 1976, Harrison et al., 1984). However, other studies have shown no response (Gwazdauskas et al., 1979). The incidence of endometritis, and cystic ovaries was reduced following selenium supplementation (Harrison et al., 1984), while first service conception rates were improved (McClure et al., 1986), although the mechanisms are not entirely clear. Whitaker (1999) is critical of the work of McClure et al. (1986) for using aggregated data and low numbers to show a significant benefit on first service pregnancy rates whilst excluding from their analysis cows inseminated less than 60 days after calving. Tasker (1987) can also be criticised for using non-return rate to assess pregnancy, as this method of assessing fertility is

likely to be unreliable. Wichtel (1998) is of the opinion that reproductive dysfunction due to selenium deficiency may not be as important as previously thought. Most recently, in 2006, a large retrospective study in France by Enjalbert et al., found that abortion, perinatal immunity, retained placenta, metritis, delayed conception, poor fecundation and cystic ovarian disease are all associated with low or marginal selenium status. They cited the studies of Orr and Blakley (1997) who suggested myocardial necrosis and heart failure in foetuses as being a possible cause of abortion, and Corah and Ives (1991) in relation to cystic ovarian disease.

1.2.2.1. Assessment of selenium status

There is a well-recognised association between selenium, iodine (Section 1.2.3) and thyroid function; Beckett et al. (1987) demonstrated impaired hepatic conversion of thyroxine (T4 or tetraiodothyronine) to triiodothyronine (T3) in selenium deficient rats and Arthur et al. (1988) demonstrated a similar biochemical effect in cattle. Arthur et al. (1990) have further elucidated the key role of selenoproteins in the deiodination of T4 (effectively a prohormone) to produce the more metabolically active T3; a process that in farm livestock principally occurs at tissue level outside the thyroid gland (mainly in the liver). Zagrodzki et al. (1998) have shown compensatory changes in thyroidal seleno-enzymes in response to iodine deficiency. Salvatore et al. (1995) have also identified a selenium containing placental deiodinase, which is distinct from those found in other tissues. It seems possible therefore that selenium may be necessary to allow placental conversion of T4 to its more metabolically active form T3. T3 formed locally in the placenta could then exert an effect locally to up-regulate the metabolic pace on a wide range of processes necessary to the normal shedding of the foetal membranes.

So, it is clear that selenium has a key role to play within the thyroid, where selenium containing antioxidant enzymes (such as the glutathione peroxidase family) protect the thyroid cells from the oxidative damage associated with hydrogen peroxide. As well as in the production of the metabolically active hormone from its precursor at tissue level. Hence some of the effects of iodine deficiency may be compensated for by adequacies of selenium, and also some of the effects of selenium deficiency may

be mediated by disturbances in thyroid hormone metabolism. It is important to note however that although selenium and vitamin E act synergistically as anti-oxidants, this interaction and compensatory effect is only between selenium and iodine.

Illingworth et al (2005) demonstrated that the annual mean of GSHPx activity in samples taken from the UK dairy herd increased from 1995 (77± 31.9 (sd) U/ml PCV) to 2004 (145± 50.7 (sd) U/ml PCV), suggesting increased dietary intakes and/or supplementation by farmers. Furthermore, Kendall et al. (2017) found in an abattoir survey of the mineral content of cattle livers that although there were animals with deficient or marginal status, particularly in beef breeds, there were a higher proportion of dairy animals with liver selenium above the normal range (up to 21.9% in the Holstein Friesian breed). These liver selenium levels correlated closely with liver Cu status in this survey (R=0.73) suggesting the effects were associated with mineral supplementation or feeding practices. An adequate physiological level of selenium is probably different to an optimal production level; Hidiroglou et al. (1985) found a low incidence of myopathy in calves, but no growth retardation at selenium blood levels below 20µg/l, Fraser et al. (1987) found no depression in milk-fat until levels fell below 12µg/l, yet McClure et al. (1986) claimed fertility responsiveness up to 87µg/l, but this was not confirmed by Wichtel et al. (1994). In the large retrospective study reported in 2006 by Enjalbert et al. (2006), the deficient range was taken as <75 units/g Hb of GSHPx activity, marginal as 75-150 units/g Hb, and low-adequate as 150-220 units/g Hb and they reported significant odds ratios for a variety of maternal and neonatal conditions. Experiences in the field of bovine embryo transfer (Christie, W.B., 2008, Personal Communication) concur, with yields of transferable day-7 embryos increasing as GSHPx levels rise above 330 units/g Hb.

Soil type and management affects aeration, drainage, and organic matter content. This has an effect on mineral availability to forages and therefore in turn to ruminants. Application of fertilisers can reduce the availability of selenium, and species of plants have varying abilities to uptake selenium (Suttle and Underwood 1999). High iron content also reduces the availability of selenium, while there are thought to be complex interactions with other trace elements resulting in poor fertility.

Selenium is particularly important for male fertility and either deficiency or excess is associated with testicular tissue development and reduced sperm viability (Ahsan et al., 2014, Marin-Guzman, 1997). It is required for normal spermatozoa development, probably because it is incorporated into the sperm mitochondria capsule and therefore can affect sperm function (Hansen and Deguchi, 1996). Both the testis and the spermatozoa produced are sensitive to reactive oxygen species (ROS) which Se and selenoproteins provide protection against (Aprioku, 2013). There is also evidence of an association between antioxidants, such as selenium, vitamin E and zinc on sperm quality at the actual point of fertilisation (Ross et al., 2010). Selenium and GSHPx are found in the seminal plasma of bulls and GSHPx activity in bovine semen is correlated with higher motility (Vaisberg et al., 2005) and fewer morphological abnormalities (Stradaioli et al., 2009) of spermatozoa. Hurley and Doane, (1989) have shown a role for selenium in sperm transport, fertilisation and uterine contractions during oestrus particularly in super-ovulated animals. Clearly calving intervals will be improved by reducing the incidence of retained placentas and endometritis, but there are likely to be additional mechanisms. For example, heifer conception rates can also be improved by selenium supplementation (Macpherson et al., 1987), and increases in embryo mortality have been found in selenium deficient sheep around the time of implantation (Peper et al., 1980). Disturbed thyroid function may account for poor expression of oestrus behaviour, or even immune suppression, which is believed to be associated with selenium deficiency in humans as reviewed by Stabel and Spears (1993). However, Meglia et al. (2001) associate a periparturient leukocytosis (mainly explained by neutrophilia, but also by monocytosis) with increased levels of Se, Cu and Na, but significantly reduced concentrations of vitamins A and E, and of Zn, Ca and P. This concurs with Politis et al. (1995) who showed that vitamin E supplementation of dairy cows prevented suppression of blood neutrophil and macrophage function during the early postpartum period. White Muscle Disease is a common manifestation of selenium and/or vitamin E deficiency associated with free-radical damage – affected calves exhibit muscular stiffness, arrhythmia, tachycardia and abnormal breathing associated with damage to the intercostal muscles and diaphragm (Hidiroglou et al., 1985). This disease can occur from birth, and it is suggested that the foetus can also be affected.

The form of Se fed as a supplement to cows has been shown to affect gene expression profiles in some tissues (Matthews et al., 2014). When the same range of Se forms (organic, inorganic and a 50% organic:50% inorganic mix) were fed to cows, there was a significant effect on the expression of over 850 genes in the testes of their offspring, 17 of which mRNAs are thought to be associated with steroidogenesis and spermatogenesis (Cerny et al., 2016b) suggesting that the type of Se fed to dams will affect the subsequent fertility of their male offspring by affecting these processes. Furthermore, Cerny et al. (2016a) also found that supplementing with the same Se forms as above had no effect on oestradiol concentrations, but did have a significant effect on progesterone levels at Day 6 after an induced oestrus ($P<0.05$), and a tendency to affect the size of the Day 8 dominant follicle ($P<0.1$).

Selenium toxicity is recorded under specific natural conditions – known as “alkali disease” or “blind staggers” – these conditions of chronic selenosis are not likely to occur in the UK, unless by overzealous use of selenium supplementation. However, there is evidence that increasing selenium supplementation may have a detrimental effect on fertility. Mohammed et al. (1991) suggest that there was an increased risk ($P<0.05$) of cystic ovaries in cattle when blood selenium levels were greater than 169 µg/l, while there was no increased risk under 108 µg/l. However, the type of cystic ovarian disease is not clearly defined. Jukola et al. (1996) found that the incidence of anoestrus or suboestrus significantly increased ($P<0.05$) when GSHPx levels rose above that equivalent to 180 µg/l of selenium in whole blood. However, the incidence of cystic ovaries or first insemination rates did not increase significantly, and the authors comment that their findings may be associated with confounding factors such as herd or cow production. Larson et al. (1980) found a positive correlation ($P<0.05$) between mean serum selenium concentrations (described as adequate to marginally excessive) and services per conception and days open. However, again confounding factors could explain these findings, and the authors themselves conclude that the ratio of the blood components may be as critical as concentration, and that there may be an optimal concentration for each blood component with detriment above or below that. Hartmann and van Ryssen (1997) have shown that hepatic copper concentrations in sheep were increased when the diet was supplemented with 1 mg

Se/kg DM – and if this was similar in cattle, this would be particularly relevant in cases of copper toxicity (Section 1.2.4.2).

1.2.3. Iodine

Underwood (1962) credits Courtois in 1811 with the first discovery of iodine in plant and animal tissues by identifying its presence in seaweed ash and Prevost in 1820 was the first to suggest that iodine deficiency was the cause of goitre. Harington and Barger, (1927) revealed the structure and synthesis of thyroxine and ultimately demonstrated its essential role in human and animal life.

The thyroid gland is thought to be unique as a tissue in its ability to trap iodine by oxidation of iodide in plasma and converting it to elemental iodine before incorporation into organic compounds. The importance of hypothyroidism in ruminants was emphasised in a review by Wilson (1975) and the role of iodine specifically in the dairy cow in reviews by several authors notably Hemken (1970) and Miller et al. (1975). It has been suggested that lowered milk production is a feature of deficiency whilst Robertson et al. (1957) suggest that ketosis may involve an adreno-cortical insufficiency induced by hypothyroidism. More recently the effect of season, lactation, age and pregnancy on thyroid function has been demonstrated (Refsal et al., 1984). Serum levels of thyroxine (T4) and triiodothyronine (T3) are positively related to days post-partum, negatively related to daily milk production in early lactation cows and inversely related to gestation length in dry cows. There is also a tendency for T4 and T3 to fall with age and for levels to be higher in winter. The interaction between iodine, thyroid function and selenium has also become clearer.

In man, primary hypothyroidism has been shown to affect the immune system, an effect manifested by a reduction in the number and functional activity of T-lymphocytes (Epishin et al., 1991). Disturbances in the endocrine system may also cause abnormalities of both cell mediated and antibody-associated immunity in animals (Greco and Harpold, 1994). Rogers (1991) mentions a reduced state of herd immunity as a possible clinical sign of iodine deficiency but fails to quote evidence to substantiate this assertion. More recently, the importance of thyroid hormones in

both the development and function of the immune system in cattle is discussed in a review by Klimiene et al. (2008). Anecdotally, in practice in Cumbria this condition is recognised, whereby herd immunity appears low, (increased mastitis, lameness, delayed wound healing etc.) yet is responsive to iodine supplementation (Paragon Veterinary Group, 2017, Personal Communication).

The classic manifestation of hypothyroidism due to primary iodine deficiency has been recognised for many years. However, because it was assumed that many organic goitrogens were destroyed in the rumen, their role was not established until later (Ahlin et al., 1994, Hemken, 1970). And more recently still, interactions with other trace elements such as selenium have been shown (see below).

Reduced reproductive performance has been associated with iodine deficiency for many years and Calderbank (1963) quotes several references to loss of libido in male animals and sub-oestrus in cows associated with hypothyroidism or thyroidectomy. Jamieson and Harbour (1947) suspected iodine deficiency as the cause of foetal goitre in stillborn lambs, as did Watson et al (1962). Allcroft et al (1954) associated a high herd incidence of stillbirth, abortion and weakly calves with low maternal plasma protein bound iodine and above average foetal thyroid weight, which showed hyperplasia on histology. However, it was Marine and Lenhart (1909) who demonstrated that histologically abnormal glands were significantly heavier than normal glands which was confirmed by the findings of Loosmore et al. (1962), Mee (1993) and Smyth et al. (1996).

Hernandez et al. (1972) shed some light on the cause of goitrous stillbirth when they found that the thyroxine level in normal bovine foetal serum was approximately twice the maternal level and was high enough to saturate the thyroid binding globulin. The maternal thyroid binding globulin was only two-thirds saturated thus providing a gradient for the transfer of thyroxine from foetus to dam. They suggested this high level of foetal thyroid activity was a homeostatic mechanism to increase calf survival in adverse conditions. Nathanie (1969), Nathanie et al. (1974) and Slebodzinski and Lipczak (1984) have also reported high neonatal thyroid activity in calves and lambs.

Mee (1991b) and Smyth et al. (1996) have linked stillbirths and peri-natal weak calf syndrome with iodine deficiency. However, although McCoy et al. (1997) were unable to reproduce the syndrome experimentally using iodine deficient diets based on wheat and soya-bean meal, pathological changes were noted in the thyroid glands of the calves from the dams fed deficient diets, and the authors do comment on the complex multi-factorial nature of this syndrome.

Moberg (1959, 1961) showed a reduction in the incidence of retained placentae in Finnish housed cattle of 47% in 257 herds compared to 127 control herds using iodine administered by sublimation and inhalation. Improved conception rates to first service were also recorded. Smirnova (1964) overcame the effects of an iodine deficient diet which was causing anovulatory oestrus by adding iodine to the diet, and pregnancy rates in repeat breeder cows grazed on iodine deficient pasture have also been shown to improve by adding iodine to the diet (McDonald *et al* 1961). Although the exact mechanisms behind these effects are unclear, they seem to affect the oestrus cycle. Hidiroglou (1979) suggests that the beneficial effects of iodine on reproductive performance may involve stimulation of the anterior pituitary gonadotrophin secretion mediated through the thyroid gland. Some support for this comes from the findings of Grunert et al. (1973) that intrauterine injections of iodine lengthened the oestrus cycle, although the exact mechanism for this was not demonstrated. Brown-Grant (1966) also suggests from work in the rat that lengthening of the oestrus cycle has an effect of iodine hormone secretion on thyrotropin releasing factor, which may in turn stimulate prolactin secretion. Iodine and protein-bound iodine concentrations are reduced in cows with cystic ovarian disease and in cows as they enter oestrus (Afiefy et al., 1970) although cause or effect was not clearly established. Miller et al. (1975) reported that oestrogens may also influence thyroid function possibly because the gonadotrophins, which stimulate oestrogen secretion, also have a direct effect on the thyroid gland.

1.2.3.1. Assessment of iodine status

The thyroid gland is the only known tissue to utilise iodine for a physiological function and iodine is primarily located in this gland. Both nutritional (plasma iodide, urinary I, plasma Se, I content in colostrum) and functional (thyrotropin, thyroid hormones)

markers of iodine status can be measured (Guyot et al., 2011). Wilson (1975) states that the thyroid contains 70-80% of total body iodine and that 90% of circulating iodine is in the form of T4. Serum T4 levels have been used extensively in the diagnosis of iodine deficiency in the field but there are several problems with this and interpretation may be difficult.

i. T4 levels vary with physiological events such as parturition, negative energy balance and lactation intensity as well as environmental variables such as ambient temperature (Refsal et al., 1984).

ii. Diets high in goitrogens can influence uptake and utilisation of iodine by the thyroid and hence influence T4 production (Stanley and Astwood, 1948, Taurog et al., 1947). Some goitrogens can competitively inhibit the uptake of iodine, such as thiocyanate and precursors which are present in commonly fed cattle feeds, such as rape, kale, soya bean, or beet pulp (Castro et al., 2011). Others affect the metabolism or release of T4 itself. These various actions may influence the measurement of status and strategies to overcome them (Kendall, N.K., 2017, Personal Communication).

iii. During periods of iodine deficiency qualitative changes may occur in thyroid secretions with reduced synthesis of T4 and enhanced production of T3 (Beckers and Delange 1980).

iv. Zagrodzki et al. (1998) proposed that although adequate selenium status is important for thyroid function and tissue conversion of T4 in cattle, it may also help compensate for dietary deficiencies of iodine. This is supported by work in sheep by Voudouri et al. (2003) who showed that there are compensatory mechanisms which can retain T3 levels, both by *de novo* synthesis, and by peripheral deiodination of T4, in situations where sheep are both Se and I deficient.

v. Controversy exists regarding the normal range of serum T4 in cattle. Whittaker (1999) suggests a lower limit of 20nmol/l as opposed to the 50nmol/l currently used by the Veterinary Laboratories Agency (currently known as the Animal & Plant Health Agency (APHA)) and that abortions are only seen below a maternal level of 10nmol/l and stillbirths below 15nmol/l. These proposed levels are based on data from 3940 samples analysed as part of the Dairy Herd Health and Productivity Scheme at the University of Edinburgh and on this basis Whittaker (1999) predicts that hypothyroidism is over-diagnosed in cattle in the United Kingdom.

vi. McCoy et al. (1997) recorded unchanged T4 levels in heifers fed iodine deficient diets, which showed other clinico-pathological and pathological changes consistent with iodine deficiency and these heifers went on to produce clinically normal calves. These findings support those of McCoy et al. (1995) who found serum T4 to be of limited diagnostic value when investigating iodine status in the field in pregnant cattle.

Plasma inorganic iodine (PII) is however a very sensitive indicator of current iodine intake in cattle (Rogers et al., 1996). It should be noted however that it is not a measure of thyroid function only of iodine supply. Rogers et al (1998) quote a normal range of 105-285µg/l but they accept that critical blood levels are controversial, a point also noted by Mee et al. (1994). Rogers in a personal communication however suggests a lower baseline for plasma inorganic iodine of 60µg/l and would only expect to encounter stillbirth problems below a level of 40µg/l. This view is broadly in agreement with levels suggested by McCoy et al. (1997). Plasma inorganic iodine levels have been found to normalise within 24 hours of supplementation but fall to control values within four to fifteen days of withdrawal of supplementation (Mee et al., 1993, Rogers et al., 1996). Mee et al. (1993) also found no variation in PII levels associated with parturition in spring calving dairy cows. They also stress the importance of sampling dry cows when assessing iodine status. Thus, to maintain normal levels of PII, supplements must be given regularly or on a continuous basis. The plasma inorganic iodine assay in plasma as described by Aumont and Tressol

(1987) was not widely used until the 1990's because the assay is time consuming to perform, requires extraction processes and has to be carried out under strict iodine free conditions by skilled staff. So, although it has been shown to be highly repeatable, it is expensive. Analysis of urine can give the same results, and is cheaper to perform as no extraction is required. Urine samples can be collected reasonably easily from cattle, with manual stimulation of urination, and although often successful, can be time consuming. As an alternative, urethral catheterisation is also possible.

1.2.4. Copper and interacting elements

Copper deficiency has been described as the second most common mineral deficiency in the world, behind phosphorus deficiency (Wikse et al., 1992). APHA (2016) reports over 100 cases per year in the UK since 2018, although this has been reducing since 2013, to 52 cases in 2015, but this may be an effect of lower submission rates to APHA. In the 1920s, "pot ale", a by-product of distilling was already being fed to livestock and stockmen noted then that it added extra "bloom and colour" to the black coats of cattle. This by-product was further concentrated in the 1960s into "pot ale syrup" (Black, H.L. et al., 1991) and became more widely used, being fed in troughs to cattle and sheep. However, sheep fed dark grains (draff and pot ale syrup) were healthy outdoors, but died if fed this product while indoors particularly if stressed – it was attributed at the time to increased iron intake while at pasture which was having a protective effect (Black, H.L., Personal Communication). This was later concurred with by Gould and Kendall (2011) who discussed the effects of water and soil and the protective effect of iron when thiomolybdates are present in the rumen. From these studies, and others initiated in the 1950s which demonstrated how closely the absorption, utilisation, and excretion of copper were linked to other dietary elements – in particular molybdenum and iron - it was suggested two types of copper deficiency existed in practice; primary and secondary. Historically, primary deficiency has been described as resulting from the simple deficiency of copper in the diet, and secondary deficiency as resulting from the depression of copper utilisation through the antagonistic effects of molybdenum, sulphur and iron. However, many clinicians and some authors now believe that this "copper responsive" condition is actually thiomolybdate toxicity (Telfer et al., 2003, Telfer et al., 2004, Black and French, 2004).

Or more accurately, as later described by Gould and Kendall (2011), a combination of low copper, antagonists preventing copper absorption, and thiomoybdates being produced and absorbed. Experimentally, in sheep, in the absence of interference from molybdenum or iron, the absolute dietary requirement for copper has been shown to be extremely low; less than 1.6mg/kg of Dry Matter (Moeini, 1997).

The principal mechanism of copper depletion is accepted to be its formation of unabsorbable complexes with tri- and tetrathiomolybdates in the rumen, which then bind to the solid phase of the digesta, while iron and sulphur also complex with copper rendering it even less available, although the exact chemistry of this reaction is unclear. Gould and Kendall (2011) examined in detail the role of the rumen in copper and thiomolybdate absorption, and neatly summarise the mechanisms in Figure 1.1.

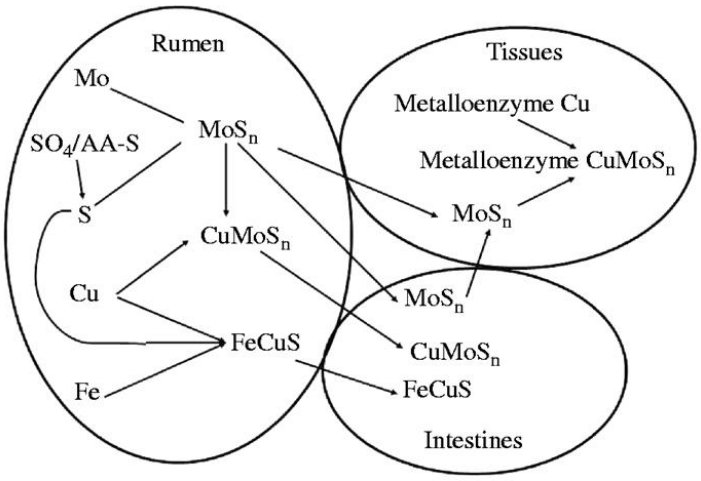


Figure 1.1. Example ruminal mechanism for the interaction of Cu, Mo, S and Fe and routes of absorption for the interaction products. MoS_n is used diagrammatically to represent the thiomolybdate series – *adapted from Gould and Kendall (2011).*

Gould and Kendall (2011) conclude that all classes of thiomolybdates can be formed in the rumen, and that as the sulphur:molybdenum ratio increases (sulphates are reduced to sulphides by rumen micro-organisms) the dynamic is driven towards the more potent tetrathiomolybdate (TTM). This is dynamic and it is unlikely to reach equilibrium because substrates are available and there is constant removal of

thiomoybdate, as it a) complexes with free copper in the rumen if it is present in adequate quantities, b) is absorbed through the rumen wall, or c) passes into the small intestine.

Other dietary factors also seem to be important. Sinclair et al. (2017) compared dairy cows being fed on grass silage or maize silage based forage rations with and without sulphur and molybdenum supplementation. After addition of S and Mo, hepatic copper and iron concentrations decreased in both grass and maize fed groups but much more so in the grass-fed animals. This was associated with lower dry matter intakes, reduced milk yield and an increased somatic cell count in the grass silage fed groups while no changes in these parameters were seen in the maize silage fed animals. The causes of these changes are unclear, and although there may be ruminal flora or rumen content consistency effects, it is also possibly due to the different bioavailability of the organically bound S and Mo in grass silage against the inorganic elements used to balance diets for total S and Mo.

Reduced fertility associated with copper deficiency has been widely believed for many years but there are differing, occasionally conflicting, reports in the literature on the effect of low copper status on reproduction in cattle (Siciliano-Jones et al., 2008, Stahlhut et al., 2006, Phillippo et al., 1987a, Black and French, 2004). The classic manifestations of copper deficiency are well recognised, such as unthrifty calves with rough faded coats and diarrhoea, but because of the importance of copper in several enzymes including cytochrome oxidase, superoxide dismutase, caeruloplasmin, lysyl oxidase and tyrosinase, a deficiency can result in many different physiological dysfunctions, ultimately producing many other clinical and subclinical syndromes. Tetrathiomolybdate has been shown to inhibit these enzymes in vitro (Chidambaram et al., 1984) so it is reasonable to suggest that this might be the cause of the changes seen. Correlations between low serum plasma copper and clinical signs have sometimes been contradictory (Rowlands et al., 1977, Mee, 1991a), Suttle 2010). Phillippo et al. (1987a, 1987b) elegantly demonstrated that hypocupraemia and liver copper levels in cattle could be induced using combinations of either sulphur and molybdenum or sulphur and iron, yet clinical signs (delayed onset of puberty, lower

weight gain, and reduced fertility) were only evident in the molybdenum-supplemented group. These fertility effects were reversed when the molybdenum and iron supplementation of the two groups were reversed, associated with a decreased rate and size of LH pulsatility in the molybdenum-supplemented cattle.

This mechanism affecting LH has been further investigated, and there appear to be several aspects to it. Copper-histidine complexes have been shown to be necessary for GnRH release (Haywood et al., 2004), while ionic copper and histidine individually had no effect. In sheep with ovarian autotransplants, Kendall et al (2005), demonstrated that infusion with tetrathiomolybdate altered copper status (for example CP:PlCu ration fell over the infusion period), and ovarian function was disturbed, associated with reductions in reproductive hormones. In particular there was a suggestion that post TTM infusion progesterone levels were lower after ovulation which could have an effect on early embryonic development, reducing interferon τ (tau) production which is required to signal to the dam that she is pregnant. It has been demonstrated that in vitro both FSH-induced differentiation of bovine granulosa cells (Kendall et al., 2003) and LH-induced differentiation of bovine theca cells (Kendall et al., 2006) can be blocked by tetrathiomolybdates but these effects can be ameliorated by copper supplementation. These studies support the hypothesis that copper-responsive subfertility results from thiomolybdate induced perturbation of the normal pattern of ovulatory follicle growth and the normal pattern of ovarian steroidogenesis. Pathological changes in the pituitary of sheep have been reported after parenteral administration of thiomolybdates (Haywood et al., 2004) and dietary inclusion of molybdenum (Williams 2004). This latter study also found changes in ACTH concentrations in the pituitary gland, which was dose dependant and suggested some sort of block to the release of ACTH. Although this mechanism is not yet understood, there may be involvement of a copper dependant enzyme, peptidylglycine α-amidating monooxygenase (PAM) (Steveson et al., 2003). Work by Frank et al. (2002) investigating a wasting disease in Swedish moose showed that cytochrome c-oxidase activity and copper content were both reduced in the myocardium of affected animals. They suggested that these findings supported the cause of the disease being “molybdenosis”. This enzyme is required for oxidative

phosphorylation and energy production in the cell, and this mechanism may be the explanation for increased milk yields being recorded in dairy cows after copper supplementation.

Anchordoquy et al. (2017) found no difference in copper concentrations in bovine oviduct fluid despite blood copper levels that were described as adequate, marginal or deficient. However, in an IVP system, the addition of 40 µg/dL Cu to IVF medium enhanced several sperm functions including motility, viability and zona binding. The additional copper did not improve sperm cell acrosome integrity or oocyte cleavage rates after IVF, but it did impair blastocyst rates. Copper uptake into cells is facilitated by a protein transporter SLC31A1 (CTR1) and this was detected in the acrosome of bovine sperm, and in the plasma membrane of in vitro matured oocytes. So, although in this study the plasma copper levels did not affect pregnancy rates 60 days after fixed time artificial insemination, the presence of CTR1 in oocytes and sperm implies an important role of Cu during fertilization.

Iron can further reduce copper availability and although neither hypothesis is proven, there are two possible mechanisms; in the rumen iron can react with sulphide and copper to produce either a) an iron-copper-sulphur complex which is not absorbed, or b) iron sulphide forms which then exchanges with copper to form copper sulphide (Gould and Kendall, 2011).

Soil type and soil pH in particular affects trace element availability. In contrast to most trace minerals, Mo is more available to plants at higher soil pH concentrations (Smith et al 1997); in more acidic soils (pH <5·5) Mo becomes less available as anion adsorption to soil oxides increases (Reddy et al., 1997). Soil contamination of forages can be significant, depending on weather conditions and storage, particularly of silage. Grazing soil intakes increases when grass is short (dry summer, late autumn, early spring) and ground is either wet (when soil splashes the foliage) or very dry (when dust contaminates the foliage). Recently fertilisers have increasingly included sulphur which gives higher forage yields and may increase palatability. All these components can combine to reduce copper availability or increase the chances of tetrathiomolybdate formation in the rumen.

1.2.4.1. Assessment of copper status

Assessment of copper status is problematic, with no single method accepted as reliable (Laven and Livesey, 2005). The liver, being the main storage organ for copper, is considered to be the most accurate tissue for assessment of copper status, particularly of copper accumulation/loading (Kendall et al, 2015). Blood copper concentrations (whole blood, plasma or serum) have been used extensively, but normal plasma Cu concentrations can be maintained until liver levels fall below 40mg/kg dry matter (Claypool et al., 1975), and plasma Cu concentrations are higher than serum Cu concentrations. In some situations, ruminants can tolerate subnormal plasma copper levels with no signs of illness or malfunction (Woolliams et al., 1983, Wiener et al., 1984, Phillippo et al., 1987b) and plasma or serum copper levels may falsely indicate adequacy when diets are high in molybdenum.

Plasma or serum copper levels have been used extensively in the diagnosis and monitoring of copper deficiency, but there are several problems with this. Suttle (1991) suggested that in such cases a proportion of the blood copper is physiologically unavailable to the animal as it is bound up as a Cu-thiomolybdate-albumin complex, which is insoluble in trichloroacetic acid (TCA). In *in vitro* studies, Dick et al. (1975) have shown that increasing amounts of di-, tri-, or tetra-thiomolybdates in plasma resulted in a decreasing amount of copper in the TCA-soluble fraction, until at a level of 3ug/ml of TTM in plasma, all the copper was found in the TCA-insoluble fraction. However, addition of molybdate with or without sulphate resulted in negligible copper in the TCA-insoluble precipitate, suggesting that the measurement of TCA-insoluble copper can be used as an indication of thiomolybdate presence.

Caeruloplasmin (CP) activity has been shown to be closely related to blood copper concentrations and in both serum and plasma. It was found to be more suitable for assessing 'marginal' blood Cu status in cattle than serum Cu concentration because of the variability in losses of Cu in clotting (Laven et al., 2007). Because CP is a protein enzyme containing 6 copper atoms per molecule, if one of these atoms is chelated by thiomolybdate the enzyme is rendered inactive. Chidambaram et al. (1984) showed that caeruloplasmin and tetra-thiomolybdate treated caeruloplasmin both contained

six copper atoms, suggesting that tetra-thiomolybdate does not actually remove Cu from caeruloplasmin to inactivate it. Therefore, attack by thiomolybdate may result in an uneven effect, reducing the enzyme activity some 6 times faster than the copper content of plasma. However, as caeruloplasmin is an acute phase protein, released from the liver in response to inflammation, it is likely that the traumas of calving, mixing with the herd etc. around calving would cause some release, but this would only be expected for a few days. So, although caeruloplasmin activity may be a more accurate method of assessing copper status, especially when high Mo levels are involved, being an acute phase protein, concentrations are affected by individual disease states. Wang et al. (1988) showed that caeruloplasmin and TCA-soluble copper concentrations decreased while total Cu increased in the plasma of calves fed a diet high in S and Mo. Telfer et al. (1996) and MacKenzie et al. (1996) have suggested that an accurate predictor of likely response to copper supplementation is the ratio of caeruloplasmin (CP) activity to plasma copper (Pl-Cu) concentration, with the presence of thiomolybdates reducing the ratio below 1.9, a level which is considered optimal. Caeruloplasmin has a turnover of only 2-3 days, and therefore providing supplemental copper for thiomolybdate to bind with in the rumen and small intestine would effectively 'spare' the caeruloplasmin from attack by thiomolybdate and the CP:Pl-Cu ratio would rise again.

Erythrocyte superoxide dismutase is a copper-containing enzyme whose activity remains low in situations of long-standing Cu deficiency or thiomolybdate challenge (Kendall et al., 2015), and in sheep has been shown to be depressed by short term tetrathiomolybdate infusion (Kendall et al, 2005).

1.2.4.2. Copper toxicity

There has been much debate recently about copper toxicity in dairy cattle in the UK, with cases per year in the UK ranging recently between 17 and 24 (APHA, 2016) and this incidence prompting an industry-led working group to investigate the causes and produce a guidance note (Bone et al 2011). Further to this report a survey of liver copper samples from 510 cull cattle was undertaken in the UK (Kendall et al., 2015) and the authors considered the importance of copper loading, defined as the

accumulation of copper in the liver which may be a precursor to chronic copper toxicity. Almost 40% of UK dairy cows had liver copper levels above the AHVLA reference range of 8000 μmol/kg dry matter. These cases are likely to be due to overzealous use of inappropriate copper supplementation in the presence of molybdenum and iron (Sinclair and Atkins, 2015).

1.2.5. Mineral supplementation

The requirements of minerals in ruminants is not disputed but exactly when, how and where they should be supplanted can be complex (Kendall 2014). Mineral supplementation in cattle can be undertaken in several ways and tailored to the situation and element(s) required;

i. soil or forage application of mineral compounds – this can be measured by soil or forage analysis but uptake will be affected by many environmental factors
ii. free access minerals, either as a powder, treacle or block – this method cannot control individual supplementation and intakes will vary depending on social grouping and environmental conditions
iii. addition to feed, for example in a total mixed ration (TMR) – this ensures an average daily consumption related to the dry matter intake of an individual, but again variation is inevitable. If given in the feed, it is best to be in the forage component to avoid sorting and selection, and to give more even intakes, which should not be related to production as may be the case with parlour or out of parlour concentrate feeding. This route is not suitable for purely grazing systems
iv. addition to water – this is affected by the solubility of the minerals and is less effective during grazing seasons, and in differing weather conditions, as water intakes vary
v. individual oral supplementation by drenches – this is more controlled per animal but would require frequent treatments as many minerals tend to be absorbed slowly and one-off oral drenches are unlikely to be absorbed rapidly enough to be stored

vi. individual oral supplementation by boluses – these are designed to reside in the reticulum and may be either metal matrix or glass They are designed to release minerals over a period of time, although the rate of release will be affected by rumen pH and rumen outflow rates

vii. topical application so that the animal or its cohorts ingests the supplement by grooming and possibly by some skin absorption – this is again a very inexact method although relatively easy to apply

viii. injection of a slow release compound usually subcutaneously – an exact dose per animal can be given but again will be metabolised at different rates in different types of animal.

Allen and Mallinson (1984) concluded that parenteral mineral supplements had either to provide a source of the element over a long period of time, or to increase the animal's physiological stores of it. These aims required introduction of a large amount of the element in a single dose that would not be toxic, and therefore had to be constrained either chemically or physically.

When giving copper supplements, if sacrificial copper is not supplied to the rumen to bind excess thiomolybdates they will still be absorbed, prior to copper being absorbed further down the digestive tract which may not fully compensate for the toxic effects of the thiomolybdates. For example, copper oxide is only solubilised in the acidic pH of the abomasum and copper chelates are designed to largely bypass the rumen, although there may be some tertrathiomolybdate binding in the rumen. And the most common copper salt used is copper sulphate, whereby the sulphates may also be driving the equilibrium towards the more toxic tetrathiomolybdate (Section 1.2.4). From this understanding that the copper-molybdenum interaction was located in the gut, it was suggested that the preferred means of administering copper was by injection in cases of secondary (molybdenum induced) copper deficiency. Other methods of copper therapy have been based on slow-release ruminal boluses.

A study conducted by Black and French (2004) compared 3 approaches; subcutaneous injections of copper and selenium, matrix intraruminal boluses (All Trace®, Agrimin, Carnforth, UK) and glass intraruminal boluses (CoSeCure®, Telsol, Leeds UK). When the data from all 3 study farms were combined, there was a significant difference between treatment groups ($P<0.001$). Cows treated with the glass bolus conceived at a rate 1.8 times greater than those treated by injection ($P<0.001$), and at a rate 1.5 times greater than those treated by matrix bolus ($P<0.01$). This was associated with significantly higher likelihood of service resulting in a conception than in the injection treated group ($P<0.01$). Black and French (2004) acknowledge that it is not possible to categorically state that copper was the only reason for the findings, and indeed the only significant blood parameter finding was a difference in GSHPx levels. However, it is believed that this relatively small difference in GSHPx levels was unlikely to account for such large fertility differences, and that the method of copper supplementation was a possible explanation. The glass bolus releases copper and cobalt in an ionic association with a phosphate group; the negatively charged oxygen ions of the phosphate hold the positive copper ions in a complex that is released into solution as the glass dissolves. Thiomolybdates have a very high affinity constant for copper and hence attract the copper away from the phosphate and form copper thiomolybdate complexes (Telfer S.B., 1984, Telfer S.B., 2012, Personal Communication).

As discussed in Section 1.2.2.1, selenium has been shown to have a synergistic effect on iodine uptake and utilisation by the thyroid gland in cattle. It is logical therefore that the manufacturers of commercial supplements would seek to incorporate both elements in a single product, as well as copper and other trace elements.

Rogers (1991) quotes dietary requirements of iodine for adult cows in the range of 12-15mg per head per day to prevent simple iodine deficiency rising to 30-60mg per head per day to prevent secondary deficiency due to intake of goitrogens. Mee et al (1996) also found these levels necessary to maintain PII in the range 105-285µg/l in Irish forage fed cows. Guyot et al. (2011) undertook a study to look at the longer-term effects of combined iodine and selenium supplementation in pregnant cattle and the

subsequent effect on their calves, using both nutritional and functional markers. They showed that supplementation of the dam was effective at improving the iodine status of newborn calves, and urged caution when interpreting plasma selenium status during high iodine supplementation as it can impact on selenium metabolism when both are supplemented together.

Numerous methods of iodine supplementation have been advocated and were reviewed by Rogers (1991). Ellis et al (1983) developed a slow release intra-ruminal device for sheep, which released iodine over a period of three years. However, until recently this concept has been little exploited in cattle. The All Trace® (Agrimin, Carnforth, UK) multiple trace element matrix bolus treatment for cattle is designed to supply iodine along with other minerals and vitamins, but as Rogers (1991) points out, at the claimed release rate of 2.2mg iodine per day from two boluses, about 22 boluses are needed to supply a cow with 25mg iodine per day.

Rogers et al (1998) showed significant increases in PII and glutathione peroxidase (GSHPx) using a zinc-ballasted matrix bolus containing 3400mg iodine, 500mg selenium and 350mg of cobalt (Ionox®, Bayer, Leverkusen, Germany) of up to 23 and 33 weeks respectively in Irish cattle using two boluses per animal. Although the numbers used in the original trial in 1996 were small at seven animals per group this was followed by a field trial in the United Kingdom (UK), reported in 1998 involving 47 commercial herds, where administration of one bolus produced an increase in plasma inorganic iodine of 117+/- 10.7µg/l relative to controls ($P<0.001$) over a one-month period.

A clinical trial by Cook and Green, (2007) investigated the use of an intra-ruminal bolus and was undertaken on four commercial dairy herds to compare the effect of iodine, selenium and cobalt supplementation with no treatment on farms known to be marginal in iodine. There was a significant effect of treatment on the likelihood of an animal retaining its foetal membranes, a control cow being more likely to retain her membranes (approximately 10%) than a treated cow (approximately 4%) ($P<0.05$). Despite the increased incidence of retained membranes in the control group

significant effects on fertility between treatment groups were not observed, but this was probably due to effective veterinary intervention, and the fact that numbers were insufficient to have a significant effect on fertility. It was concluded that in this trial, treatment with the bolus (Ionox®, Bayer, Leverkusen, Germany) reduced the risk of a cow retaining foetal membranes. However, these boluses also contained selenium and cobalt, so the effect cannot be attributed only to the iodine supplementation. Further analysis of the same data set Cook and Green (2010) found that supplementation with iodine, selenium and cobalt increased the milk production of dairy cows in the first 100 days of lactation. The value of this is limited, as this data was from one farm with a known marginal iodine status, but this is the first published data to show an increase in milk production from the use of a combined iodine, selenium and cobalt bolus.

In a small study Black et al (1996) comparing glass ruminal mineral boluses from the same manufacturer, one of which contained additional iodine (Cosecure® and Coseicure®, Telsol, Leeds UK), there were no significant effects on fertility. The group treated with the higher iodine boluses did have significantly increased haemoglobin (Hb) levels – which is likely to positively affect health and production – but it is likely that the iodine containing boluses dissolved faster releasing higher amounts of all three elements.

Importantly, In the studies described above investigating mineral supplementation, it has not been possible to have enough statistical power to demonstrate significant differences in some parameters, although trends were identified. In many studies of this nature, several farms, or epidemiologically separate groups of animals have to be used to achieve large enough numbers which in itself introduces many more confounding variables. This demonstrates a need for more controlled experiments on fewer animals under similar environmental conditions.

1.2.6. Summary

This part of the review has only been able to consider the relevance in bovine reproduction of selenium and iodine, as well as copper and its interactions with

molybdenum, sulphur and iron. And there are many more trace elements that are likely to be having an effect in cattle. It is clear that the knowledge of the modes of action of micro-minerals, their interactions, assessment and supplementation is patchy and incomplete. Examples of important, yet poorly understood areas in the bovine include; -

i. the anti-oxidative mechanisms by which selenium and Vitamin E act in relation to bovine fertility and the way they can partially compensate for each other
ii. the interactions between iodine and selenium in the bovine, and their interdependence
iii. the unresolved debate about whether copper responsive conditions seen in the bovine are true copper deficiencies or thiomolybdate toxicities

Furthermore, there has been little work to date on the effects of trace elements on the ovarian dynamics (follicles and corpora lutea), the oocyte, early embryo development or the uterine environment.

Further tools are required to be able to study these trace elements in cattle, both at the level of reproductive biology, but also at a more macro level, and ideally without the need for lengthy, expensive trials involving large sample sizes. Better understanding of these trace elements, and applying this knowledge is likely to have a significant impact on the health, welfare and productivity of domestic cattle. Utilising OPU/IVP as a research platform has many advantages which are discussed in the next part of this literature review.

1.3. Fertility, oocyte quality and bovine assisted reproduction

1.3.1. Introduction

Currently trials in cattle to investigate fertility, health or production traits, or interventions such as medicines or dietary manipulations, need to be large scale to provide enough statistical power to prove differences, so it is expensive to test interventions against control groups. The outcome of many of these trials is often a confirmed established pregnancy. For example, to prove a difference in pregnancy rate of 10% between two groups with a confidence level of 95%, using a two-tailed

test would require 496 attempts (by insemination or embryo transfer) to establish a pregnancy in each group (Chapman and Seidel, 2008).

Since the birth of the first calf after transfer of an embryo produced by in-vitro fertilisation (IVF) was reported by Brackett et al. (1982), advances have been made in the development of relatively simple methods for producing bovine embryos in-vitro as reviewed by Thompson (1996). The development of a technique known as ovum pick-up (OPU), which facilitateds the recovery of pre-ovulatory oocytes from live donors (Pieterse et al., 1988) was largely responsible for the expansion in commercial application of IVF.

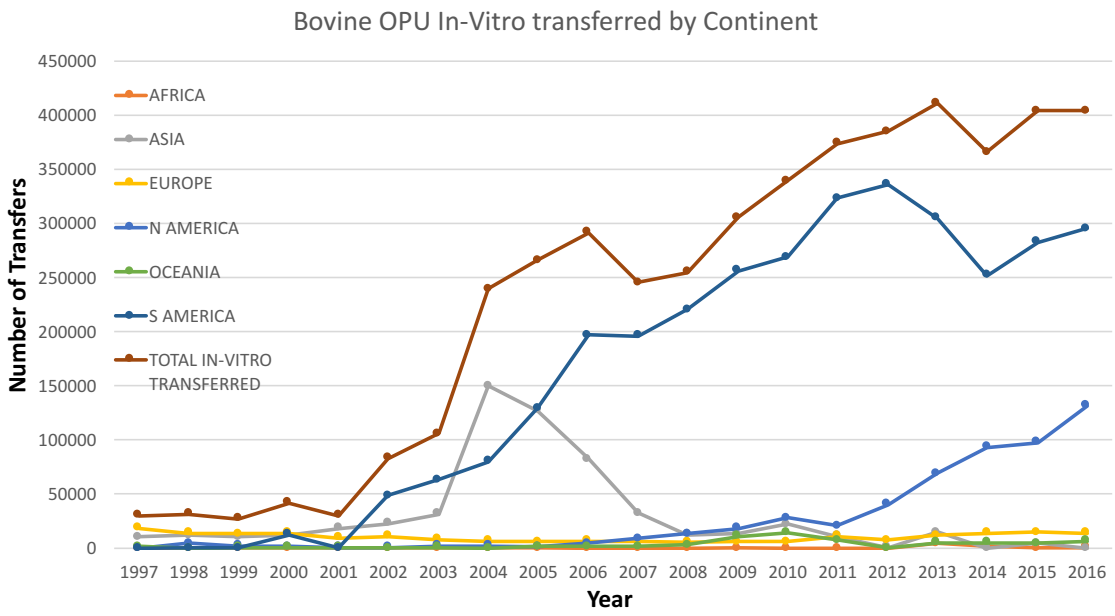


Figure 1.2. The number of OPU/IVP embryos transferred worldwide and by continent, between 1997 and 2016 (adapted from Perry 2017)

Since 2005 there has been a steady decline in the number of *in vivo* produced embryos, from around 800,000 to 632,638 in 2016, while the number of *in vitro* produced embryos has steadily increased to 632,958 in 2016 (Perry, 2017), overtaking the number of *in vivo* produced embryos per year for the first time. This has been largely driven by a steady, increase in the number of transfers since 2001 in South America (especially with *Bos indicus* cattle which seem more suited to these techniques). Since 2011 there has been an average 30% annual increase in the number

of OPU/IVP embryos transferred in North America, yet there has been little change in the number transferred in Europe (Perry, 2017). See Figure 1.2.

1.3.2. Advantages of OPU/IVP

The techniques of Ovum Pickup (OPU) and In-Vitro Embryo Production (IVP) have significant advantages over both traditional breeding programmes and the use of conventional multiple ovulation embryo transfer (MOET);

i. The process is non-surgical and requires either no follicle stimulating hormone (FSH) or lower doses of FSH than for conventional ET
ii. OPU collections can be performed more frequently (weekly or fortnightly), so more oocytes can be collected in a shorter period
iii. Oocytes can be collected from both juvenile heifers, and pregnant donors during the first trimester, extending the number of potential embryos which can be produced
iv. The technique can be used on animals with a range of reproductive disorders which might not otherwise be able to continue breeding
v. Less semen is used per fertilisation so multiple donors can be fertilised with a single straw - saving money and utilising limited semen stocks
vi. A wide range of bulls can be used, due to the frequency of collection, giving greater scope for genetic improvement

1.3.3. OPU/IVP to amplify genetic gain

To achieve the increased health, welfare and productivity of animals producing protein, as well as to reduce the impact livestock farming might have on climate change, greenhouse gas (GHG) emissions and the demand for fresh water, there is a plethora of existing and on-going research. It is imperative that there is a greater amplification and distribution of these outputs. Cattle breeders recognise that it is important to generate as many offspring as possible from genetically superior or important animals, and although the widespread use of artificial insemination has led to very significant improvements in the genetic merit of cattle, there is a need to amplify female genetic lines as well. The rate of genetic selection for quantitative traits

can be increased by using advanced breeding technologies such as multiple ovulation embryo transfer (MOET) or ovum pickup and in-vitro fertilisation OPU/IVP (Hansen and Block, 2004). This is achieved by improving the accuracy and intensity of selection, and OPU/IVP can at the same time significantly reduce the generation interval (see Figure 1.3) as it can be used in much younger heifers and can utilise semen from a selection of young genomic sires. New technological developments are likely to further enhance the technique. With the bovine genome now mapped to over 3 billion base pairs, or 22,000 genes (Larkin, 2011) our emerging understanding of genotypes will further enhance our ability to select parents to use in OPU/IVP programmes, and even of individual embryos prior to transfer. The adoption of genomic screening of heifers as a robust scientific method for selecting animals with superior genetic potential in combination with advanced breeding technologies (OPU/IVP-ET) will advance the rate of genetic improvement in traits of commercial importance.

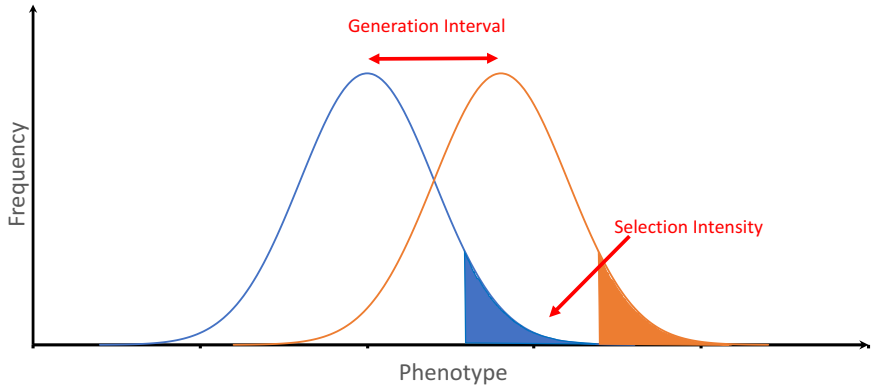


Figure 1.3. The genetic gain equation and normal distribution curves of a population, with the frequency of a trait within a breeding population (y-axis) against the phenotype (x-axis) showing schematically how reducing generation interval and increasing selection intensity by selecting animals at one extreme of the population can increase genetic gain.

1.3.4. IVP as an experimental tool

If parameters from within an IVP program such as oocyte collection rates, fertilisation rates, cleavage rates or blastocyst rates, could be correlated with subsequent pregnancy, then in the future these markers of fertility would facilitate smaller and faster studies being undertaken. It is also anticipated that specific genes in the cumulus cells may be markers of oocyte quality (Section 1.3.7.2) and this would give an even quicker assessment of intervention effects. If crossover trials of small groups of animals were to be possible, this would greatly enhance the statistical power of intervention studies.

1.3.5. The Potential of OPU/IVP

Further technological developments are likely to further enhance advanced breeding technologies. The fact that bona fide oocytes have been produced by putative germ cells in bone marrow and peripheral blood, (Johnson et al., 2005) and that oocytes and granulosa cells (GCs) have been produced in vitro from murine embryonic stem cells (ESCs) - their identity confirmed by electron microscopy (Hubner et al., 2011) - could mean that the pool of gametes from selected individuals in the future is technically limitless.

In most dairy systems, farmers require replacement heifers for their herd of a breeding that they desire, so bull calves are unwanted and wasteful, unless they can be of a cross that will be suitable for fattening. The advances in reliability of sexed semen for conventional artificial insemination (AI) have led to an interest in this being used in IVP systems. Studies in Texas by Stewart et al. (2011) have shown that by using sexed semen to produce in vitro embryos that were transferred fresh to lactating dairy cows there was an increased percentage of cows that became pregnant and also of cows giving birth to a live heifer compared with percentages from AI with conventional semen. Sexing of embryos by biopsy of the 7-day embryo using a micro-blade has been used successfully commercially (Lacaze et al., 2008), usually to select female embryos but is time consuming on farm, and there is wastage of the male embryos. Use of sexed semen means that 98% of all embryos produced are of the desired gender, and hence the number of embryos suitable for transfer is effectively doubled.

With declining pregnancy rates in dairy cattle (Royal et al., 2000, Dobson et al., 2007), any solutions to improve fertility are attractive to farmers, and OPU/IVP has been suggested as a means of bypassing, or at least limiting some of the known problems. Although Sartori et al. (2006) found that embryo transfer did not improve overall pregnancy rates compared with artificial insemination in lactating dairy cows there was an apparent benefit of ET when single ovulating follicles were small. Yet Demetrio et al. (2007) concluded that the transfer of fresh embryos did increase the probability of conception of lactating Holstein cows and suggested it was because ET can bypass the negative effects of increased milk production and low progesterone on the early embryo. This effect was most evident in high-producing cows and is thought to be associated with the increased dry matter intakes associated with higher milk production resulting in lower circulating progesterone, possibly as a result of increased hepatic blood flow and therefore faster metabolic clearance (Vasconcelos et al., 2003). They also demonstrated that high body temperature measured on day 7 had a negative effect on conception rates and embryonic retention. This links to the findings of a review paper by Rutledge (2001) which suggested that a major pathway is in the effects of maternal heat stress on the early cleavage stage embryo. So therefore, higher pregnancy rates can be obtained with transfer of late cleavage stage embryos. Another solution to heat stress in Japan was to utilise ET after AI, which was found to significantly improve pregnancy rates, but the effects were reduced by higher rates of foetal loss (Tani et al., 2011).

The OPU/IVP technique is also useful in individual infertile cows, where the causes are failure of ovulation or fallopian transport or where the uterine environment will not support a pregnancy (such as low-grade endometritis) or in situations of early embryonic death (Hansen, 2006). An essential part of the establishment of pregnancy is the production of interferon τ (IFNT) by the elongating blastocyst. IFNT is detectable in uterine fluid from days 12 to 25 and in the cow, it is thought that it inhibits oxytocin receptor up-regulation, which in turn suppresses luteolytic pulses of prostaglandin F2α (Robinson et al., 2010). This is the basis behind the technique of implanting "support" or "cowstopper" embryos (blastocysts) one week after an insemination; part of their effect being to create an additional source of IFNT and therefore improve maternal recognition of pregnancy. IVP embryos could be produced relatively cheaply

from abattoir ovaries to act as "support" embryos – unpublished results (Mullan, J.S., 2011, Personal Communication) suggest that around 70% of calves born assisted by this method are the dam's own, the others either being the implanted "support" embryo or twins. If it was undesirable to have the chance of an implanted "support" embryo developing, then parthenotes (activated, unfertilised oocytes) could be produced and implanted. Indeed, it has been shown by investigating gene expression and measuring mRNA by RT-PCR that early parthenotes produce more IFNT than fertilised embryos (Labrecque and Sirard, 2011).

Crossbreeding has been widely used in the beef industry for decades and there has been a trend towards more crossbreeding in the dairy herd recently, particularly to avoid dystocia problems with Holstein heifers (Olson et al., 2009). At least 10% heterosis can be expected for total genetic merit, mainly due to increased longevity and improvement of functional traits. There is however some evidence of recombinant loss, and it is critical for long-term crossbreeding that genetic gain within the parental breeds is not reduced (Sorensen et al., 2008). So, IVP is likely to have a place not only in amplifying purebred genetics, but also in creating F1 embryos for crossbreeding programmes, especially as the use of single-nucleotide polymorphism (SNP) chips of low density make genomic selection applications economically more feasible (Ibanez-Escriche and Gonzalez-Recio, 2011).

1.3.6. OPU/IVP as a basis for other technologies

Nuclear cloning and transgenesis are possible, but are currently limited largely by societal concerns, which have swung from initial debate about the potential cloning of humans to that of using human embryos to produce stem cells for research (Wadman 2007) – however these techniques will also benefit from improved OPU/IVP technologies, and are likely to become a breeding tool of the future (Campbell et al., 2007).

Intracytoplasmic sperm injection (ICSI) is a technique where a single sperm cell, with acrosome and sperm membrane intact is directly injected into a metaphase II oocyte, and then cultured in-vitro. Although it is a technique most widely used as a "last resort" in human assisted reproduction, it yields relatively poor blastocyst numbers and pregnancy results in livestock. However, it may be a technique to be used for

genetic salvage, transgenic production, or to improve efficiencies in IVP systems especially when using sexed semen, which is less robust than conventional semen (Garcia-Rosello et al., 2009).

1.3.7. Current understanding and limitations of OPU/IVP

1.3.7.1. Ovum pickup (OPU)

Ultrasound guided transvaginal aspiration of bovine oocytes is now a relatively well described and repeatable technique, although manual syringe aspiration of slaughterhouse ovaries tends to give higher recovery rates (Hashimoto et al., 1999a). For optimal oocyte quality, there are mechanical factors such as the resolution of the ultrasound image associated with different frequencies (Hashimoto et al., 1999b), aspiration procedure, needle type, and the influence of the aspiration vacuum on cumulus oocyte complex (COC) morphology (Hashimoto et al., 1998, Jaskowski, 2001). There are also biological influences such as hormonal priming prior to follicle aspiration (Hashimoto et al., 2000, Seneda et al., 2005), timing of the procedure within the oestrus cycle, age, breed, and body condition of the donor (Bols, 2005). Initially, in the UK, the preferred combination was a 7.5 MHz probe, 19-gauge needle and 80mm Hg of aspiration pressure (Mullan, J.S., 2011, Personal Communication), but this has now been superseded as described in Chapter 3. Although it has been shown that the recovery rate of oocytes is better from follicles less than or equal to 4 mm in diameter, quality, cleavage rate and blastocyst development did not differ between different follicle sizes (Seneda et al., 2001).

OPU/IVP can be undertaken with no hormonal intervention; OPU being conducted weekly or even twice weekly. However, cumulus-oocyte complexes (COCs) are not all developmentally competent. Blondin (2002) describes a method of “coasting” and he states that with this technique “never has in vitro technology been so close to producing 100% developmentally competent COCs”. It is also understood that optimal oocyte competence in the bovine develops between the follicle stimulation hormone (FSH) surge and the pre-ovulation luteinizing hormone (LH) surge (Sirard et al. 2006). ‘Coasting’ therefore refers to FSH stimulation, at a lower dose than used in MOET, followed by FSH withdrawal (typically 48 h) prior to OPU.

1.3.7.2. Oocyte selection

The standard procedure for selecting oocytes after OPU aspiration is to identify the COCs in the aspirate, grade by the presence and abundance of cumulus cells, wash and place in maturation medium. However, the assessment of oocyte quality is critical (Goovaerts et al., 2010). Various reviews of IVP and ET concur that good quality oocytes are imperative to achieve good blastocyst rates (Merton et al., 2003, Galli et al., 2001), and there is a need for reliable non-invasive techniques to assess oocyte quality (Goovaerts et al., 2010). There is general agreement that the quality of the COC, and specifically the intact cumulus cell investment (Tanghe et al., 2002) and the homogeneity of the cytoplasm (Boni et al., 2002) are very important and an ability to select those with the best developmental potential would improve the blastocyst production rate which is currently limited to around 30-40%. Assessment of COC using light microscopy is the most common technique although the visualisation of structures within the COC are hampered by the dark cumulus cells of bovine COCs compared to the translucency of human equivalents. COCs are usually grouped in relation to their quality grade as proposed by de Wit et al. (2000); 1) COC-A: compact and bright, 2) COC-B: less compact and dark, and 3) COC-C: strongly expanded cumulus with dark spots. These studies suggest that in the bovine the morphological criteria for oocyte classification are related to developmental competence. If the COCs are further classified it has been shown that those complexes that are just at the beginning of expansion in outer cumulus layers and have slight granulations in the ooplasm developed past the 16-cell stage significantly more than oocytes with a compact and complete cumulus or oocytes with incomplete and/or expanded cumulus. This is thought to be because these oocytes are from more atretic follicles therefore in later phases of follicular development, meaning that these oocytes, having been subjected longer to the follicular microenvironment, are likely to be more differentiated (Blondin and Sirard, 1995). Another method of assessing COC competence utilises the fact that glucose-6-phosphate dehydrogenase (G6PDH) is an enzyme which plays a role in cell energy supply, and reduces in activity when the oocyte nears maturity. Brilliant cresyl blue (BCB) is a vital blue dye that is reduced to a colourless substance by the action of G6PDH, so the cytoplasm of mature oocytes will remain blue. Although it has been suggested that this may be an excellent non-

invasive technique for selecting oocytes, the technique is time consuming and does not increase blastocyst rates above those obtained by morphological selection alone (Goovaerts et al., 2010). It has also been shown that plasma membrane Ca^{2+} current in the immature oocyte is related to developmental potential, and that calcium stores are related to developmental competence in mature oocytes (Boni et al., 2002) while amino acid turnover has also been suggested as a potential measure of likely embryo viability (Sturmey et al., 2008). However, none of these techniques are suitable for rapid or "cow-side" selection of those competent oocytes most likely to ultimately result in a pregnancy. So, other characteristics of COCs are being evaluated. The "Quiet Embryo Theory" (Leese et al., 2008) is based on the idea that embryos with higher viability have lower metabolic rates so there is potential to develop objective tests associated these findings. Also, it is known that the oocyte is key in regulating cumulus cell function (Matzuk et al., 2002), and in turn the cumulus oophorus plays an important role in controlling meiosis, supporting cytoplasmic development and creating a microenvironment conducive to spermatic capacitation and penetration (Tanghe et al., 2002, Tanghe et al., 2003). Hence there is a growing interest in identifying genes in cumulus cells as markers of oocyte quality using quantitative RT-PCR (Assidi et al., 2008, Bettegowda et al., 2008).

1.3.7.3 Oocyte maturation

There is a finite time during which an oocyte is capable of fertilization or activation, and the nature of collecting oocytes from ovaries, or live donors and then transporting them to a laboratory means that oocytes will be at varying stages of maturation when fertilized.

It has been shown that the absence of a dominant follicle, whether naturally absent or removed by ultrasound guided aspiration (dominant follicle removal, DFR) improved the viability of IVP embryos produced in Nellore cattle (Gradela et al., 2000). Similarly, in Holstein cows DFR prior to FSH stimulation improved follicular growth, ovulation and embryo production rates (Kim et al., 2001). This technique in conjunction with "coasting" therefore goes some way towards the aspirated COCs being at a similar and more competent stage of development.

Recent techniques using a protein synthesis inhibitor, cyclohexamide (CHX), to arrest the oocyte in meiosis at the germinal-vesicle stage, did not reduce the ability of those oocytes to develop (Hashimoto, 2009) and this could have significant technical advantages in the field. Utilising CHX would allow oocytes to be transported over greater distances, and OPU collections at various times and locations to be synchronised when entering the culture systems. Hence optimisation of media components and inter-oocyte communication can occur (Section 1.3.7.5).

1.3.7.4. Sperm

The sperm used in IVP systems is also critical, especially if using sexed semen, because of its inherently poorer performance (Wilson et al., 2006). It has been shown that development to blastocysts on day 7 was significantly influenced by sperm, and hence bulls have a differing capacity to produce embryos (Palma and Sinowatz, 2004). Hansen (2006) suggests that the reasons for this may be associated with the interaction between sperm and capacitation factors in the maturation media and/or genes being transmitted by the bull that might influence embryonic development. For example, a bovine analogue of the preimplantation embryonic development (ped) gene has been identified – this gene has been shown in the mouse to control embryonic development (Xie et al., 2010).

1.3.7.5. Embryo culture systems

Embryos in vitro are subjected to stresses that they do not experience in vivo, and this can compromise the physiology, gene expression and the development of the embryo (Gardner and Lane, 2005). The process of embryo culture has several interacting components – laboratory processes and techniques including embryo manipulation, microenvironment including pH, buffering capacity and temperature, atmospheric gas components, media constituents at various stages and quality control throughout. So, it is imperative that the whole "system" is considered as opposed to simply the media (Gardner, 2008). The varying stages of collection, maturation, fertilisation, development and cryopreservation require differing media components, and the intricacies are too many to cover in this review; however, there are some important principals, and components. Any laboratory disposables such as dishes, pipettes and

oils should be assumed to be embryotoxic unless proven otherwise by bioassay – suboptimal contact supplies are more difficult to identify than contact that is downright toxic, as blastocysts may well develop but be less robust and less capable of further development. The incubation system used is critical, to ensure that a low oxygen tension environment is maintained (usually between 5 and 10%), and quickly regained after the environment is breached when the door is opened. The carbon dioxide (CO_2) concentration is also crucial to help maintain the media pH. These systems can be either a) multigas chambers with CO_2 sensors, b) constant flow chambers using premixed gasses, or c) closed systems that are purged with premixed gas. Temperatures fluctuations must be avoided throughout the process, and measurements should be made of actual media and atmosphere rather than the warming surfaces themselves due to heat conductivity differences. The media itself has several components including; -

i. Water as the predominant component must be ultrapure (Wiemer et al., 1998) and several salts including sodium chloride and potassium phosphate which are included in embryo media must be reagent quality.

ii. Energy sources are usually in the form of glucose or pyruvate, although embryos can survive for several hours in holding media with no energy supplied. Glucose is required in relatively low concentrations (approximately 0.5mM) during early development, but this rises to approximately 2.0 mM by the morula stage (Hasler, 2010)

iii. Macromolecules are required for their surfactant properties, reducing the opportunity for embryos to float or stick to surfaces. Serum and bovine serum albumin (BSA) have both been successfully used as components of culture and freezing media, also having properties which support chelation, colloidal osmotic regulation and pH regulation. However, both present a risk of pathogen contamination (Givens and Marley, 2008) and will reduce shelf-life of unfrozen media. So, use of recombinant albumin overcomes the risks of prion or virus transfer and has been shown to be equally effective as serum albumin (Bavister et al., 2003). Serum also contains other beneficial components and was used for many years until it became associated with

large offspring syndrome (LOS) (Farin et al., 2001). Recent work on day 14 elongated embryos has suggested that serum improves blastocyst rates by altering gene expression but with some embryos overexpressing some genes, which may be the cause of LOS (Angulo et al., 2011). Hylauronic acid (HA) is a glucosaminoglycan which is naturally found in oviduct, follicular and uterine fluids (Lee and Ax, 1984) and is involved in gene expression regulation and cell development. It has been shown to improve blastocyst production and increase hatching rates (Block et al., 2009). The same authors found that embryo survival post vitrification was improved when hyaluronic acid was included in the culture media. Some commercial flush media contain no components of animal origin and claim good results – in these media polyvinyl alcohol (PVA) is usually added as the required surfactant.

iv. Amino acids are found in follicular, oviductal and uterine fluids (Harris et al., 2005, Orsi et al., 2005), and in particular high concentrations of the nutritionally non-essential amino acids (as defined by Eagle, 1959); alanine, aspartate, glutamate, glycine and serine, along with taurine. Various studies are cited by Gardner (2008) that demonstrate that the inclusion of various amino acids in embryo culture systems for various mammalian species improve blastocyst rates. However, the requirements change throughout embryo development. Non-essential amino acids and glutamine are prerequisites for early embryo development, significantly increasing cleavage rates up to the eight-cell stage, while at this time essential amino acids are inhibitory. This effect is attributed to the amino acids acting not only as energy substrates, but also as intracellular pH regulators and osmolytes (particularly glycine) (Steeves et al., 2003), hence substituting for some of the effects attributed to serum. After the eight-cell stage, non-essential amino acids and glutamine no longer stimulate cleavage rates but significantly increase blastocoel development and blastocyst hatching. However, essential amino acids at this stage do increase cleavage rates as well as stimulating development of the inner cell mass (ICM) of the resultant blastocysts. This addition of essential amino acids also improves post-transfer foetal

development. Hence it has been suggested that optimal development in vitro is achieved when embryos are cultured with non-essential amino acids and glutamine to the eight-cell stage followed by development to the blastocyst stage in the presence of all 20 amino acids (Lane and Gardner, 1997).

v. Antioxidants such as glutathione, pyruvate, ascorbic acid, catalase and BSA may be included to reduce free radical formation. Chelators to bind heavy metal ions should not be required in a completely pure media, but impurities in water and salts mean that they are often included in the form of transferrin, EDTA, or BSA (which may already be included for its other properties – see above)

vi. Buffers are essential in any embryo culture system. It is generally agreed that bovine systems should be run between pH 7.2 and 7.4 – this seems to be based on evidence that the intracellular pH of in-vitro derived bovine embryos is 7.2 +/- 0.02 (Lane and Bavister, 1999) while a study of rabbit embryo hatching found an optimal pH of 7.2 to 7.4 with severely deleterious effects below 7.0 and above 7.8 (Kane, 1974). A common buffer, PBS (phosphate buffered saline) was compared against 3 zwitterionic buffers (zwitterions are neutrally charged molecules with positive and negative electrical charges at different locations within the molecule); MOPS (3-[N-morpholino] propanesulphonic acid), HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) and TES (2-[[1,3-dihydroxy-2-(hydroxymethyl) propan-2-yl]amino]ethanesulfonic acid) in tyrode albumin medium modified with pyruvate (TALP). At a pH of 7.2 exposure to TES or PBS for 41 minutes significantly reduced the blastocyst development rate compared with MOPS or HEPES (which are the most commonly used in embryo culture media), these effects being explained by differing (mostly reduced) mRNA expression (Palasz et al., 2008). It has been suggested that buffering capacity is as critical as actual pH (Sinclair, K.D. 2011, Personal Communication)

In the bovine, group culture systems remain a prerequisite to achieve acceptable blastocyst rates (Ferry et al., 1994), thought to be the result of autocrine and paracrine communication between oocytes and embryos. Growth factors that improve in vitro

development include interferon τ, epidermal growth factor, platelet-activating factor, insulin like growth factors I and II and transforming growth factors α and β (Goovaerts et al., 2010).

As the requirements of the oocyte and embryo change throughout development, and because there is a need for refreshing of nutrients and other factors, and a removal of toxic substances, such as ammonia from amino acid breakdown, it is likely that static and semi-static embryo culture systems with manual translocation of embryos into different media will be superseded by more dynamic culture systems (Goovaerts et al., 2010, Gardner, 2008).

Although there has been a great deal of research undertaken on the interactions between the follicle itself, the oocyte and the cumulus cells, there is also a need for a reliable single oocyte culture system so that individual oocytes can be tracked back to individual follicles (Carolan et al., 1996).

It is also important to understand that much of the work done on embryo culture systems, media etc., is based on the ability of the system to produce morphologically acceptable embryos rather than pregnancies. Similarly, it is important to assess how robust embryos are and in particular their ability to survive cryopreservation (see below) and maintain post thaw pregnancy rates – so the important figure is based on the number of embryos from the entire cohort (both fresh and frozen embryos) that are capable of producing a live birth (Lane and Gardner, 2007).

1.3.7.6. Embryo cryopreservation

Cryopreservation of embryos is necessary so that embryos can be readily stored (indefinitely) and transported in liquid nitrogen, prior to thawing/warming and transfer into recipients. The most commonly used method of cryopreservation of bovine embryos is still slow controlled (or equilibrium) freezing, whereby embryos are drawn into a small volume of media in a straw before a computer controlled freezer decreases their temperature in line with a pre-programmed curve from a seeding temperature of -5^{o} to -7 ^{o} to between -30 ^{o} and -35 ^{o} at which point the embryos are plunged in liquid nitrogen. Originally glycerol or DMSO were used as cryoprotectants along with serum, but this required a cow-side multi-stage thawing and equilibrating procedure which was laborious and time-consuming. In 1992 ethylene glycol (EG) was

introduced as a cryoprotectant (Voelkel and Hu, 1992), and its low molecular weight means that embryos in an isotonic environment thaw, with EG exiting the embryonic cells and water entering at similar rates, thus maintaining an osmotic equilibrium without damaging the cells. Similar pregnancy rates between glycerol and ethylene glycol frozen embryos (Leibo and Mapletoft 1998) has resulted in EG direct transfer embryos now being standard practice.

Vitrification is the process whereby water or water-based solutions are solidified without forming ice crystals (Vajta et al., 2009), and was first described being used successfully for mammalian (mouse) embryos in 1985 (Rall and Fahy, 1985). The terms freezing and thawing should correctly only relate to situations where ice crystals form (as in slow controlled freezing) whereas vitrification involves cooling and warming. Usually the technique involves highly concentrated aqueous solutions of cryoprotectants, ideally in very small quantities, and very rapid cooling, but to date a totally satisfactory method of warming vitrified embryos for direct transfer has not been established. A technique called open pulled straw (OPS) has been described (Vajta et al., 1998) which can in theory facilitate a one-step thaw, but does require a high degree of operator skill. More recently a modification of the Cryotop® vitrification system (Chian et al., 2004) has been described (Inaba et al., 2011). The Cryotop® system further reduces the volume of cryoprotectant required and utilises a fine transparent polypropylene film attached to a handle, and equipped with a cover straw, in which oocytes can be loaded. Inaba et al (2011) compared the Cryotop® vitrification-straw dilution (CVSD) system with an in-straw vitrification-dilution (ISVD) technique and conventional slow freezing, outside dilution of the straw (SFODS). They showed that IVP embryos from abattoir derived ovaries cryopreserved with either the CVSD or ISVD vitrification techniques hatched at a significantly greater rate than those cryopreserved by the conventional SFODS technique. In addition, when CVSD and ISVD preserved OPU/IVP embryos were transferred to recipients, similar conception rates to freshly transferred OPU/IVP embryos were achieved. This is the first time that in-straw dilution of cryoprotectants on warmed embryos using the Cryotop® system has been reported resulting in successful pregnancies.

1.3.7.7. Donors and recipients

Although there is little published data, it is intuitive that the selection of donors and recipients in an OPU/IVP program is critical. Ideally animals should be in good general health, nutritionally balanced with both macro and micronutrients, cycling normally and with normal reproductive tracts when examined rectally by ultrasound. Donors giving the highest number of oocytes per collection do not necessarily result in the highest number of transferrable blastocysts (Duszewska et al., 2003). The yield of oocytes/COCs is also influenced by the frequency of collection; collecting at 7-day intervals significantly increases the number of COCs collected compared to a 3- or 4-day interval, but the quality of COCs and blastocyst rate are both significantly reduced (Merton et al., 2003). Some recent work on genetic evaluations (Merton et al., 2009) suggests that some OPU/IVP traits such as number of COCs, number of embryos at day 7, and number of transferable embryos at day 7, could be of potential value for selection of dams and sires for OPU/IVP programs, which would enhance the number of offspring from superior donors as well as improve the cost efficiency of such programs. Oocyte yield in the Nelores breed has also been shown to be affected by certain gene sequences, with possible variations of up to 7.4 +/- 1.1 oocytes when 3 genes are analysed (GDF9, FGF8 and BMP15) while poly(A) mRNA abundance is associated with differing morphological qualities of oocytes (Pontes et al., 2010, Biase et al., 2008). However, despite these studies, it is still not clear why *Bos indicus* breeds produce significantly higher oocyte yields than *Bos Taurus*, with *indicus-taurus* cross dams giving intermediate results. It may be because of a higher number of germinal cells in the foetus, a longer period of mitosis, different atresia mechanisms, or even follicular renewal (Pontes et al., 2010). Recipients, as with donors, should be healthy, cycling and nutritionally adequate – improved results are claimed if the donor and recipients are under identical management and nutrition regimes (Mullan, J.S., 2011, Personal Communication). Embryos can be transferred following observation of a natural heat, or after a variety of synchronisation and fixed ET programs which may result in a higher number of recipients receiving an embryo, thereby improving overall pregnancy rates and simplifying management of large groups of recipients (Rodrigues et al., 2010, Baruselli et al., 2010).

1.3.8. Summary

This review has considered the current state of IVP, and addressed some of the shortfalls and hurdles to it being used more routinely. If these can be overcome, the opportunities and potential for IVP are significant; both as a means of amplifying desired genetic traits and as an experimental tool, to investigate interventions such as medicines or trace element manipulation. This review considered the need for;

i. a robust, repeatable media and culture system, that works under UK conditions
ii. reliable and predictable results using sexed semen, as this is likely to be used much more commonly in the future
iii. a cryopreservation system with a one-step "on-farm" thaw, so that simple, rapid embryo transfers can be performed by technicians and vets

A more intimate understanding of the biological processes that occur during IVP, for example the impact of therapeutic pre-stimulation on follicle population and quality, the factors that influence oocyte maturation, and the selection of the embryos most likely to result in pregnancy will further enhance the outcomes of this project, and inform future work.

1.4. Chapter summary and general conclusions

A successful outcome for this study would be to overcome current OPU/IVP limitations, so that blastocyst output from a range of ages and breeds of cattle can be increased. By achieving this, it was further anticipated that markers of future fertility of an individual, or group of individuals, might be identified such as number of oocytes collected and blastocyst yields. Reliable predictors could then be exploited in small group intervention trials where, for example, mineral supplementation or pharmaceutical treatments could be compared and their effects on subsequent embryo development established. For this to be achieved, reliable and reproducible protocols and techniques must be developed at all stages to remove as many confounding variables as possible from the experimental design. Crossover trials would further enhance the statistical power.

1.4.1. Working hypothesis

An aspiration of industry might be to use OPU/IVP to assess fertility responses (i.e. oocyte quality) in intervention studies such as those investigating effects of mineral and trace elements. In order to realise this aspiration robust OPU/IVP systems need to be developed for use in UK cattle breeding. The working hypothesis was that this can be best achieved by combining emerging techniques for ovarian stimulation with state-of-the-art systems for bovine oocyte maturation and embryo culture.

The study therefore sought to establish industry knowledge base and requirements for mineral and trace element interventions whilst simultaneously establishing a commercially-based system for OPU/IVP and ET.

1.4.2. Aims

i. Assess the attitudes and approach to trace element diagnosis and treatment in the UK
ii. Optimise and standardise OPU/IVP under UK conditions
iii. Establish a robust OPU/IVP system and identify key markers in the system
iv. Investigate FSH/LH stimulation and coasting protocols under UK conditions
v. As proof of concept use a crossover trial utilising OPU/IVP to compare interventions that may affect oocyte quality

Chapter 2: Survey of cattle mineral knowledge, advice and interventions in the UK

2.1. Introduction

There have been many reviews of bovine mineral nutrition, and its importance, both in the UK (Suttle, 2010), and under different systems, for example tropical (Prasad and Gowda, 2005) or extensive (Knowles and Grace, 2014). Advice is widely available on how to approach on-farm assessment of micro-nutrient status (Suttle, 2004, Bone, 2007, Bone and Kendall, 2011), yet questions remain around how this is undertaken in the UK (Sinclair et al., 2015), and why there is evidence that in many situations, cattle are being over-supplemented (Sinclair and Atkins, 2015, Campbell, 2015). Similar results in California (Castillo et al., 2013) showed that minerals are being oversupplied by between 1.1 times (selenium) and 26 times (iron) compared to NRC (2005) requirements. Copper is of particular concern given the increasing reports of copper toxicity in cattle (Bone et al, 2011., Kendall et al., 2015). In both the UK and USA surveys mentioned above copper was being over supplied on many farms, and this was justified by some producers as being required to overcome high levels of antagonists, yet these were not found to be present. Although Sinclair et al. (2015) call for one person to be responsible on the farm for mineral nutrition, there are several sources of minerals and often several individuals who potentially have an influence. For example, in the same study of mineral status on a subset of UK farms, it was shown that for the upper 90th percentile of farms, parlour concentrates alone were accounting for more than the total recommended requirements of copper, zinc and manganese. To investigate these influences, an online survey was carried out in the UK, of veterinary surgeons, nutritionists and other advisers who give nutritional advice on farm, to ascertain how important they felt various mineral deficiencies and toxicities were in relation to bovine health and fertility, and how well they understood the various interactions. The survey also assessed how they identified, confirmed and treated these mineral imbalances.

2.2 Materials and methods

The on-line survey was designed using a web-based tool (SurveyMonkey® - www.surveymonkey.com) which allows the design of surveys, and the collection and basic analysis of responses. The survey was notified to relevant individuals who might be advising on cattle nutrition and/or mineral supplementation (including, vets in practice or industry, nutritionists, feed and mineral salespeople) via professional organisations, at conferences, on websites, etc. and completion was encouraged by the chance of winning a voucher.

The survey was split into sections and respondents were asked questions on their;

i. perceived importance of minerals and how often mineral imbalances were recognised
ii. methods to identify and/or diagnose mineral imbalances
iii. understanding of the mechanisms, clinical signs, and preferred treatments of deficiencies or toxicities of;
    - copper, selenium, iodine and zinc
iv. approach to the use of in-feed supplementation
v. demographic information

There were 33 questions in total covering sections i to iv above, and a further 5 questions to establish the demographics of the respondents (the full survey is in Appendix 1)

For the purposes of the survey, respondents were given the following definitions;

i. It is assumed that two types of deficiency exist in practice; "primary", resulting from the simple deficiency of a mineral in the diet, and "secondary", resulting from the depression of utilisation through the antagonistic effects of other substances,
ii. "Copper deficiency" can be taken to mean any copper responsive problem

iii. "Selenium deficiency" can be taken to mean a deficiency of selenium, or Vitamin E, or both

The raw data were downloaded and further analysed using MS Excel (Microsoft, 2007), and were presented using “bubble charts”. The “bubbles” were scaled in all the charts so that their area represented the third dimension of data for each point. Where the questionnaire used a scale of 1 (extremely unlikely) to 10 (highly likely) the Net Promoter Score (NPS) system as described by Reichheld (2006) was adapted where 1 to 6 was classified as low, 7 or 8 as medium and 9 or 10 as high.

2.3. Results

The survey had 173 respondents, 78% of which were vets and 22% non-veterinary paraprofessionals (nutritionists, feed advisors, academics, salespeople). The first question asked, “How important do you think minerals are in cattle nutrition?” (Figure 2.1). Vets were more evenly distributed (low 33%, medium 37%, high 30%) while non-vets were more likely to score medium importance (low 17%, medium 48%, high 35%).

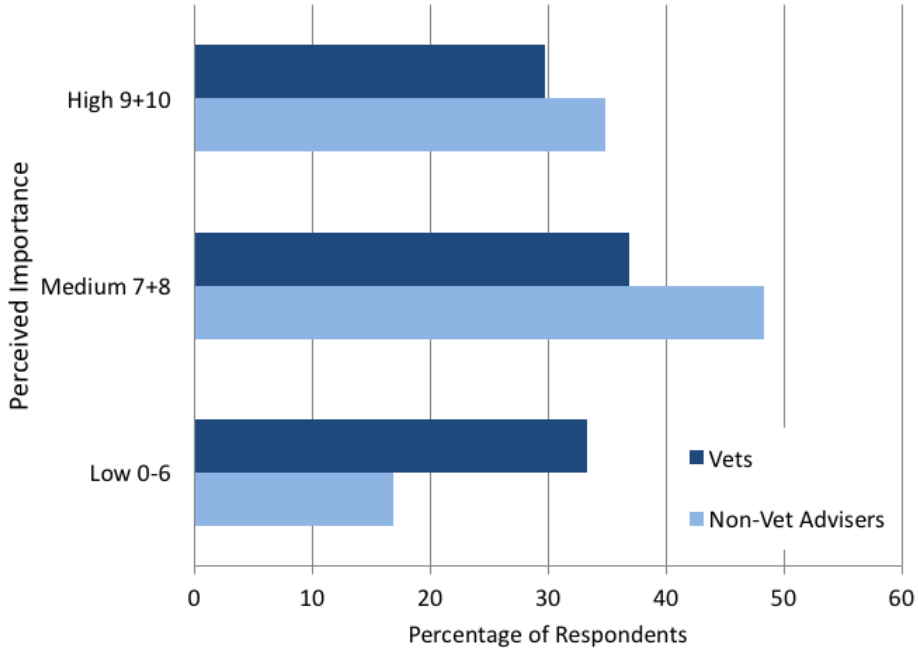


Figure 2.1. Perceived importance by vets (dark blue bars) and non-vet advisers (light blue bars) of minerals in cattle nutrition in the UK shown as the proportion in each Net Promoter Score band.

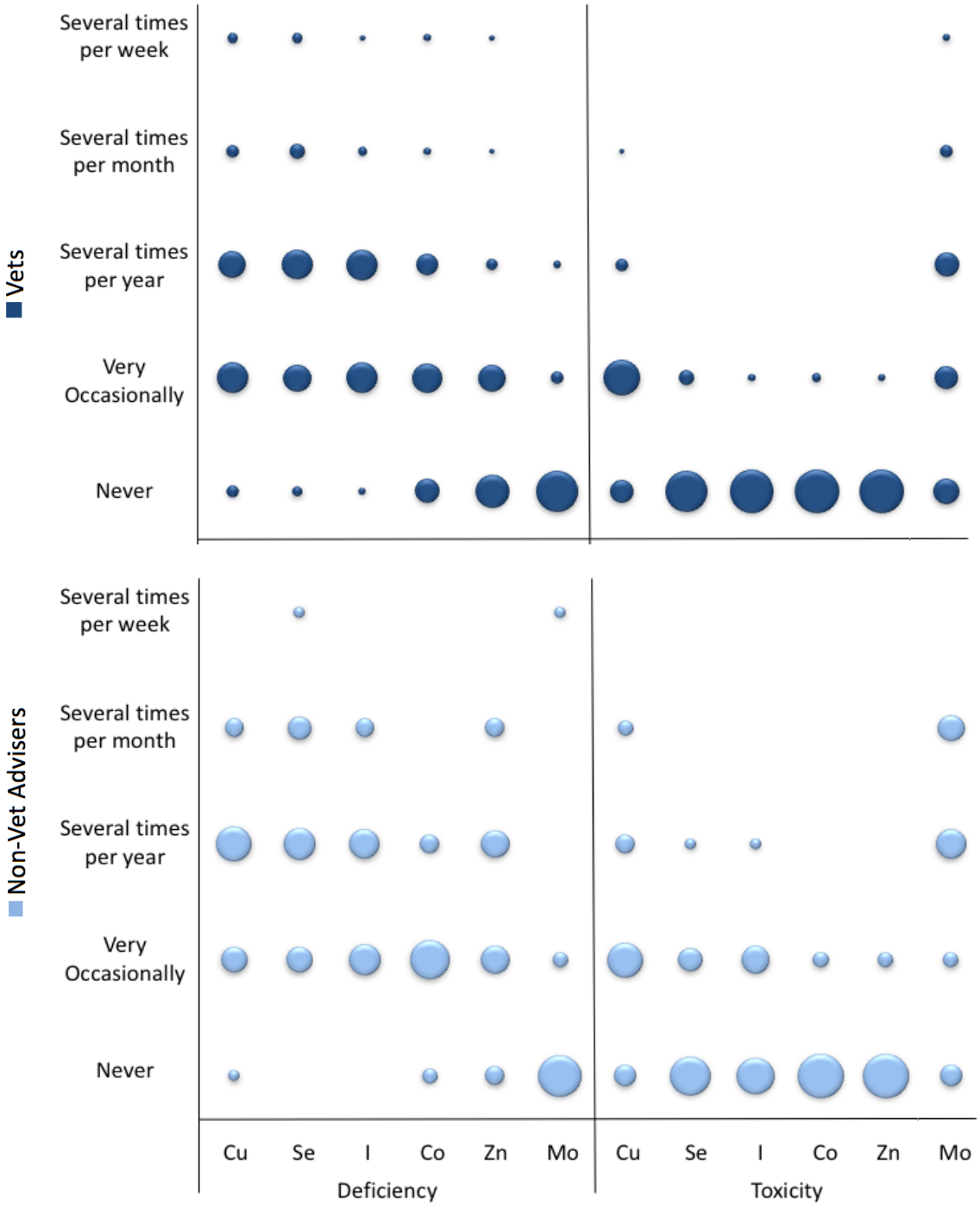


Figure 2.2. Relative frequency of identification of deficiency and toxicity by vets (dark blue upper chart) and non-vet advisers (light blue lower chart) of copper, selenium, iodine, cobalt, zinc and molybdenum. The proportion of responses for each condition is represented by the area of the bubbles.

A “bubble chart” distribution technique was used to demonstrate the relative frequency of identification of deficiencies and toxicities of copper (Cu), selenium (Se), iodine (I), cobalt (Co), zinc (Zn), and molybdenum (Mo). There was a close similarity in the relative frequency of identification of various deficiencies and toxicities between vets and non-vet advisers (Figure 2.2), with the most frequently identified deficiencies across all professionals being selenium, copper and iodine, while the most commonly identified toxicity was molybdenum. There was a tendency for non-vet advisers to identify selenium and zinc deficiencies, and molybdenum toxicities, more frequently than vets, relative to other conditions, while vets tended to identify cobalt deficiency relatively more frequently.

Respondents were also asked to select statements that most suited their understanding of the frequency and mechanisms of deficiencies of copper, selenium, iodine and zinc (Figures 2.3, 2.4, 2.5, and 2.6). The range of responses demonstrated the wide variation in the understanding of the various deficiencies both between and within the groups of professional advisers;

There is variability in what vets and advisers understand by “copper deficiency” – of the options given, 33% of vets and 43% of non-vet advisers chose “Primary copper deficiency is common in the UK, and is exacerbated by the presence of other substances which can antagonise the copper”, while “Primary copper deficiency is extremely rare in the UK – a) it is usually antagonism from other substances that causes a secondary deficiency”; was chosen by 33 % of vets and 19% of non-vet advisers, and - b) it is usually molybdenum interaction that causes a problem”; was chosen by 23% of vets and 33% of non-vet advisers (Figure 2.3).

Although the majority of respondents agreed that “Primary selenium deficiency is common the UK” 26% of vets and 25% of non-vet advisers felt that this was “exacerbated by the presence of other substances which can antagonise the selenium”, while 40% of vets and 38% of non-vet advisers chose the response that stated it “is not associated with antagonism from other substances” (Figure 2.4).

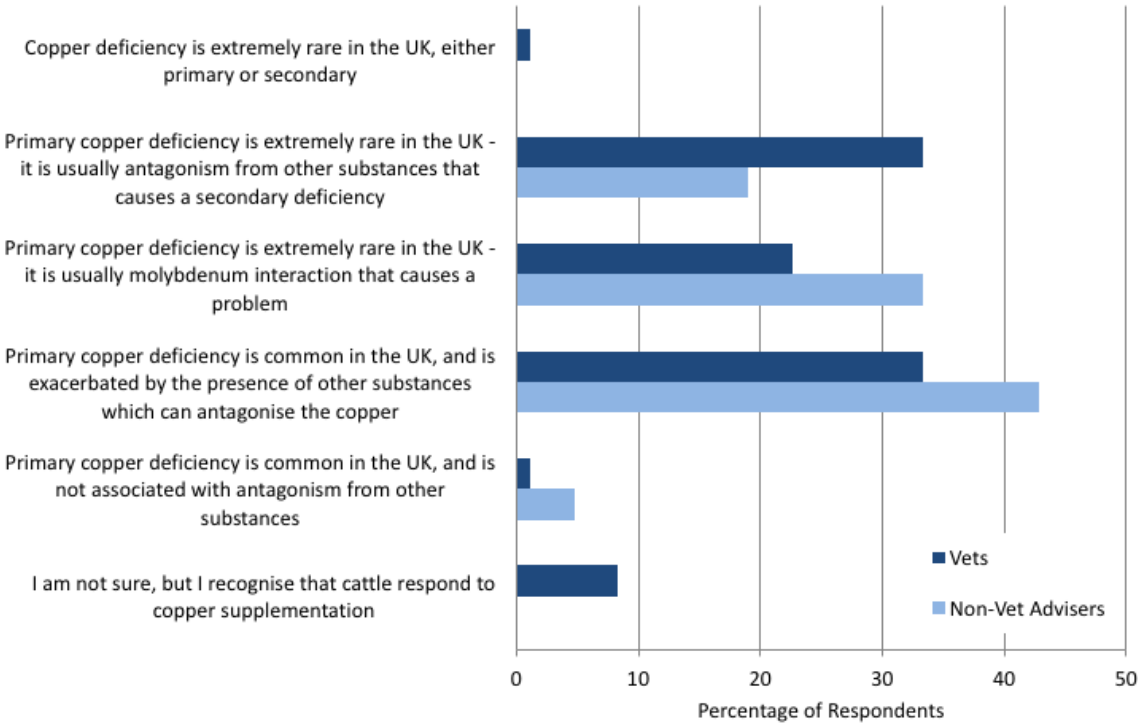


Figure 2.3. Understanding of Copper Deficiency by vets (dark blue bars) and non-vet advisers (light blue bars) shown as the proportion selecting each statement

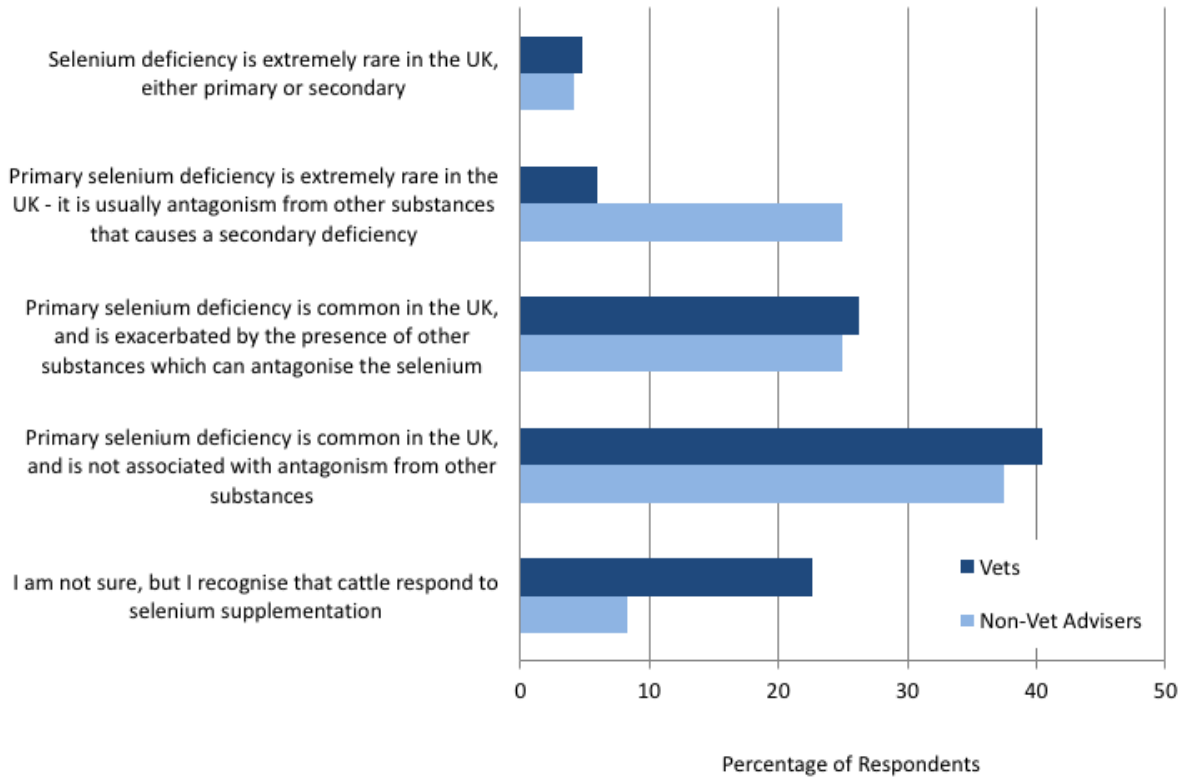


Figure 2.4. Understanding of Selenium Deficiency by vets (dark blue bars) and non-vet advisers (light blue bars) shown as the proportion selecting each statement

When the understanding of iodine deficiency was analysed (Figure 2.5), it was shown that there was a divergence between the vets, 46% of whom understood that "Primary iodine deficiency is common in the UK, and is not associated with antagonism from other substances", and the non-vet advisers, of whom only 19% chose this statement. Conversely only 11% of vets chose "Iodine deficiency is extremely rare in the UK, either primary or secondary" compared with 24% of non-vet advisers.

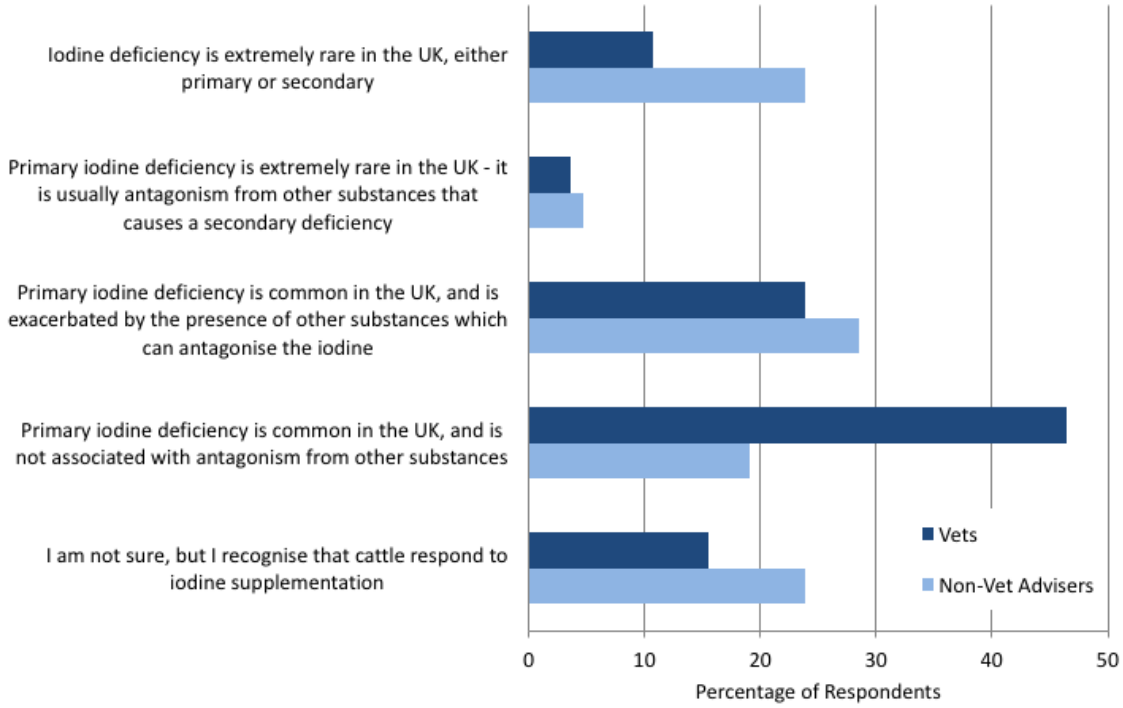


Figure 2.5. Understanding of Iodine by vets (dark blue bars) and non-vet advisers (light blue bars) shown as the proportion selecting each statement

This was reversed when respondents were asked about zinc deficiency; 62% of vets understood that "zinc deficiency is extremely rare in the UK" (total of both categories) compared with 34% of non-vet advisers, while 52% of non-vet advisers believed that "primary zinc deficiency is common in the UK" compared with only 8% of vets (Figure 2.6).

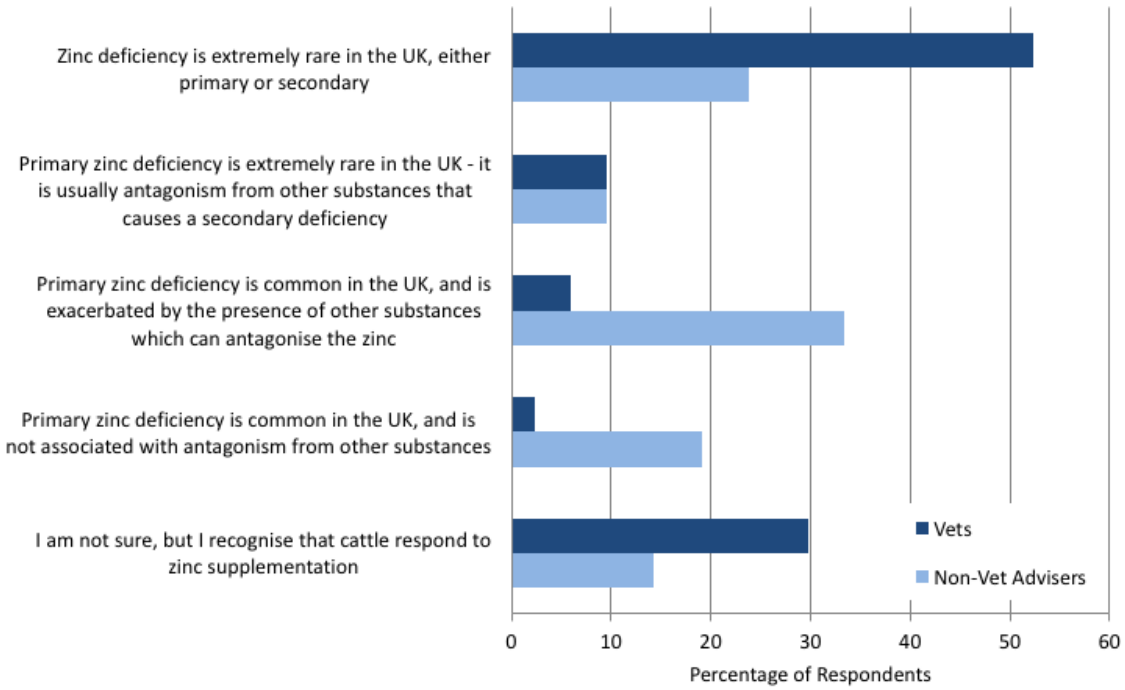


Figure 2.6. Understanding of Zinc Deficiency by vets (dark blue bars) and non-vet advisers (light blue bars) shown as the proportion selecting each statement

The survey also investigated the relative frequency of various treatment options depending on the options available for treatment of that particular deficiency. In this paper, the treatments for "copper deficiency", which was defined in the survey as "any copper responsive problem", are shown (Figure 2.7) as an example of one these sets of responses. The options given for treatment were; alter ration, in-feed supplementation, in-water supplementation, copper injection, copper needles, mineral drench, matrix bolus or glass bolus. There was a broad distribution of treatments used, with all options being chosen at least "occasionally" by both groups. The most frequently selected treatments by all advisers were glass boluses, in-feed supplementation, matrix boluses, and then copper injections, while the most infrequently selected was in-water supplementation. Non-vet advisors most often choose in feed supplementation, whereas vets most often advised the use of glass matrix boluses. Vets recommended copper injections relatively more frequently than non-vet advisers. As a treatment option, altering the ration, was "never" selected by the majority of respondents in each category.

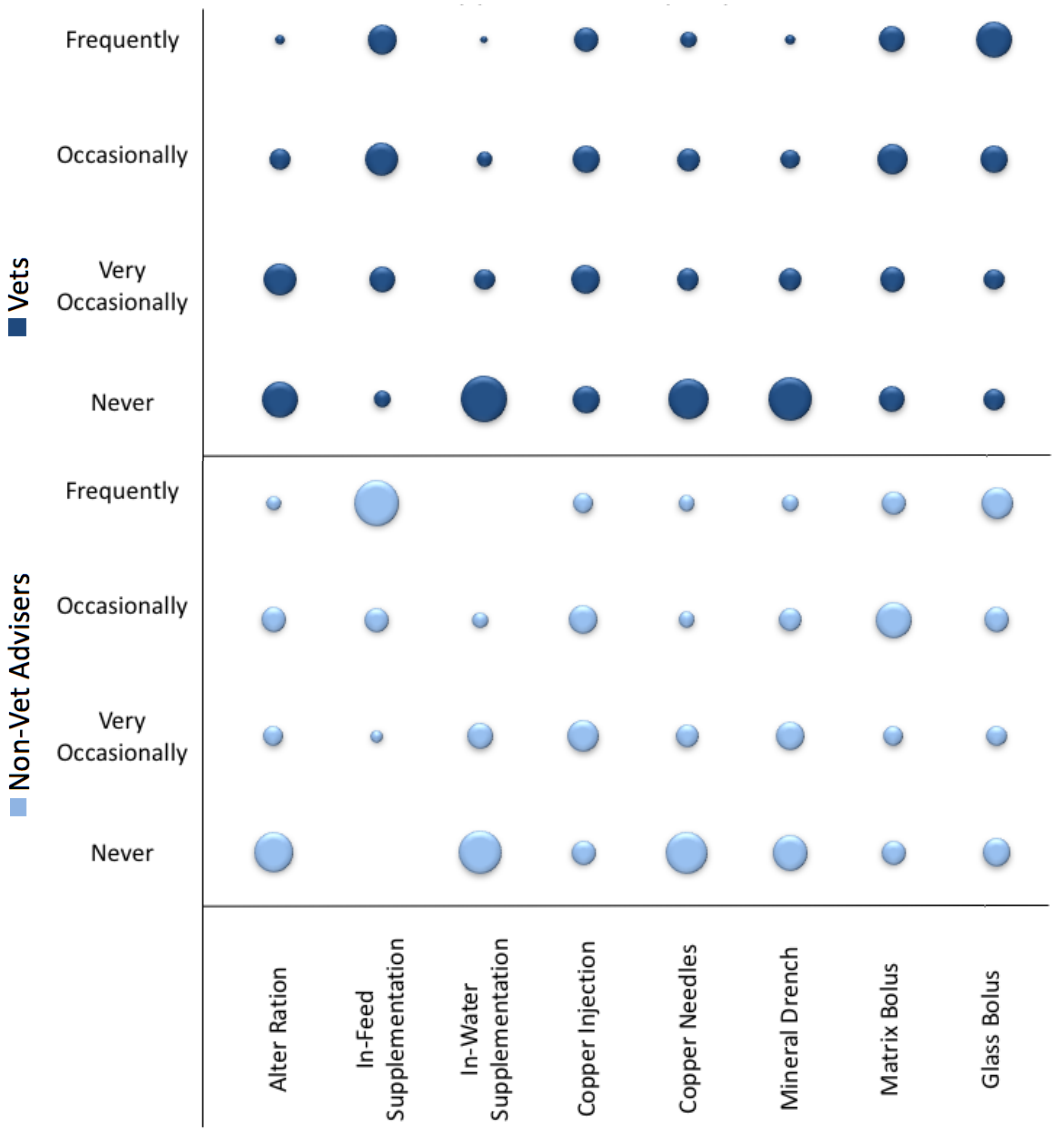


Figure 2.7. Relative frequency of treatment options advised by vets (dark blue upper chart) and non-vet advisers (light blue lower chart). The options were; alter ration, in-feed supplementation, in-water supplementation, copper injection, copper needles, mineral drench, matrix bolus or glass bolus, and the proportion of responses for each condition is represented by the area of the bubbles.

When the perceived prevalence of copper and selenium toxicity was investigated via the questionnaire, (Figure 2.8) the majority of respondents felt that copper toxicity was “very rare” (17% of vets and 11% of non-vet advisers) or “quite rare” (48% of vets and 67% of non-vet advisers), while fewer thought that is was “quite common” (32% of vets and 11% of non-vet advisers) or “very common” (4% of vets and 11% of non-vet advisers). However, the views of the prevalence of selenium toxicity were more

aligned with “very rare” being selected by 60% of vets and 33% of non-vet advisers, and “quite rare” being chosen by 35% of vets and 62% of non-vet advisers.

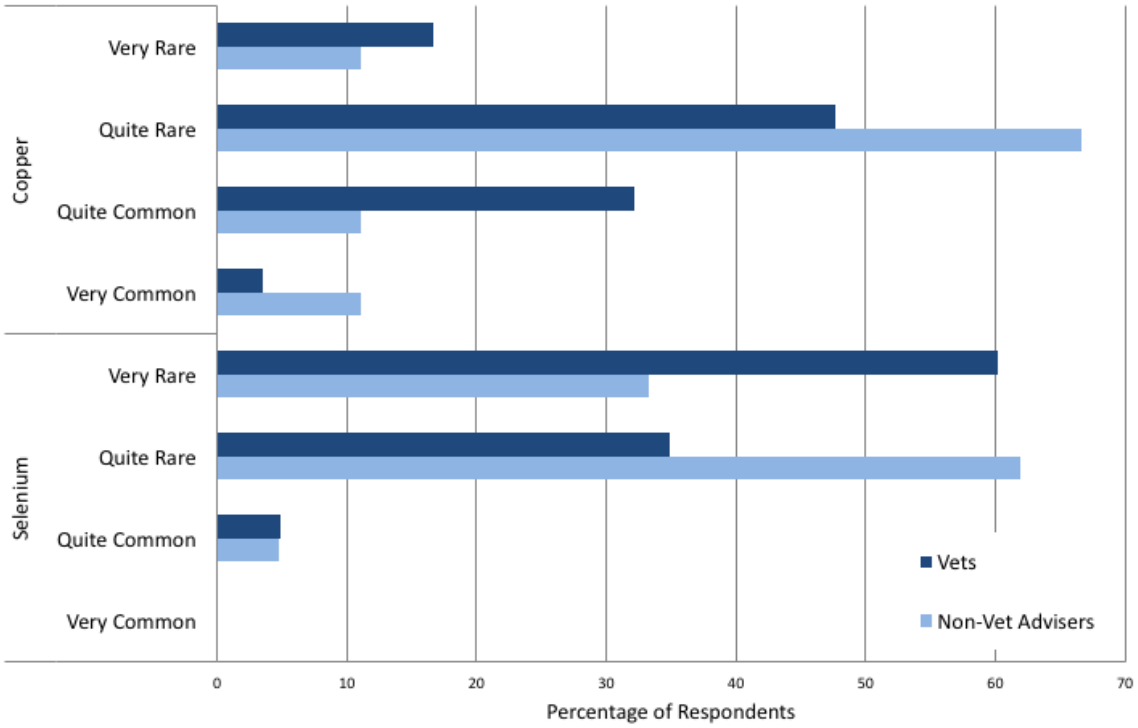


Figure 2.8. Perceived prevalence of copper and selenium toxicity by vets (dark blue bars) and non-vet advisers (light blue bars) shown as the proportion selecting each statement

For the findings associated with reduced fertility attributed to each mineral deficiency (data not shown), there was general consensus between the vets and advisers, with the “classic” signs predominating; poor pregnancy rates, anoestrus and reduced oestrus expression associated with copper (primary or secondary), retained placenta and endometritis associated with selenium, and stillbirths associated with iodine.

2.4. Discussion

This is the first survey of this nature in the UK and as far as we know globally, investigating the attitudes and approaches of advisers to mineral micro-nutrition on farms. There was very little consensus across the professional advisers in the UK, or within the vet and non-vet adviser subgroups, and not even a clear agreement on which imbalances are common or rare. The most frequently identified deficiencies

across all professionals were selenium, copper and iodine, while the most commonly identified toxicity was molybdenum. There was a disparity in the answers around mechanisms and interactions associated with deficiency, and particularly in the context of copper responsive conditions. Of the various treatments available for copper responsive conditions, all were used at least "occasionally", and the most frequently selected treatments by all advisers were glass boluses, in-feed supplementation, matrix boluses, and then copper injections. And while there was a diversity in the choice of treatments, altering the ration was relatively rarely selected.

This survey was designed to investigate information from animal health professionals who are regularly advising farmers about trace element nutrition. The questions, and answer statements selected were not designed to be "right" or "wrong", but to assess the level of understanding and agreement amongst the various advisers. Care was taken to explain the use of the terms "primary deficiency" and "secondary deficiency", particularly with reference to copper, as the term "copper deficiency" is used ubiquitously to identify various hypothesised mechanisms (Suttle, 1991, Telfer et al., 2003, Black and French, 2004, Telfer et al., 2003b). Therefore, in this study "copper deficiency" was taken to mean any "copper responsive condition", and on the understanding that many of the respondents will not recognise "copper deficiency" but rather a "molybdenum toxicity".

It is accepted that geography, working environment, cattle type etc. will affect an individual's perception of the prevalence or mechanisms of the various deficiencies or toxicities. There are also situations where producers have perceived an improvement with supplementation and believe that further supplementation will aid further, as in the copper toxicity report by Johnston et al. (2014). Advisers (and farmers) have ready access to information, (e.g. Suttle and Sinclair, 2000, AHDB Beef and Lamb, 2001, AHDB Dairy, 2011) and the opportunity to learn about the requirements, mechanisms, effects and treatments associated with these key micronutrients and so it might have been expected that there would have been more alignment in their responses. So, it appears from the survey and is particularly interesting that copper metabolism is not clearly understood.

In 2017, 3% of food incidents as reported by the Food Standards Agency in the UK were associated with heavy metals, attributed largely to lead and copper poisoning in livestock (FSA, 2017). Copper excess in the human is associated with gastrointestinal, hepatic and neurodegenerative disease, and although copper homeostasis is very effective, it can be overwhelmed or disrupted (Gaetke et al., 2014). Recent reviews of human copper nutrition call for a review of upper and lower intake limits (de Romana et al., 2011, Stern, 2010). Currently in the UK, the maximum recommended daily intake of copper is 0.16 mg/kg bw/day (equivalent to 10 mg/day in a 60 kg adult) (FSA 2003). The FSA/APHA trigger level for an incident of reportable copper toxicity in cattle is 25000 µmol/kg (1.6g/kg) of liver dry matter (DM) (Bone et al 2010), while the APHA reference range maximum is 8000 µmol/kg (0.5g/kg) DM. Assuming a liver dry matter content of 27% (Rosendo and McDowell, 2003) 70g of fresh liver from an animal at the top of the APHA reference range would equate to the maximum daily intake for a human, so working under the precautionary principle it is understandable why there are concerns about the risks to human intakes from animal toxicity events.

So with copper toxicity being a real threat to both animal and human health it is imperative that we understand the options for assessment of mineral status and treatment (Laven and Livesey, 2005, Kendall et al., 2015), and that the mechanisms as we understand them to date are communicated to the advisory professionals, particularly veterinary practitioners, who are responsible for diagnoses of clinical disease In particular in the UK, the true copper-molybdenum interaction, although now better elucidated (Gould and Kendall, 2011), is still not widely understood by those advising on supplementation with an associated risk of over supplementation (Sinclair and Atkins, 2015, Campbell, 2015). This confusion has not been assisted by the open debate between respected cattle nutrition experts in the veterinary journals, for example Telfer et al. (2004) and Suttle and Phillippo (2005).

As might be expected there is a variation in the treatments recommended and there may be some commercial aspect to this; for example, non-vet advisers may work for a feed or mineral firm and have more of a vested interest in selling a feed supplementation than a bolus, and vice-versa for vets. Because of the perceived

importance of trace elements in cattle nutrition, albeit at a micro, rather than macro level, it is surprising to note that the majority of respondents do not opt to alter the ration. Given that there may be more than one adviser on a farm, each responding to different requirements of the producer, (animal health, production, financial efficiency, breeding goals, etc.), each may have a different understanding and preferred approach to trace element nutrition. It is therefore possible that several sources of minerals are being provided or advised at the same time, without one person having an overview and ultimate responsibility. There is an argument that the current recommendations (ARC, 1980 and NRC, 2001) for maintenance, growth, lactation and the foetus, are outdated, were based on minimal (to avoid deficiency), and not optimal (to support production), requirements, and on samples from relatively small numbers of animals. There is no doubt that there have been significant advances, particularly in nutrition and genetics. This progress is likely to be enhanced by the developing molecular biology techniques whereby gene expression, immune function and cell mediation (including nutrient transport) are becoming better understood – for example the studies by Osorio et al. (2016), Batistel et al. (2017) and Arroyo et al. (2017). Nevertheless, although NRC recommendations for nutrient requirements of dairy cattle is currently being updated with a new factoral approach, and due in 2018, these remain the guidelines advisers work to.

The results of this survey support the views of Weiss, (2017) in a recent "100 Year Review". There is a need for further research into the true prevalence and importance of trace elements, and the most appropriate and accurate methods of treatment, particularly to avoid liver loading and toxicity. Molecular techniques will be of value in further elucidating the various mechanisms and interactions.

These data and findings were accepted and presented as a poster in Santiago, Chile at the World Buiatrics Congress in 2010 – see Appendix 2

Chapter 3: Key factors affecting OPU/IVP in the UK

3.1. Introduction

There are three key areas that must be addressed and optimised for a successful commercial OPU/IVP operation; the donor (management, preparation and oocyte collection), the IVP laboratory processes, and the recipients (management and embryo transfer) (Blondin et al., 2015). The donor must be prepared to ensure that as many competent oocytes are presented for aspiration as possible, which means managing the size and dynamics of follicles of that particular animal (Blondin et al., 2015, Blondin et al., 2012, Bo and Mapletoft, 2014). The mechanical aspiration techniques of both the dominant follicle and the pre-ovulatory oocytes can also impact on the quality and blastocyst yield, requiring optimum equipment, environment and operator expertise to insult the oocytes as little as possible (Boni, 2012).

It is recognised that *Bos indicus* cattle respond more favourably to OPU/IVP than *Bos taurus* (Pontes et al., 2010, Gimenes et al., 2015, Watanabe et al., 2017), the reasons for which are not fully understood. Retrospective studies in other countries working with *Bos taurus* cattle have shown improvements in embryo yields by using stimulation regimes (Blondin et al., 2015, De Roover et al., 2008, Merton et al., 2003) and the effects of these on UK cattle were investigated.

Within the in-vitro maturation, fertilisation and culture area Blondin et al. (2015) state that “there is no miracle media or additives”, but they insist that in the commercial IVP lab environment consistency and quality control is key to success, and this is supported by Lane et al. (2008) and Watanabe et al. (2017). Similarly, Lonergan and Fair, (2016) conclude that the media system only has a “modest influence” on the development potential of the embryo, and that the key factor is the quality of the oocyte itself, while Vajta et al. (2010) argue that although mammalian in-vitro culture systems have improved dramatically in recent years, they have not yet reached biological limits and further improvement is possible. There are indeed hundreds of scientific papers every year investigating various aspects of mammalian IVP, but in this study the aim was to establish a robust OPU and IVP system that works consistently under UK conditions. To do this it was necessary to start working with a basic system

and by a series of iterations improve the processes. Furthermore, it was necessary to establish key variability drivers within the system and to minimise the impact of these wherever possible. This study was undertaken as part of a series of packages within an Innovate UK match-funded industry-led project, which are described in Appendix 3, and which also addressed a final aspect of success which is to ensure that the communications between the OPU and ET transfer field teams and the IVP laboratory are very effective (Blondin, 2017).

3.2 Materials and Methods

3.2.1. Dataset

Two IVP laboratories were established at the outset of the Innovate UK project (IVP 1 and IVP 2), both of which had some experience of embryo culture, but not on a commercial basis. Over the course of the study, six separate OPU teams were trained (OPU 1 to 6), and these were phased in over the 5 years of the Innovate UK project. IVP 2 processed oocytes from OPU 2, while all the other OPU teams sent oocytes they collected to IVP 1. The various OPU Team/Laboratory combinations, and the number of OPU cycles each undertook during the course of the study are shown in Table 3.1 and schematically in Figure 3.1.

Donors used throughout the trial were from a variety of sources;

i. Cows belonging to the practice which were being used to produce support embryos – these are embryos implanted into recipients that have failed to become pregnant by insemination alone, one week after they have been artificially inseminated to support the natural pregnancy.

ii. Cows that were deemed to be “problem breeders”, which could not get back in calf, or would not flush by conventional MOET – these were brought by clients

iii. Batches of cows and heifers that were used as donors to produce batches of embryos for export

iv. Heifers of higher genetic value as defined by genomic screening, or by profitable lifetime index (PLI) assessment – these were used later in the

project as the earlier scanner handles were too large to be used comfortably in younger heifers with smaller vulvas and vaginas.

Table 3.1. OPU Team and IVP Laboratory combinations that contributed to the dataset, with the date of commencement, contributing data and number of OPU cycles undertaken

IVP Laboratory	OPU Team	Start Date	Number of OPU cycles
IVP 1	OPU 1	June 2011	1298
	OPU 2	June 2013	123
	OPU 3	January 2014	45
	OPU 4	April 2015	221
	OPU 5	July 2015	62
	OPU 6	March 2016	45
IVP 2	OPU 2	November 2011	405

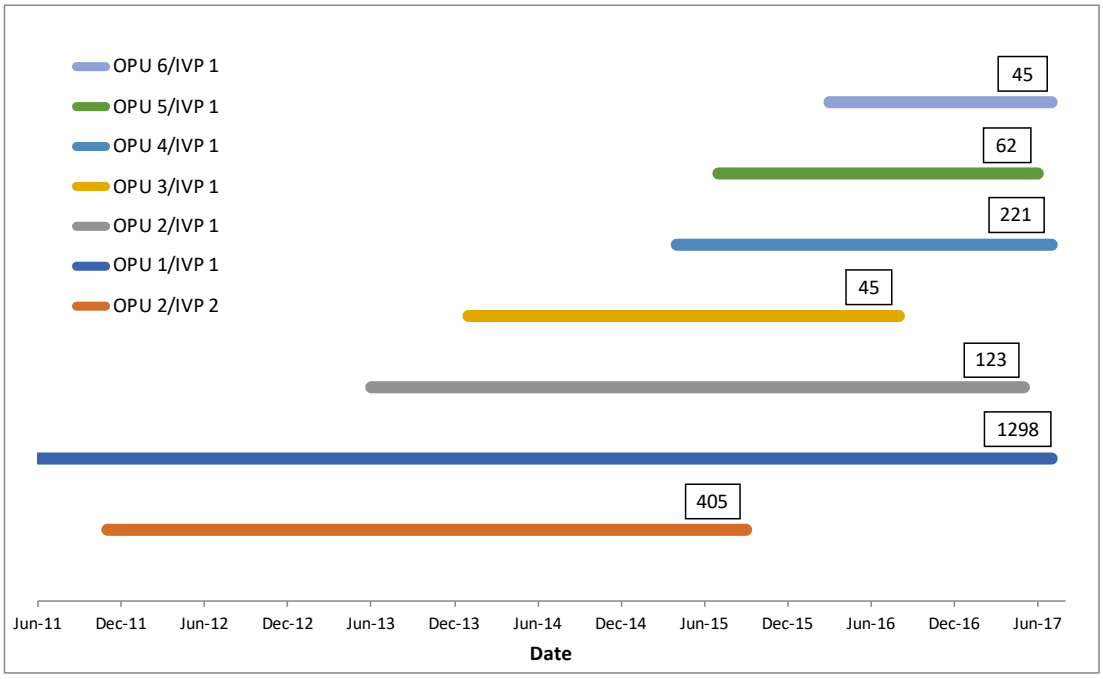


Figure 3.1. A schematic representation of the OPU Team/Laboratory combinations active throughout the project indicating dates of collections and the number of OPU cycles (numbers in black boxes).

3.2.2. Refinements to procedures

Throughout the first 4 years of the project data were analysed and changes made to procedures, both cow-side and within the laboratories, as well as in the equipment used. From 1st June 2015, these processes were standardised across all the OPU teams. The processes are described below, with a section on each describing the changes that were implemented and used from this date.

3.2.3. The OPU and cow-side procedures

3.2.3.1. Pre-treatment of the donor

Structures around the ovary, particularly the uterus (Herzog and Bollwein, 2007) become more vascular (and therefore more prone to haemorrhage) during proestrus and oestrus, while it has been shown that blood flow increases to the dominant follicle (Arashiro et al., 2013), the pre-ovulatory follicle (Acosta, 2007), and early corpus luteum (Matsui and Miyamoto, 2009). So, it was assumed that the OPU procedure carried least risk if it was carried out during metoestrus or dioestrus. In other countries where OPU is undertaken the collections are often done twice weekly without ovarian stimulation, for example Brazil (Pontes et al., 2010, Watanabe et al., 2017), so this is not an issue as the animal never reaches proestrus or oestrus. It has been shown by Petyim et al. (2001) that frequent follicle aspiration can cause slight morphological changes, with their most significant finding being a thickened ovarian tunica albuginea. However, despite these findings along with some endocrine profile changes, there did not seem to be any long-term effects on the ovarian function. However, we opted to use a less frequent collection interval and as a consequence it was preferable to control the oestrus cycle by treatment with an intra-vaginal progesterone (P4) releasing device (PRID® Delta (1.55 g of P4, Ceva Santé, Libourne, France) or CIDR® (1.38 g of P4, Zoetis, New Jersey, USA)).

3.2.3.2. Stimulation and Coasting

The preferred technique adopted after 1st June 2015 in this study was “stimulation and coasting”, as described by Blondin et al. (2002), and this is shown schematically in Figure 3.2. This is designed to stimulate antral follicle numbers and synchronise follicular growth, and the length of the coasting period is key to optimising the

follicular differentiation at the time of harvesting (Nivet et al., 2012). Firstly, dominant follicle(s) were removed (DFR) by ultrasound guided trans-vaginal aspiration around one week prior to oocyte collection; any follicle greater than 5 mm in diameter was aspirated and cumulus oocyte complexes discarded. The donor was then pre-treated with a course of hormones, injected intra-muscularly prior to aspiration. There are two commercially available products in the UK, containing different proportions of follicle stimulating hormone (FSH) and luteinising hormone (LH). These are:

i. Folltropin® (Vetoquinol SA, Lure cedex, France) – 35iu/ml FSH with "low LH activity" (as declared by the manufacturer). The dose administered for adult cows was 2.5ml (87.5iu FSH) per injection every 12 hours for 3 days. This dose was reduced for lighter weight, or younger heifers to a minimum of 1.5ml (52.5iu FSH).
ii. Pluset® – (Laboratorios Calier, SA, Barcelona, Spain) – 50iu/ml FSH and 50iu/ml of LH. The dose administered for adult cows was 2ml (100iu FSH and 100iu LH) per injection every 12 hours for 3 days. This dose was reduced for lighter weight, or younger heifers to a minimum of 1ml (50iu FSH and 50iu LH).

This was followed by the period known as "coasting" during which the donor was not supplied with external FSH/LH prior to OPU being carried out to allow a degree of *in-vivo* maturation of oocytes.

This can then be adjusted for subsequent cycles depending on follicle numbers, follicle size, and quantity and quality of the cumulus and granulosa cells. Programmes were tailored to individual donors by adjusting the coasting period to ensure the correct maturity of the COC's obtained, and product/dose was varied to maximise the number of blastocysts produced.

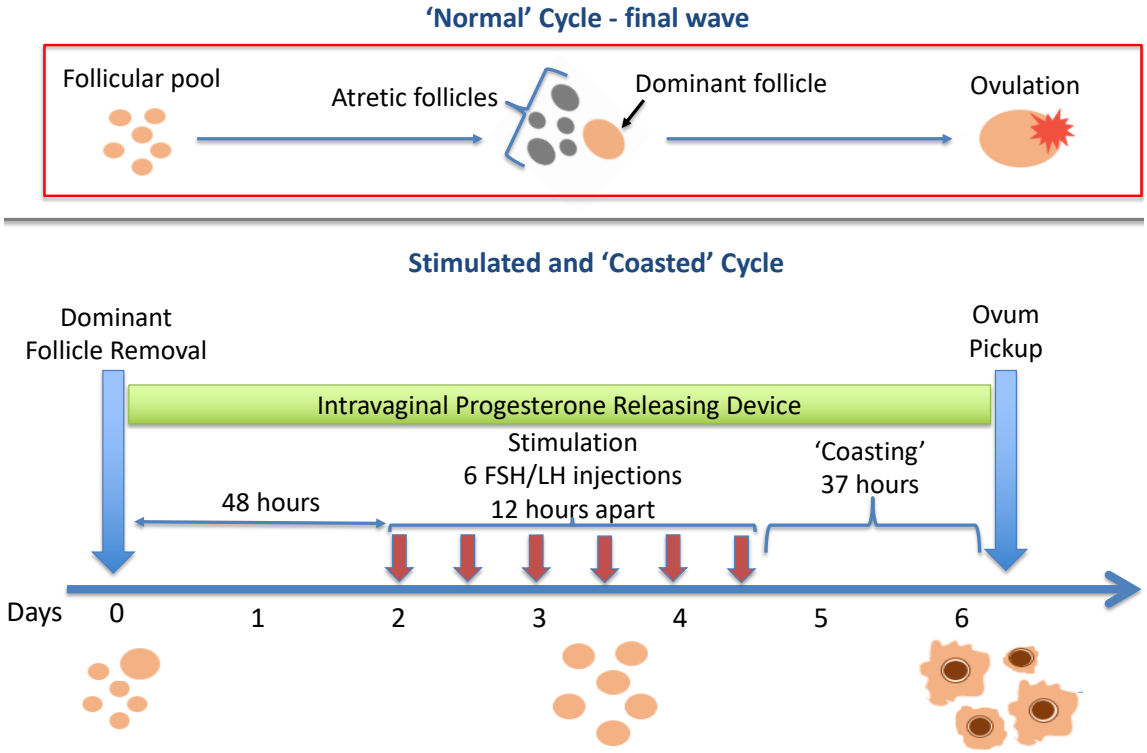


Figure 3.2. Schematic, with an approximate timeline in days, showing the final follicular wave (2nd or 3rd) of a 'normal' single dominant follicle and ovulation process (in red box) compared with a typical dominant follicle removal, FSH/LH stimulation and coasting regime utilising exogenous progesterone from an intravaginal device.

3.2.3.3. The OPU procedure:

This can be defined as the recovery of cumulus-oocyte complexes from antral follicles of the bovine ovary by transvaginal aspiration using an ultrasound guided needle, first described by Pieterse et al. (1988). The probe casing (needle guide) is designed to be taken apart for cleaning and sterilisation between sessions of use and required assembly before use.

The procedure was carried out under epidural anaesthesia (Procaine Hydrochloride 50mg/ml, Adrenacaine®, Norbrook, Newry, Northern Ireland) usually without sedation whilst the animal was restrained in a standard cattle crush suitable for fertility examinations, with good access around the rear.

Oocytes are sensitive to temperature shock and care was taken to minimise changes in temperature, both in the collection area and within the laboratory. The collection

area was maintained at close to 30°C, using a gas powered "space heater" which was manually adjusted, while the laboratory had an approximate working temperature of 28°C with heat stages for searching at 38.5°C.

The rectum was manually emptied of faeces prior to cleaning the vulva and perineum with paper towelling and surgical spirit. The original ultrasound equipment used was an Ibex Pro Ultrasound with 10-6Mhz 14mm curved linear area transducer fitted into a transvaginal needle guide device (Figure 3.3). A sector view was visualised on a grid screen with the line of needle approach shown as a dotted line. The follicle aspiration procedure was carried out by a veterinary surgeon assisted by a technician to hold equipment, flush tubing and change needles. The probe handle and needle guide was placed in the animal's vagina with the transducer head held firmly against the anterior fornix of the vagina. It was usual for the assistant to hold the needle guide in place throughout the procedure (Figures 3.4 and 3.5)

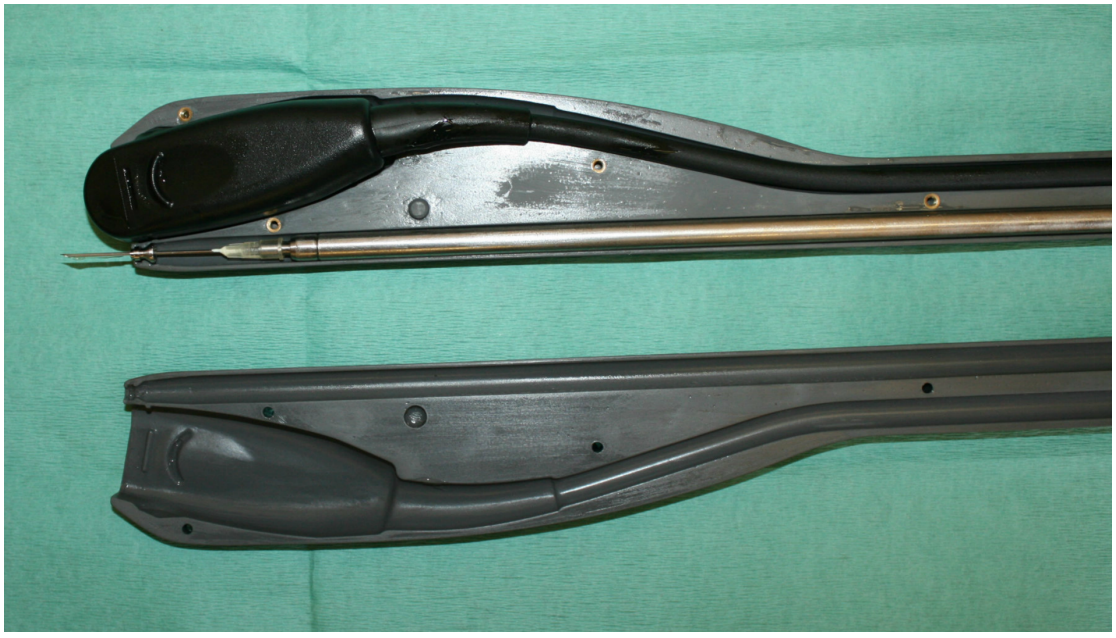


Figure 3.3. An early model transvaginal casing opened to show the positioning of the ultrasound transducer and needle guide.

Figure 3.4. The position of the veterinary surgeon and the cow-side technician showing the ultrasound scanner, and the water bath.

Figure 3.5. The position of the veterinary surgeon guiding the needle by ultrasound

The technique used was adapted from Galli et al. (2001) by Christie, W.B. (Personal Communication). The veterinary surgeon located an ovary per rectum and placed it against the transducer head enabling visualisation of the antral follicles on the scanner screen. The aspiration needle (Microlance 18G 2'', short bevel; Becton Dickinson, Franklin Lakes, New Jersey, USA) was attached to a stainless-steel introducer through which a length of tubing ran, connecting the needle (via a stainless-steel adapter) to a collection vessel, which in turn was connected to an aspiration pump, adjusted to read 75-80 mmHg via a vacuum reserve (Craft Duo-vac Suction Unit, R29660; Rocket Medical, Washington, UK). This needle and tubing were primed with approximately 10 ml of warm heparinised flush medium. The needle introducer was then passed by the assistant into the needle guide, before the veterinary surgeon took over and guided the needle into each follicle in turn with the pump activated by a foot pedal. The aspirated flush medium containing COCs was then collected in a 50ml Corning® tube held in a tube warmer (Cook Medical, Bloomington, Indiana, US). Periodically (usually after 4 to 6 follicles) the assistant would flush the tubing and its contents through with medium and the needle was replaced. The procedure was repeated for the second ovary.

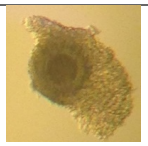
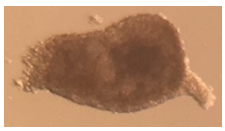
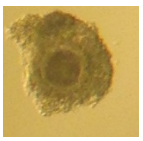
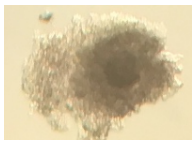
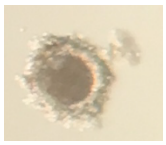
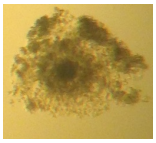
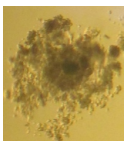
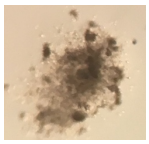
The aspirate was transported immediately to the embryology laboratory in the Corning® tubes surrounded by air-pocket insulation in a polystyrene box. Here it was washed through a pre-warmed Emcon filter with warm flush medium (Vigro® Complete Flush; Vetoquinol SA, Lure cedex, France) to remove any contaminating blood cells. Any cumulus-oocyte complexes (COCs) were thereby concentrated in approximately 10 ml of the medium in the bottom of the filter before being rinsed into a dry 90 mm searching dish that had search lines scored on the bottom.

The dish was searched under a stereo dissecting microscope (Nikon SMZ10) at 12x magnification on a heated stage (38.5^{o}C) and the COCs picked out with a micropipette and washed through a 35mm Falcon dish containing Vigro® Holding Medium (Vetoquinol SA, Lure cedex, France). Once washed the viable oocytes are transferred with a micropipette to the maturation dish containing: M199 Hepes with NaHCO3, Fetal Calf Serum, Pyruvate stock, Gentamycin, Pluset® stock, Epidermal Growth Factor (EGF), Insulin-Transferrin-Selenium (ITS), Cysteamine stock (all Sigma Aldritch®, St.

Louis, Missouri, USA) and GlutaMAX (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Once all COCs had been recovered they were graded as shown in Table 3.2 and washed through 2 clean drops of HEPES buffered maturation medium prior to being pipetted into a labelled maturation vial. Maturation vials were then immediately placed into a BioTherm™ INC-R81 portable incubator (Cryologic, Victoria, Australia) at 38.5^{O}C and transported to the relevant lab. (NB at IVP 1, when donors were collected on-site the aspirate was taken the short distance to the laboratory in an insulated polystyrene box and the COCs, once found were placed in maturation media in the main incubator)

Table 3.2. Cumulus oocyte complex grading system used at the IVP 1 laboratory (adapted from Wurth and Kruip, 1992)

COC Grade	Description	Image	
1	Thick even layers of compact and bright cumulus investment, and a translucent ooplasm.		
2	Less compact and slightly darker cumulus investment. Fewer layers of cumulus cells.		
3	Very few cumulus cells, often structures with partial denudation and darker ooplasm.		
4	Strongly expanded cumulus investment with dark spots of degenerated cumulus cells and dark ooplasm or completely denuded.		
Discarded	All degenerate structures are discarded. Denuded oocytes are carried through maturation.		

Standard Protocols adopted after 1st June 2015 included the following changes:

The temperature in the collection area was maintained at 27 to 32^{O}C having a thermostatically controlled hot air blower installed powered by a biomass boiler. The epidural anaesthetic dose was standardised to 3-5ml of 2% Procaine Hydrochloride 50mg/ml, (Adrenacaine®, Norbrook, Newry, Northern Ireland).

The vagina was lavaged using approximately 200ml of 1% Virkon S™ (Lanxess, Sudbury, UK) and rinsed thoroughly with Normal Saline through an adapted giving set External genitalia were also rinsed using dilute Virkon and wiped with paper towels. OPU equipment used was either an Easi-Scan® with Mindray 65C15EAV (5-8.5 MHz) Micro Convex Transducer (BCF, Bellshill, UK) (Figure 3.6) or a Prosound 2® with WTA OPU probe handle (Hitachi Aloka Medical, Tokyo, Japan) (Figure 3.7). The vacuum pump was upgraded to a Cook Aspiration Unit™ (Cook Medical, Bloomington, Indiana, US) with more emphasis put on control of the vacuum to reduce cumulus damage or loss during collection. Reverse bevel needles and Brazilian tubing (Partnar Animal Health, Port Huron, Michigan, USA) were introduced to help reduce follicular leaking, and to minimise the turbulence produced by changes in the tubing lumen diameter (Figure 3.8). The technique was adapted to minimise flushing between follicles unless excessive blood was aspirated.

The laboratory was redesigned and additional equipment purchased (Figure 3.9). The microscope used was an SMZ10 (Nikon, Tokyo, Japan). (NB This was further upgraded for more detailed grading purposes to an SMZ17 (Nikon, Tokyo, Japan) in January 2017). The maturation incubator was upgraded to a Miri® Benchtop Multi-room Incubator (Esco Medical, Egaa, Denmark) and the CO_2 incubator was upgraded to a Series CB60 (Binder, Tuttlingen, Germany). Transport incubators were upgraded to iQ1T models (MicroQ, Scottsdale, Arizona, USA)

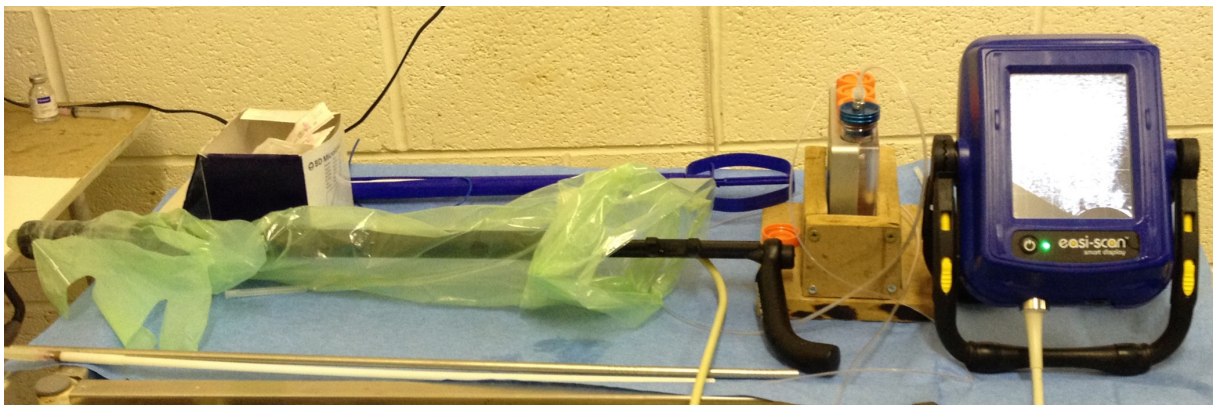


Figure 3.6. An Easy-Scan ultrasound machine showing handle prepared for transvaginal insertion, stainless steel needle guide and block heater.

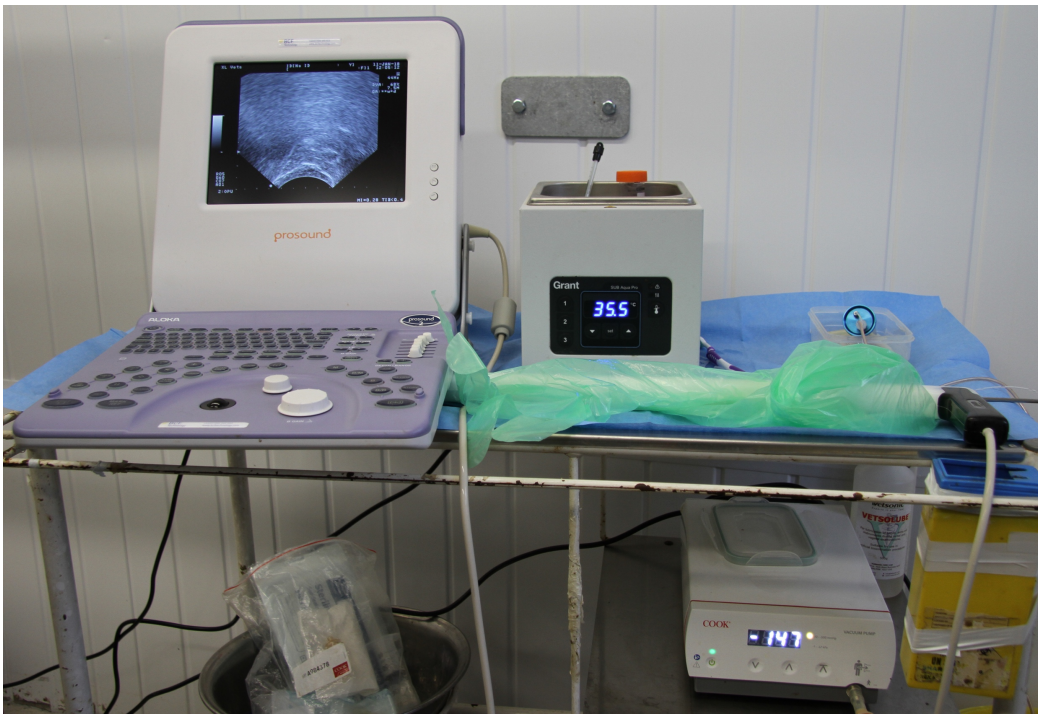


Figure 3.7. A Prosound 2® ultrasound machine showing handle prepared for transvaginal insertion, stainless steel needle guide, water bath and vacuum pump (below).

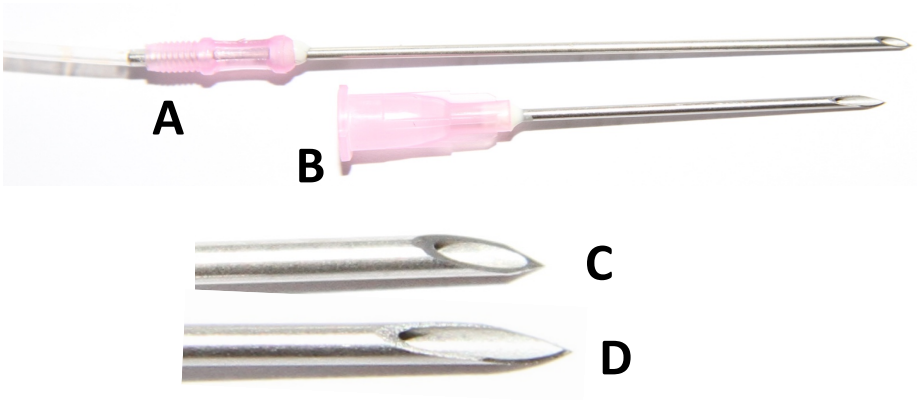


Figure 3.8. Aspiration needle connected directly to "Brazilian" tubing to minimise turbulence (A) compared to a standard disposable luer lock needle (B). Reverse short bevel straight cut tip (C) shown alongside a conventional bevel double cut needle (D).

Figure 3.9. The laboratory in November 2016, showing the incubators, microscopes, and preparation area.

3.2.4. The IVP processes

3.2.4.1. Maturation

Either fresh medium was made on the day of collection, or frozen vials of the same premade maturation medium was thawed, having been stored at -20^{o}C. All dishes and vials to be used were equilibrated for at least 2 hrs in a CO_2 incubator (5% CO_2) before adding cumulus oocyte complexes (COCs). Up to 40 COCs were placed in a well or tube.

3.2.4.2. Fertilisation

Fertilisation plates were prepared using 43µl drops of G-IVF® media covered in oil. These plates were equilibrated for a minimum of 1.5 hours before use in 5% CO_2.

Before thawing semen, oocytes were washed out of maturation through 2 x 150µl drops of warmed G-MOPS® (Vitrolife™, Warwick, UK) in a 60mm Falcon dish and placed into a wash drop in a fertilisation dish, pipetted gently to thoroughly mix & moved to a fertilisation drop. The drop was marked and the dish labelled.

Semen was then prepared in one of two ways;

i. Density Gradient

A density gradient was prepared using Bovipur® and Bovidilute® (Nidacon, Mölndal, Sweden) and one straw of thawed semen was then spun for 4 minutes in a basic centrifuge. The supernatant was drawn off, washed with

1ml Boviwash® and spun a second time for 45 seconds. This was repeated. The resulting pellet is then re-suspended in Boviwash®.

ii. Swim up

1.5ml of Boviwash® was pipetted into a labelled Migration Sedimentation Chamber (MSC) (Partnar Animal Health, Port Huron, Michigan, USA). The semen was layered carefully into the ring-well, taking care not to contaminate the medium above the well and then incubated upright in a warm box at 36-38^{o}C for 1.5 hours. The supernatant was gently drawn off with a pipette down to just below the inner rim of the ring-well and transferred to a sterile, warm tube labelled with the sire. The remaining supernatant in the base of the MSC was drawn off with a 200µl tip and added to the snap-top vial, before spinning in a basic centrifuge for 45 seconds. The sperm-pellet was visually assessed, and supernatant drawn off to an appropriate level in which it was re-suspended. All sperm pellets differ in size and more wash is left with the pellet if it is larger, therefore taking less supernatant. The approximate volumes were 50µl sperm and 250ul wash, to result in around 1 million sperm per ml. The pellet was then re-suspended in the remaining medium and counted before being added to the oocyte drop.

3.2.4.3. Sperm count

To prepare for counting, 5 µl of well mixed semen suspension was added to 95 µl of 4% NaCl diluent and mixed well. 10µl of this suspension was then placed on a haemocytometer and counted. The count of both haemocytometer grids was added, and divided by 10 to give million/ml (n). Divide 50 by n to give the dose in µl of sperm to add to a fertilisation drop for 1 million/ml dose (standard dose – 0.5 million/ml)
Fresh stock solution (pH/Hep) was made on the day of use, and equilibrated in a vial, loose-capped in the CO_2 incubator (5% CO_2) for minimum 1 hour before use. It was important that all vials, media, semen and mini centrifuge were kept at 37^{o}C in the warm box throughout.

3.2.4.4. Insemination

Prior to adding the calculated dose of semen, 2µl PH/HEP was added to the fertilisation drop. The fertilisation dish was then incubated for 5 hours or overnight. (5% CO_2, 5% O_2).

3.2.4.5. Culture

Culture plates were prepared using BBH7® (MOFA® Verona, Wisconsin, USA) as the culture medium. The drops were placed under mineral oil (Sigma Aldritch®, St. Louis, Missouri, USA) and equilibrated for at least 1.5 hours before use (5% CO_2). 18-24 hrs after the start of fertilisation, the oocytes were moved to a wash drop of culture medium and cumulus cells removed with a stripping pipette prior to being moved to a fresh drop of culture medium.

Culture was then undertaken in a humidified atmosphere of 5% CO_2, 5% O_2 at 39^{o}C for 8 days. On day 7 and again on day 8 development was assessed and viable morula or blastocysts selected for transfer or cryopreservation.

As of 1st June 2015, the protocols were standardised and along with some smaller iterative process adaptions the key changes were;

A new set of media were introduced, sourced from Boviteq (St Hyacinthe, Quebec, Canada), with very little media preparation now required locally. However, some components were added either at batch preparation (BSA) or on the day of use including, antibiotics and energy supplements, with foetal calf serum (FCS), oestradiol and lyophilised LH/FSH also being added to the maturation medium.

Maturation vials were pre-prepared in batches and were then stored at -20°C before thawing on the day of use. This allowed for easier satellite team involvement.

The fertilisation protocol was modified such that fertilisation dishes were equilibrated under oil in humidified atmosphere at 5.6% CO_2 and 20% O_2.

3.2.4.6. Preparing for fresh embryo transfer

For fresh transfer embryos were placed into Holding Medium (HM) made up of G-MOPS™ (Vitrolife®) and FCS and then using a 1ml syringe attached to a straw adapter were loaded into 0.25ml straws as follows;

A 5cm column of HM was drawn into the straw followed by 0.5cm air, 1cm HM and another 0.5cm air bubble. The embryo was then drawn into the straw in a 1-3cm column of HM followed by a third 0.5cm air bubble and then HM is drawn in to fill the straw, making sure the cotton wadding is thoroughly wetted (Figure 3.10). This embryo-loaded straw was now ready for fresh transfer.

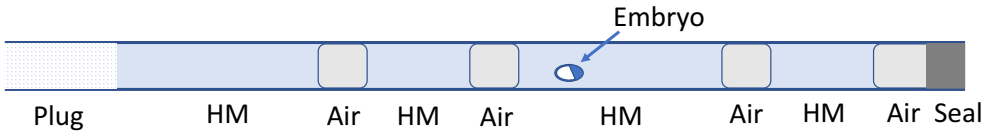


Figure 3.10. A schematic of a loaded transfer straw showing the plug, columns of holding medium, air bubbles and seal

3.2.4.7. Cryopreservation

Two techniques were used in this study;

Embryo Freezing - conventional in-vivo produced early stage cattle embryos (late morulae and blastocysts with an intact zona pellucida) are suitable for preservation by deep-freezing in liquid nitrogen (cryopreservation).

In this study embryos were vigorously washed through flushing medium, before being placed directly into Emcare Freezing Medium (ICPbio Reproduction, Spring valley, Wisconsin, USA) for a minimum of 10 minutes and maximum of 20 minutes. While the embryos were equilibrating they were loaded into straws as above (Figure 3.10), using ethylene glycol (EG) instead of holding medium, and sealed with a heat sealer. This embryo-loaded straw was now ready for cryopreservation.

The straws are placed into the chamber of a programmable freezing machine (Freeze-Control CL856 or CL5500) at a temperature just below the freezing point of the freezing medium (–7 degrees C) and ice formation is initiated by touching the straw with a cooled swab (seeding). The straws are then cooled at a rate of 0.5^{o}C per minute to – 32^{o}C and then plunged into liquid nitrogen for storage.

Embryo Vitrification - various techniques were undertaken at Nottingham as part of the Innovate study although these are not reported here. The technique opted for was using a hemi-straw protocol.

Embryos were transferred through a series of solutions;

i. Vitrification Holding Medium (VHM) composed of GMOPS+ and FCS
ii. VHM, EG (Ethylene Glycol), and DMSO (Dimethyl Sulphoxide)
iii. VitSucrose, EG, and DMSO

The embryo was then loaded with 1µl of the final solution into the tip of a hemi straw and plunged directly into liquid nitrogen, stirring to minimise the Leidenfrost effect.

From 1st June 2015, the quality of embryos being produced had improved to a standard whereby they were able to be cryopreserved by freezing as described above and with the one-step thaw being so much more practicable on farm this technique was adopted throughout.

3.2.5. Transfer of bovine embryos

This involves the placement of an early stage embryo (usually 6 – 8 days gestation) into the uterus of a recipient cow or heifer whose oestrus cycle has been synchronised with that of the donor.

Potential recipients were screened and selected to ensure that they were of a suitable breed, age and size to calve normally after receiving an embryo from the donor cow. Advice about nutrition and general management was given to optimise fertility as much as possible.

Occasionally embryos were transferred after a natural heat simply by observing and recording oestrus in a group of recipients but more often oestrus was synchronised in a group of recipients using techniques involving CIDR®, PRID® and prostaglandin treatments. Embryos were transferred 6.5 to 7.5 days after the onset of observed standing heat.

If using conventionally frozen (direct transfer) embryos, these were “quick thawed”, by removing the embryo straw from the transport goblet using a pair of dressing forceps, thawed in air at ambient temperature for 4 seconds and then plunged rapidly

into 20^{o}C water for 10 seconds. The straw was then dried with clean paper towelling and transferred as below.

If using vitrified embryos – they were thawed through a 3-step process of reducing concentrations of "Warmsucrose" solution (sucrose (sigma s7903) 3.664G and VHM) before being assessed, and if suitable, loaded into a transfer straw as above and transferred.

For transfer, the piston/stilette of a Cassou embryo transfer gun (cow or heifer size as appropriate) is withdrawn 15cm and the loaded straw placed, wadding first, into the barrel of the gun. If a cryopreserved embryo was being transferred it was necessary to remove the sealed end of the straw using sharp scissors. The straw was held in place by sliding a sterile sheath over the gun and straw, bedding it down firmly and then fixing it by downward pressure on the plastic ring at its base. The gun was then made ready for the transfer by covering with a plastic chemise labelled with the embryo identity.

From this point, all transfers were the same technique. The recipient was restrained in a standard cattle crush or AI stall, suitable for fertility examinations. A low epidural anaesthetic was administered (3-5 ml of 2% Procaine Hydrochloride 50mg/ml, Adrenacaine®, Norbrook, Newry, Northern Ireland injected into the epidural space between the last sacral and the first caudal vertebrae). The rectum was manually emptied of faeces and the ovaries gently palpated per rectum to determine the presence of a normal corpus luteum and the side of ovulation. Recipients were rejected if they had not ovulated, were cystic or where other reproductive problems were detected.

After cleaning of the vulva and perineum the insemination gun was inserted into the vagina and advanced into the external os, through the cervix and up the lumen of the uterine horn on the same side as the ovary with the corpus luteum.

3.2.6. Database and data analysis

A web-based software solution was commissioned (http://www.arrow-web.co.uk), which is comprised of a relational database management system (RDBMS) and a bespoke front-end web application. MySQL Community Edition (https://www.mysql.com/) was used as the RDBMS. As per standard database design, tables were joined using foreign keys to ensure data consistency and accuracy. The database schema also defined required fields, but most validation was done at the web application layer.

The web application is a bespoke piece of software written in PHP (http://php.net/). It is built on the Laravel framework (https://laravel.com/). It also uses JavaScript to add some interactive elements. The web application implements much of the complex validation, ensuring that all data is accurate and correctly linked.

The web application includes numerous reports that interrogate the data and display it in an easy to view format. In addition, the system also provides a full download. This joins all the relevant tables to produce a single comma separated values (CSV) file. The CSV file contains all information held in relation to a donor animal, flush and embryos produced (Table 3.3)

This CSV file was then downloaded into MS Excel (Excel for Mac, Version 15.41, Microsoft, 2017) and the data checked prior to statistical analysis being performed using Genstat version 11.1.0.1504 (VSN International Ltd., Hemel-Hempstead, UK).

3.2.6.1. Statistical analyses

Data were analysed using REML Generalised Linear Mixed Models within Genstat (Genstat 18th ed, VSNi, Rothamsted, UK). Embryo development (proportions) assumed binomial-error distributions with logit-link functions. Four datasets were analysed comprising different OPU Team/Laboratory combinations active throughout the project (Figure 3.1). For each set of analyses the term ‘Donor ID’ was fitted to the random model to account for multiple cycles and oocytes per donor. For Dataset 1 terms fitted to the fixed model were ‘OPU/Lab’ and ‘OPU/IVP protocol’ (i.e. ‘OPU

1/IVP 1 stimulated' vs 'OPU 1/IVP 1 non-stimulated' vs 'OPU 2/IVP 2 non-stimulated'). For Dataset 2, terms fitted to the fixed model were + 'Semen Type' ('Sexed' vs 'Conventional') + 'Stimulated Folltropin' vs 'Stimulated Pluset®' vs 'Non-stimulated' + 'OPU team' (OPU 1, OPU 2, OPU 3, OPU 4, OPU 5, OPU 6,). For Dataset 3, the terms fitted were as for Dataset 2 but restricted to the following OPU teams: OPU 1, OPU 2 and OPU 4. Finally, for Dataset 4 fixed terms fitted in the initial analysis were 'Semen type' ('Sexed' vs 'Conventional') + 'OPU/IVP protocol' ('Non-stimulated' (with or without DFR) vs 'Stimulated' (Folltropin with or without DFR vs Pluset with DFR)) + 'Collection date' (number of days from start of study). Embryo development data are presented as back transformed means. Differences between individual means were established from predicted least-significant differences (LSDs).

Table 3.3. The parameters recorded on the database

Animal Data	**DFR/OPU Data**	**Laboratory Data**	**Outcome Data**
organisation_name	dfr_date	coc_grade_1	total_embryos_produced
client_id	flush_id	coc_grade_2	embryos_discarded
animal_id	collection_date	coc_grade_3	embryos_frozen_day_7
dob	coasting_regime	coc_grade_4	embryos_frozen_day_8
donor_name	coasting_doseage	coc_collected_total	freezing_method
donor_ear_tag	fsh_batch	coc_discarded	embryo_id
donor_dna_number	collection_site	coc_onto_maturation	embryo_number
donor_hb_number	collected_by	time_coc_onto_maturation	embryo_location
donor_breed	temp_at_collection	semen_prep_method	grade_on_thaw
donor_clinical_history	temp_outside	semen_dose_straws	grade_on_recovery
donor_repro_status	time_collecn_completed	heparin_concn_ug_ml	embryos_transferred
donor_parity	follicle_number_left	fertilisation_date_1	transfer_date
lactational_status	follicle_number_right	fertilisation_date_2	embryo_operator
donor_travel_time	follicle_average_size	no_cleaved_day_4	recipient_breed
sire_1	aspiration_medium_type	more_than_4_cells	Recipient_age
sire_2		poor_quality_morulas	recipient_id
sire_3		good_quality_morulas	pregnancy_diagnosis

3.3. Results

3.3.1. Dataset 1

These were the data from all the OPU cycles undertaken before 1st June 2015 which was the point at which all protocols were standardised having optimised the system. Data from OPU 3/IVP 1 and OPU 4/IVP 1 were not included as these groups were still in the early training phase.

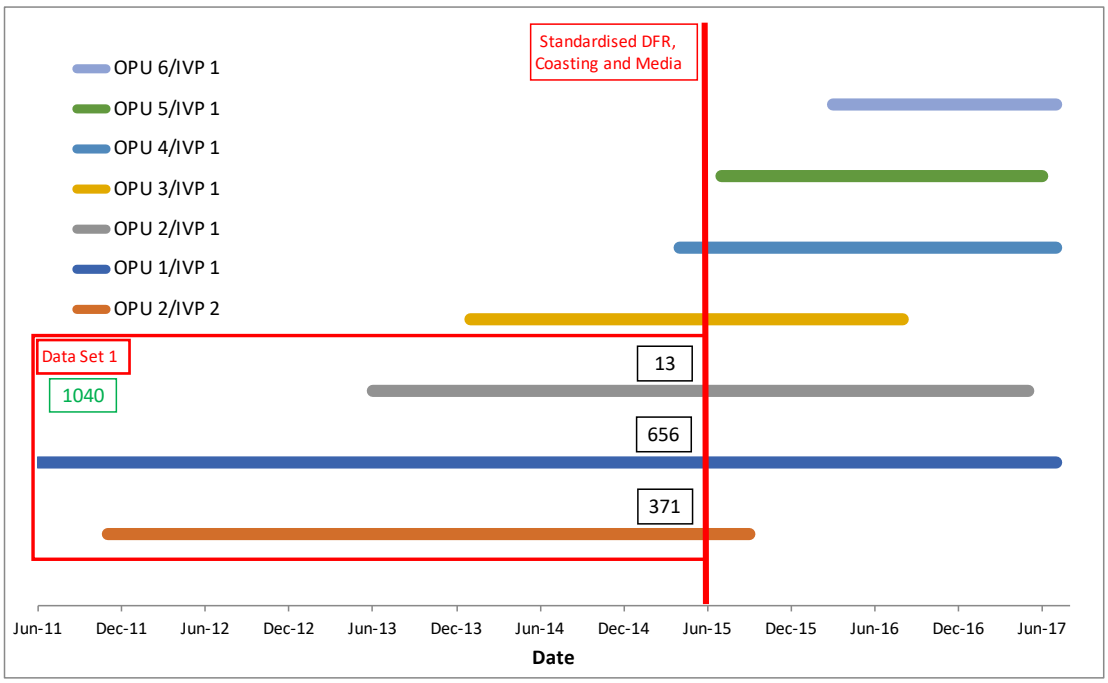


Figure 3.11. From Figure 3.1, Dataset 1 is superimposed as the red box, and includes data from OPU 2/IVP 2, OPU 1/IVP 1 and OPU 2/IVP 1 for the first 4 years of the project, prior to standardised protocols for OPU, cow-side and laboratory processes being introduced on 1st June 2015 (shown as a vertical red line). The number of cycles in the total dataset (green box) and from each OPU team (black boxes) are shown.

It can be seen in Table 3.4 that there were no stimulated cycles at the OPU 2/IVP 2 site, while all the collections at OPU 2/IVP 2 and OPU 2/IVP 1 were undertaken by the vet identified as C1. Although this reduces the risk of an operator being a confounding variable within that dataset, it has the risk of introducing a systemic effect within that subset. At OPU 1/IVP 1 there were 3 main OPU vets within this dataset, but several others were training at this site so the data are likely to be less consistent. The Holstein was by far the predominant breed at OPU 2/IVP 2, while at OPU 1/IVP 1 oocytes were collected from several other dairy breeds, because cattle owned by the practice

tended to be Ayrshire at that time. At both centres, the ratio of dairy to beef was approximately 6 to 1. Although the OPU team and equipment were the same as at OPU 2/IVP 2, because the number of animals processed by the OPU 2/OPU 1 was small (at only 13), it was decided to also exclude this subset from the subsequent statistical analysis of Dataset 1.

Table 3.4. A breakdown of data subsets from Dataset 1 including the total number of OPU cycles, the distribution of stimulated and non-stimulated cycles, the OPU vets involved, the main breeds collected, and the number of different sires used by centre.

OPU Team/Lab	OPU 1/IVP 1	OPU 2/IVP 1	OPU 2/IVP 2
Number of Cycles	656	13	371
Stimulated			
Yes	403	4	
No	253	9	371
OPU Vets			
C1		13	371
P1	347		
P2	153		
P3	70		
Others	146		
Breeds			
Holstein	226	11	312
Other Dairy	327	2	8
Beef	103		51
Number of Sires	115	6	85

Differences in oocyte recovery and embryo development were observed between OPU/Lab site combinations, and between stimulated and non-stimulated cycles at the OPU 1/IVP 1 site (Table 3.5). Key findings are summarised below. Some of these (e.g. oocyte recovery) will be purely an OPU effect and some a combined OPU/IVP effect due to cow-side and/or laboratory factors.

The OPU 2 team did not record the number of follicles aspirated but collected more ($P<0.001$) oocytes than the OPU 1 team (for both stimulated and non-stimulated cycles), while OPU 1 collected more oocytes from stimulated cycles than non-stimulated (Table 3.5). The OPU 2 team collected more ($P<0.001$) oocytes suitable for insemination than did the OPU 1 team (for both stimulated and non-stimulated cycles), while the OPU 1/IVP 1 team combination produced more oocytes that went onto insemination from stimulated animals. Although data were only available from the OPU 1/IVP 1 grouping, the proportion of oocytes suitable for insemination per follicle was greater ($P=0.002$) from stimulated than non- stimulated cycles. The proportion cleaved of inseminated oocytes was greater ($P<0.001$) at IVP 2 than at IVP 1. The proportion cleaved of inseminated oocytes was greater ($P<0.05$) for stimulated than non-stimulated animals for the OPU 1/IVP 1 group.

The OPU 2/IVP 2 lab-combination produced more ($P<0.001$) blastocysts per cycle (2.02) than did the OPU 1/IVP 1 lab-combination (0.80 stimulated, 0.47 non-stimulated) (Table 3.5). Within the OPU 1/IVP 1 grouping, more blastocysts per cycle were produced from stimulated than non-stimulated cycles (0.80 vs 0.47, $P<0.05$). Although again only OPU 1/IVP 1 data are available, there were more blastocysts produced per follicle from stimulated than non-stimulated cycles. Blastocysts of cleaved were greater ($P=0.001$) for stimulated (0.56) than non-stimulated cycles; the latter were similar between sites (0.42 vs 0.43 for OPU 2/IVP 2 and OPU 1/IVP 1 sites respectively).

Table 3.5. Summary results from mixed effects statistical models to quantify the impact of ovarian stimulation protocol on OPU (follicles and oocytes collected) and IVP (oocytes inseminated, cleavage proportions and blastocyst production). Results of OPU/IVP combination on stimulated and non-stimulated cycles within Dataset 1.

OPU/Lab	OPU 1/IVP 1		OPU 2/IVP 2	(P)
OPU/IVP Protocol	Stimulated	Non-stimulated	Non-stimulated	
Total No Cycles	403	253	371	
Response Variate				
		OPU		
No. Follicles Aspirated per Cycle	10.88[a]	8.60[b]	*	<0.001
No. Oocytes Collected per Cycle	6.74[b]	5.32[c]	10.82[a]	<0.001
Oocytes per Follicle	0.60	0.58	*	NS
		IVP		
No. Oocytes Inseminated per Cycle	5.50[b]	3.92[c]	9.08[a]	<0.001
Inseminated per Follicle	0.48[a]	0.42[b]	*	0.002
Inseminated per Oocyte	0.82[a]	0.74[b]	0.84[a]	<0.001
Cleaved of Inseminated	0.35[b]	0.29[c]	0.56[a]	<0.001
≥4 cells of Cleaved	0.70[b]	0.60 [b]	0.77[a]	0.003
Total No. Blastocysts per Cycle	0.80[b]	0.47[c]	2.02[a]	<0.001
Blastocysts per Follicle	0.07[a]	0.05[b]	*	0.019
Blastocysts per Oocyte	0.12[b]	0.09[b]	0.19[a]	<0.001
Blastocysts of Inseminated	0.15[b]	0.12 [b]	0.23[a]	<0.001
Blastocysts of Cleaved	0.56[a]	0.43[b]	0.42[b]	0.001

Data were log transformed prior to analysis with back transformed means shown. Letters in superscript indicate significant differences within a row (P<0.05).

3.3.2. Dataset 2

Because there were two laboratories operating, individuals were being recruited and trained, and frequent changes were being made to the protocols, the data were very confounded. In an attempt to reduce some of these variables, Dataset 2 was analysed which contains the data from all the OPU cycles undertaken after 1st June 2015; this was the point at which all protocols were standardised having optimised the system.

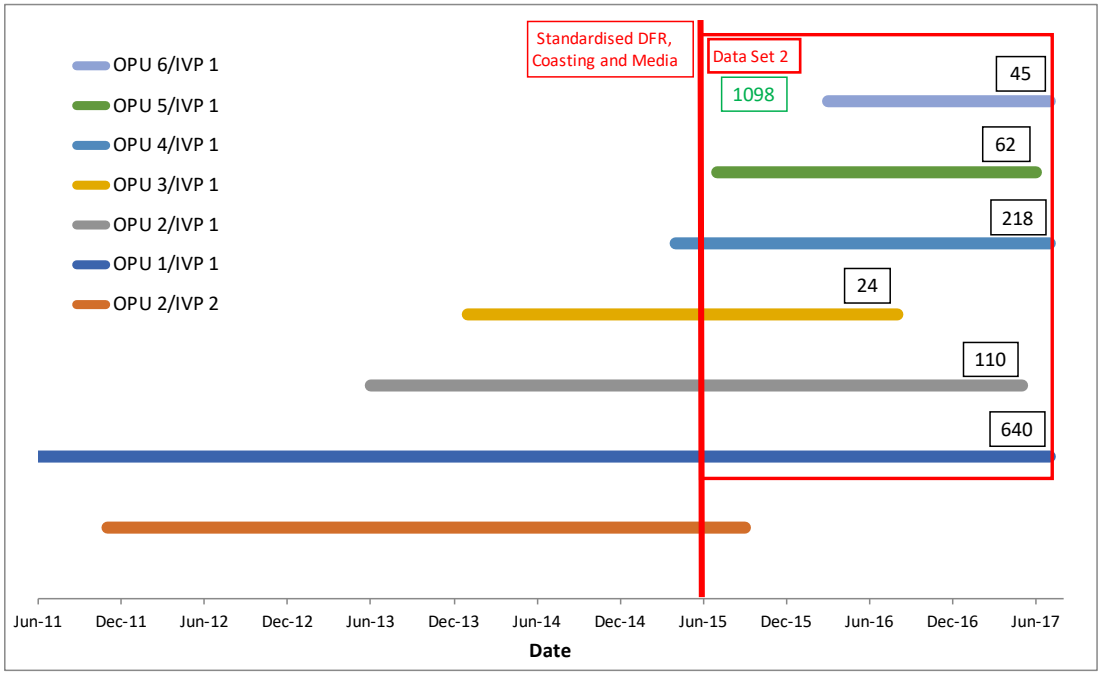


Figure 3.12. From Figure 3.1, Dataset 2 is superimposed as the red box, and includes data from all 6 OPU teams, using the IVP 1 laboratory for the final 25 months of the project, after standardised protocols for OPU, cow-side and laboratory processes were introduced on 1st June 2015 (shown as a vertical red line). The number of cycles in the total dataset (green box) and from each OPU team (black boxes) are shown.

Table 3.6. A breakdown of data subsets from Dataset 2 including the total number of OPU cycles, the percentage distribution of stimulated and non-stimulated cycles, the OPU vets involved as a percentage of cycles undertaken at that centre, the percentage of breeds collected, the minimum, maximum, mean and median ages of donors from each team, and the number of different sires used by centre.

OPU Team	OPU 1	OPU 2	OPU 3	OPU 4	OPU 5	OPU 6
Number of Cycles	640	110	24	218	62	45
Stimulated (%)						
Folltropin®	85.6	25.5	100	91.3	85.5	88.9
Pluset®	10.6	17.3	0	2.3	1.6	11.1
None	3.8	57.3	0	6.4	12.9	0
OPU Vets (%)						
P1	5.0					
P2	1.1					
P3	53.4					
P4	26.9					
P5	8.6					
C1		100				46.7
R1			100			
N1				99.1		
B1					77.4	
B2					22.6	
T1						26.7
T2						8.9
Others	5.0	0.0	17.8	0.9	0.0	0.0
Breeds (%)						
Holstein	63.3	51.8	45.8	12.4	1.6	20.0
Other Dairy	13.4	11.8	0.0	1.8	16.1	8.9
Beef	23.3	36.4	54.2	85.8	82.3	71.1
Number of Sires	146	46	5	76	24	9
Ages*						
Minimum	0y 6m	1y 1m	2y 0m	1y 3m	2y 2m	1y 0m
Maximum	16y 9m	13y 11m	5y 3m	20y 0m	18y 2m	11y 1m
Mean	4y 1m	2y 9m	3y 2m	7y 4m	6y 8m	4y 11m
Median	3y 4m	2y 9m	2y 3m	7y 5m	6y 8m	5y 10m

**Dates of birth were not recorded for every donor at the start of the project*

At least 85% of cycles within Dataset 2 were Folltropin® stimulated cycles (Table 3.6) except at OPU 2 which only stimulated 42.8% of cycles in total, and only 25.5% were with Folltropin®. Most centres had one or two OPU Vets doing most of the collections, except OPU 1, which was the main training centre – by the time of this dataset vets P1 and P2 were doing fewer OPU collections than in Dataset 1 (Table 3.4) while younger vets in the team became more experienced. It can be seen that the region and type of practice in which the OPU team was based affects the distribution of breeds, with OPU 1 and OPU 2 predominantly dairy and the others more beef orientated. The OPU 1 team worked with much younger animals, largely due to the smaller scanner probe purchased by that team allowing collection from younger heifers. Those teams with more dairy animals tended to work with younger heifers – this is almost certainly an effect of genomic testing becoming more common, especially in the dairy sector, encouraging farmers to breed with younger animals.

This is an imbalanced set of data in that seven-times more conventional semen than sexed semen was used, and with several confounding factors such as breed and age of donor, and the fact that the choice of semen used was determined by the farmer. Higher numbers of oocytes per follicle and of oocytes that were selected for insemination were seen with sexed semen (Table 3.7). There were no significant effects of semen type (conventional vs sexed) on proportion cleaved or that formed blastocysts. Similarly, the number of pregnancies per embryo transfer was unaffected by semen type.

Table 3.7. Summary results from mixed effects statistical models to quantify the impact of semen type (conventional or sexed) on oocytes per follicle and inseminated per follicle within Dataset 2

Semen Type	Conventional	Sexed	(P)
Number of Observations	960	139	
Response Variate			
Oocytes per Follicle	0.63	0.70	0.011
Inseminated per Follicle	0.51	0.56	0.045

Data were log transformed prior to analysis with back transformed means shown.

Results at various stages of the OPU/IVP process were compared across all 6 OPU teams, with IVP undertaken centrally at IVP 1, comparing those cycles that had been stimulated with Folltropin® or Pluset® and then coasted, against cycles that were not stimulated or coasted (Table 3.8).

There were differences (P<0.001) between all 3 groups with more follicles aspirated from Pluset® (12.32) than Folltropin® (10.25) and from non-stimulated (8.53). There were more oocytes collected (P=0.001) from Pluset® stimulated (7.02) than Folltropin® stimulated (5.43) or non-stimulated (5.45), between which there was no difference. More oocytes were suitable for insemination (P<0.001) from Pluset® treated cycles than either of the two other groups, there was no difference between the Folltropin® stimulated, and non-stimulated cycles (Table 3.8).

Stimulating with either Pluset® or Folltropin® produced more oocytes that were inseminated per follicle (0.61 and 0.55 respectively, P=0.005)) compared with those cycles that were not stimulated (0.46) (Table 3.8). There were differences (P<0.001) between all 3 groups with more inseminated per oocyte from Pluset® (0.92) than Folltropin® (0.88) and from not coasted (0.81). From those OPU cycles that were stimulated with Pluset®, more oocytes cleaved (P<0.05) of those that were inseminated than those on Folltropin®.

There were more embryos produced per cycle (P<0.001) following Pluset® stimulation and coasting (1.52) than following Folltropin® stimulation and coasting (0.79), or from those that were not stimulated (0.78) (Table 3.8). This Pluset® effect was associated with more blastocysts per follicle (P=0.007), and a higher proportion of blastocysts from those inseminated (P=0.028), than with Folltropin® stimulation. It was also seen that with Pluset® there were more blastocysts per oocyte (P<0.001).

Although there were numerically more pregnancies per embryo transferred when Folltropin® stimulation and coasting was used (0.41), than when Pluset® was used (0.28) or in the absence of stimulation (0.25), there were insufficient data to show a statistically significant difference (Table 3.8).

Table 3.8. Summary results from mixed effects statistical models to quantify the impact of ovarian stimulation protocols using different products (Folltropin® and Pluset®) on OPU (follicles and oocytes collected), IVP (on oocytes inseminated, cleavage proportions and blastocyst production) and ET (pregnancies) within Dataset 2.

OPU/IVP Protocol	Stimulated		Non-Stimulated	(P)
	Folltropin®	Pluset®		
Total No. Cycles	892	98	143	
Response Variate				
OPU				
No. Follicles Aspirated per Cycle	10.25^{b}	12.32^{a}	8.53^{c}	<0.001
No. Oocytes Collected per Cycle	5.43^{b}	7.02^{a}	5.45^{b}	0.001
Oocytes per Follicle	0.64	0.65	0.70	NS
IVP				
No. Oocytes Inseminated per Cycle	4.70^{b}	6.57^{a}	4.19^{b}	<0.001
Inseminated per Follicle	0.55^{a}	0.61^{a}	0.46^{b}	0.005
Inseminated per Oocyte	0.88^{b}	0.92^{a}	0.81^{c}	<0.001
Cleaved of Inseminated	0.63^{b}	0.75^{a}	0.68^{ab}	0.019
≥4 cells of Cleaved	0.59	0.63	0.64	NS
Total No. Blastocysts per Cycle	0.79^{b}	1.52^{a}	0.78^{b}	<0.001
Blastocysts per Follicle	0.09^{b}	0.14^{a}	0.10^{ab}	0.007
Blastocysts per Oocyte	0.13^{b}	0.23^{a}	0.14^{b}	<0.001
Blastocysts of Inseminated	0.17^{b}	0.26^{a}	0.21^{ab}	0.028
Blastocysts of Cleaved	0.28	0.35	0.34	NS
ET				
No. Pregnancies per Transfer	0.41	0.28	0.25	NS

Data were log transformed prior to analysis with back transformed means shown. Letters in superscript indicate significant differences within a row (P<0.05).

Table 3.9. Summary results from mixed effects statistical models to quantify the impact of six different OPU teams within Dataset 2 on OPU (follicles and oocytes collected), IVP (oocytes inseminated, cleavage proportions and blastocyst production) and ET (pregnancies).

OPU Team	OPU 1	OPU 2	OPU 3	OPU 4	OPU 5	OPU 6	(P)
Total No. Cycles	640	110	24	218	62	45	
Response Variate							
OPU							
No. Follicles Aspirated per Cycle	11.36^{b}	14.87^{a}	7.94^{bc}	10.15^{b}	6.51^{c}	13.11^{ab}	<0.001
No. Oocytes Collected per Cycle	6.93^{b}	12.40^{a}	4.03^{c}	7.58^{b}	3.91^{c}	4.20^{c}	<0.001
Oocytes per Follicle	0.61^{c}	0.89^{a}	0.52^{c}	0.72^{b}	0.62^{bc}	0.52^{c}	<0.001
IVP							
No. Oocytes Inseminated per Cycle	5.61^{b}	10.43^{a}	3.11^{c}	6.42^{bc}	3.65^{c}	3.93^{c}	<0.001
Inseminated per Follicle	0.46^{c}	0.76^{a}	0.39^{c}	0.60^{b}	0.55^{bc}	0.44^{c}	<0.001
Inseminated per Oocyte	0.84^{b}	0.85^{bc}	0.78^{b}	0.87^{bc}	0.94^{ac}	0.95^{a}	0.011
Cleaved of Inseminated	0.67	0.61	0.74	0.67	0.63	0.80	NS
≥4 cells of Cleaved	0.67^{a}	0.62^{a}	0.33^{b}	0.60^{a}	0.76^{a}	0.71^{a}	0.022
Total No. Blastocysts per Cycle	1.39	1.82	0.34	1.28	0.77	1.07	NS
Blastocysts per Follicle	0.12	0.12	0.05	0.12	0.12	0.13	NS
Blastocysts per Oocyte	0.22	0.14	0.09	0.18	0.20	0.29	NS (0.055)
Blastocysts of Inseminated	0.27^{a}	0.17^{b}	0.12^{b}	0.21	0.22	0.31	0.032
Blastocysts of Cleaved	0.44^{a}	0.29^{bc}	0.16^{c}	0.33^{b}	0.36^{abc}	0.41^{ab}	0.003
ET							
No. Pregnancies per Transfer	0.26	0.49	*	0.30	0.22	*	NS

Data were log transformed prior to analysis with back transformed means shown. Letters in superscript indicate significant differences within a row (P<0.05).

There were differences in the number of follicles aspirated by the various OPU teams (P<0.001) with OPU 2 the highest (14.87) and OPU 5 the lowest (6.51) (Table 3.9). The OPU 2 operator recovered more oocytes (12.40, P<0.001) than any of the other teams, with the teams in OPU 1 and OPU 4 (6.93 and 7.58 respectively) collecting more than the remaining 3 teams. More oocytes per collection and, as a result, more oocytes per follicle (P<0.001), were selected for maturation and insemination from OPU 2 than any other team, suggesting that the oocytes looked morphologically good enough to go into the IVP system.

A higher proportion of oocytes went onto maturation (P=0.011) from OPU 6 (0.95) than any other team, and more from OPU 5 (0.94) than OPU 1 (0.84) or OPU 3 (0.78) (Table 3.9). Fewer oocytes from OPU 3 than any other team had reached a stage of 4 cells or greater when examined (P=0.022). Numerically OPU 2 produced most embryos per collection but this was not statistically significant.

There was a trend (P=0.055) towards most blastocysts per oocyte from OPU 6 (0.29) and fewest from OPU 3 (0.09). There was a difference across the teams in the proportion of blastocysts of those that cleaved (P=0.003), with OPU 1 highest at 0.44 and OPU 3 lowest at 0.16 (Table 3.9).

Many of the embryos had not been transferred at the time of these analyses and pregnancy data were incomplete for those that had. These low numbers mean that the analysis is not significant. However, numerically, OPU 2 achieved more pregnancies per embryo transfer.

There remain many confounders in these data such as donor selection, operator experience and skill, stage in the project when the collections were undertaken etc. So, in an attempt to deal with some of these confounders, Dataset 3 was analysed which had the 3 most experienced OPU teams, and more evenly sized groups of OPU sessions. All IVP was undertaken at the IVP 1 lab.

3.3.3. Dataset 3

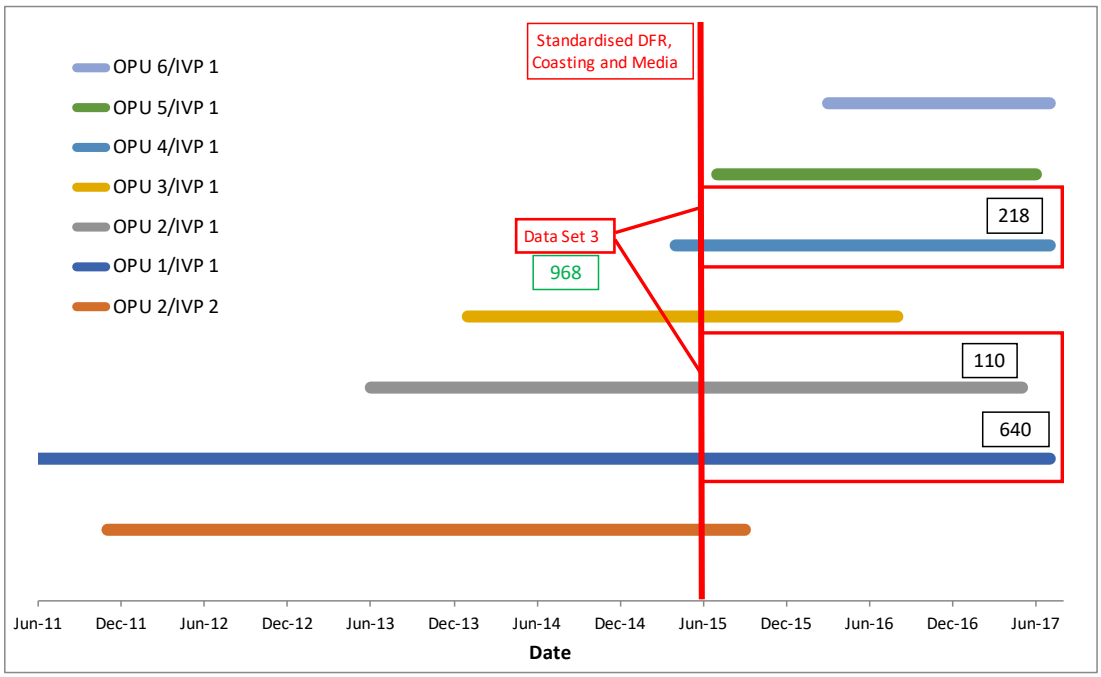


Figure 3.13. From Figure 3.1, Dataset 3 is superimposed as the red box, and includes data from the 3 most experienced OPU teams (OPU 1, OPU 2 and OPU 4), using the IVP 1 lab for the final 25 months of the project, after standardised protocols for OPU, cow-side and laboratory processes were introduced on 1st June 2015, shown as a vertical red line. The number of cycles in the total dataset (green box) and from each OPU team (black boxes) are shown.

Table 3.10. Summary results from mixed effects statistical models to quantify the impact of semen type (conventional or sexed) on oocytes per follicle and inseminated per follicle within Dataset 3

Semen Type	Conventional	Sexed	(P)
Number of Observations	831	137	
Response Variate			
Oocytes per Follicle	0.73	0.79	0.011
Inseminated per Follicle	0.60	0.65	0.042

Data were log transformed prior to analysis with back transformed means shown.

The breakdown of ovarian stimulated cycles, OPU vets, breeds and ages is described in section 3.3.2 and shown in Table 3.6. As with Dataset 2, which considered all 6 OPU teams, in these data there were more oocytes per follicle and inseminated per follicle associated with sexed semen (Table 3.10).

These data were from the 3 most experienced OPU teams by the end of the study; OPU 1 with 1298 cycles, OPU 2 with 528 cycles (405 through the IVP 2 lab and 123 through the IVP 1 lab), and OPU 4 with 221 cycles. The pattern of results is very similar to that of Dataset 2 (Table 3.8), but generally with higher numbers. There were differences between all 3 stimulation and coasting protocols ($P<0.001$) (Table 3.11) with more follicles aspirated from Pluset® treated cycles (14.2) than Folltropin® (11.5) and from not coasted (9.4). Again, there were more oocytes collected from the Pluset® group than from either of the other two groups and there was no difference between Folltropin® and non-stimulated.

The Pluset® treatment group performed better than the other treatment groups throughout all stages of embryo production (Table 3.11) with more oocytes inseminated ($P<0.001$), more inseminated per oocyte collected ($P<0.001$) and more blastocysts produced ($P<0.001$) at 2.5 per cycle compared with 1.2 and 1.2 from the Folltropin® stimulated and non-stimulated groups respectively. Interestingly, the Folltropin® group and non-stimulated group performed similarly for most parameters except the number of follicles aspirated with Folltropin® at 11.53, and non-stimulated at 9.37, ($P<0.001$) and inseminated per oocyte ($P<0.05$) where the Folltropin® group resulted in 0.85 compared with 0.78 from the non-stimulated cycles.

There was no difference (Table 3.11) in the proportion of blastocysts of cleaved, pregnancies per OPU or pregnancies per transfer although numerically there were more pregnancies from Folltropin® – but there were not enough data to give sufficient statistical power.

Table 3.11. Summary results from mixed effects statistical models to quantify the impact of ovarian stimulation protocols using different products (Folltropin® and Pluset®) on OPU (follicles and oocytes collected), IVP (on oocytes inseminated, cleavage proportions and blastocyst production) and ET (pregnancies) within Dataset 3.

OPU/IVP Protocol	Stimulated		Non-Stimulated	(P)
	Folltropin®	Pluset®		
Total No. Cycles	775	92	101	
Response Variate				
OPU				
No. Follicles Aspirated per Cycle	11.53^{b}	14.24^{a}	9.37^{c}	<0.001
No. Oocytes Collected per Cycle	7.87^{b}	10.73^{a}	8.02^{b}	<0.001
Oocytes per Follicle	0.74	0.76	0.80	NS
IVP				
No. Oocytes Inseminated per Cycle	6.61^{b}	9.84^{a}	6.07^{b}	<0.001
Inseminated per Follicle	0.62^{ab}	0.69^{a}	0.55^{b}	0.002
Inseminated per Oocyte	0.85^{b}	0.91^{a}	0.78^{c}	<0.001
Cleaved of Inseminated	0.58^{b}	0.72^{a}	0.65^{ab}	0.014
≥4 cells of Cleaved	0.60	0.64	0.65	NS
Total No. Blastocysts per Cycle	1.17^{b}	2.45^{a}	1.24^{b}	<0.001
Blastocysts per Follicle	0.10^{b}	0.17^{a}	0.12^{ab}	0.005
Blastocysts per Oocyte	0.14^{b}	0.25^{a}	0.16^{b}	0.003
Blastocysts of Inseminated	0.17^{b}	0.27^{a}	0.23^{ab}	0.029
Blastocysts of Cleaved	0.30	0.38	0.37	NS
ET				
No. Pregnancies per Transfer	0.44	0.31	0.29	NS

Data were log transformed prior to analysis with back transformed means shown. Letters in superscript indicate significant differences within a row (P<0.05).

Table 3.12. Summary results from mixed effects statistical models to quantify the impact of the three most experienced OPU teams (OPU 1, OPU 2 and OPU3) within Dataset 3 on OPU (follicles and oocytes collected), IVP (oocytes inseminated, cleavage proportions and blastocyst production) and ET (pregnancies).

OPU Team	OPU 1	OPU 2	OPU 3	(P)
Number of Observations	640	110	218	
Response Variate				
OPU				
No. Follicles Aspirated per Cycle	11.09^{b}	13.83^{a}	10.03^{b}	0.051
No. Oocytes Collected per Cycle	7.05^{b}	12.43^{a}	7.73^{b}	<0.001
Oocytes per Follicle	0.62^{b}	0.89^{a}	0.73^{c}	<0.001
IVP				
No. Oocytes Inseminated per Cycle	5.74^{b}	10.44^{a}	6.59^{b}	<0.001
Inseminated per Follicle	0.47^{b}	0.76^{a}	0.61^{c}	<0.001
Inseminated per Oocyte	0.84	0.84	0.88	NS
Cleaved of Inseminated	0.67	0.61	0.67	NS
≥4 cells of Cleaved	0.66	0.62	0.60	NS
Total No. Blastocysts per Cycle	1.46	1.82	1.35	NS
Blastocysts per Follicle	0.13	0.12	0.13	NS
Blastocysts per Oocyte	0.22^{a}	0.14^{b}	0.18^{ab}	0.036
Blastocysts of Inseminated	0.28^{a}	0.17^{b}	0.22^{ab}	0.01
Blastocysts of Cleaved	0.44^{a}	0.29^{b}	0.33^{b}	0.001
ET				
No. Pregnancies per Transfer	0.26	0.49	0.30	NS

Data were log transformed prior to analysis with back transformed means shown. Letters in superscript indicate significant differences within a row (P<0.05).

The OPU 2 OPU team aspirated more (P=0.051) follicles than the other two teams; 13.8 compared with 11.1 for the OPU 1 team and 10.0 for the OPU 4 team (Table 3.12). However, the OPU 2 team also achieved a better (P<0.001) recovery rate of oocytes per follicle. This resulted in more (P<0.001) oocytes collected in total (12.4), compared to either OPU 1 (7.1) or OPU 4 (7.7).

The increased number of oocytes collected by the OPU 2 team leads to more oocytes inseminated per follicle (P<0.001) (Table 3.12). However, there was a difference in blastocyst production per oocyte collected with the OPU 1 team collections resulting in more (0.22, P=0.036) than either OPU 4 (0.18) or OPU 2 (0.14) (Table 3.12). The OPU 1 team also achieved the highest rates of blastocysts from those that cleaved than the other teams (P=0.001). Despite the better blastocyst per oocyte proportion from the OPU 1 team over the OPU 2 team (0.28 vs 0.17, P=0.01), numerically OPU 2 achieved almost twice as many pregnancies per transfer as OPU 1 (0.49 vs 0.26) (Table 3.1~~1~~2), although with the small number of pregnancy results received by the end of the study, there is no statistical difference.

3.3.4. Dataset 4

To investigate the effects of the type of semen and of different ovarian stimulation and coasting techniques throughout the project, data were analysed from the OPU 1 OPU team where all the oocytes were processed by the IVP 1 lab to minimise variability (Figure 3.14).

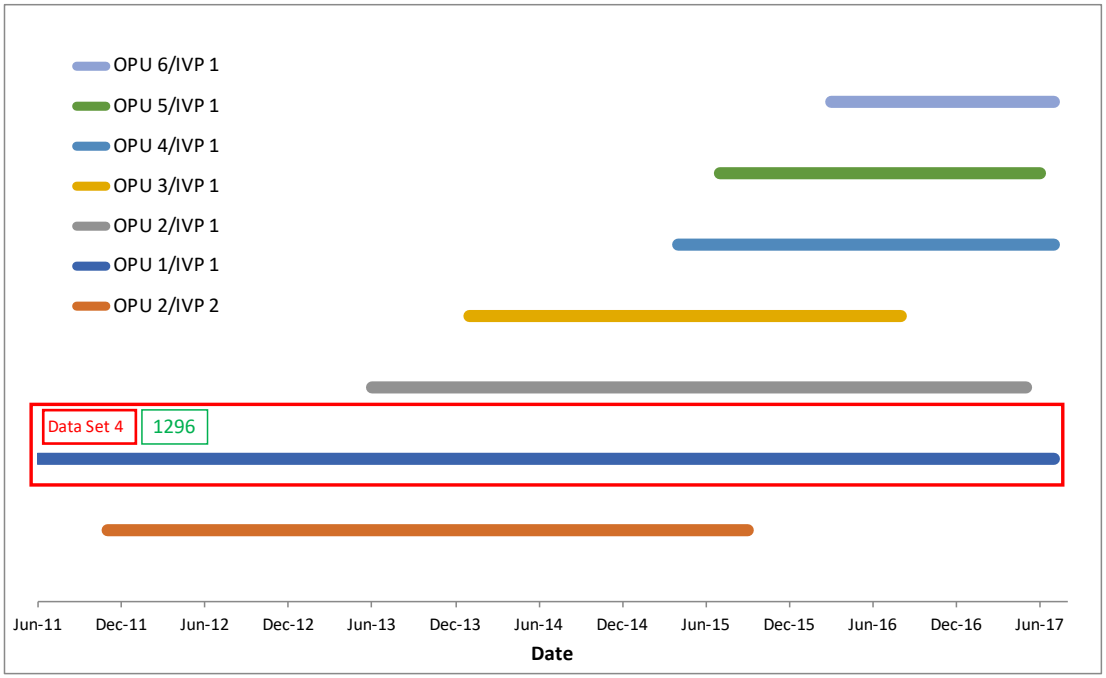


Figure 3.14. From Figure 3.1, Dataset 4 is superimposed as the red box, and includes data from the most experienced OPU Team (OPU 1), using the IVP 1 lab and throughout the whole project. The number of cycles in the total dataset are shown in the green box.

Table 3.13. A breakdown of data from Dataset 4 including the total number of OPU cycles, the percentage distribution of stimulated and non-stimulated cycles, the OPU vets involved as a percentage of cycles undertaken, the percentage of breeds collected, the minimum, maximum, mean and median ages of donors, and the most used sires.

Number of Cycles 1296					
Stimulated (%)				Semen Type	
Folltropin®		73.3		Conventional	93.1
Pluset®		5.3		Sexed	6.9
None		21.4			
OPU Vets (%)				Breeds (%)	
P1	JSM	29.6		Holstein	48.6
P2	DB	12.4		Ayrshire	21.9
P3	GD	31.7		Limousin	6.1
P4	RS	13.3		Aberdeen Angus	5.6
P5	DG	4.3		Jersey	5.0
P6	DP	3.1		British Blue	3.2
P7	PA	1.7		Dairy Shorthorn	2.2
P8	WC	1.3		Charolais	2.0
Others		2.7		Danish Red	2.0
				Others	3.4

Ages*	
Minimum	0y 6m
Maximum	16y 9m
Mean	5y 0m
Median	4y 3m

**Dates of birth were not recorded for every donor at the start of the project*

Most Used Sires (% of total of 247)	
Woodmarsh Lymetime (Holstein)	7.9
Lencrest On Time (Jersey)	4.1
De-Su Big Bang ET (Holstein)	3.1
Granite ET RDF (Holstein)	2.2
Lady's Manor La Bron (Holstein)	2.0
Most Used Sexed Semen Sire	
Progenesis Fortune ET	0.8

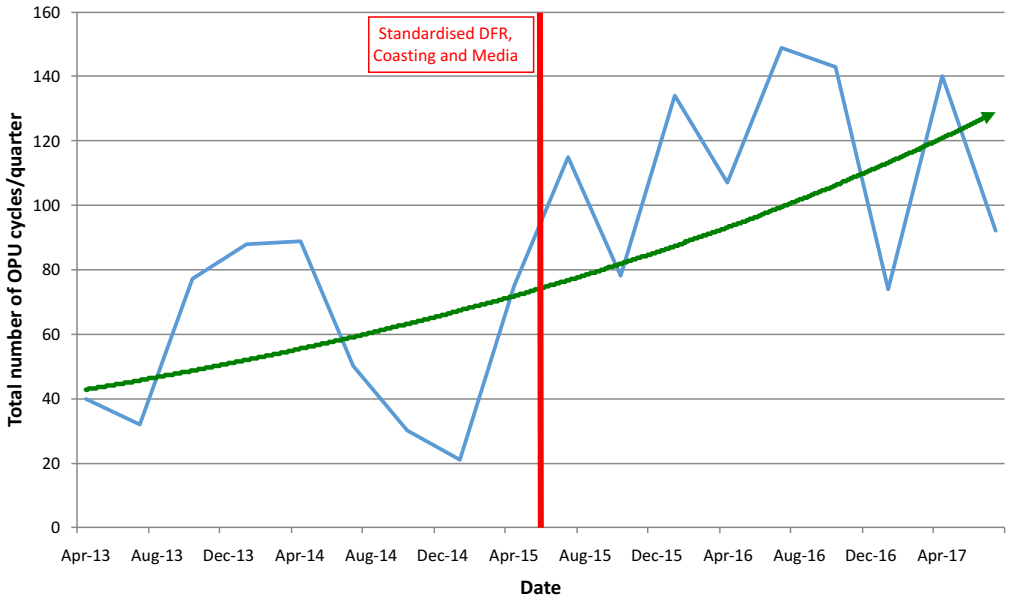


Figure 3.15. From Dataset 4, the number of OPU cycles per calendar quarter is shown from quarter 6 of the project (solid blue line) with an exponential trend line (solid green line). A vertical red line shows the point at 1st June 2015 from when standardised protocols for OPU, cow-side and laboratory processes were introduced.

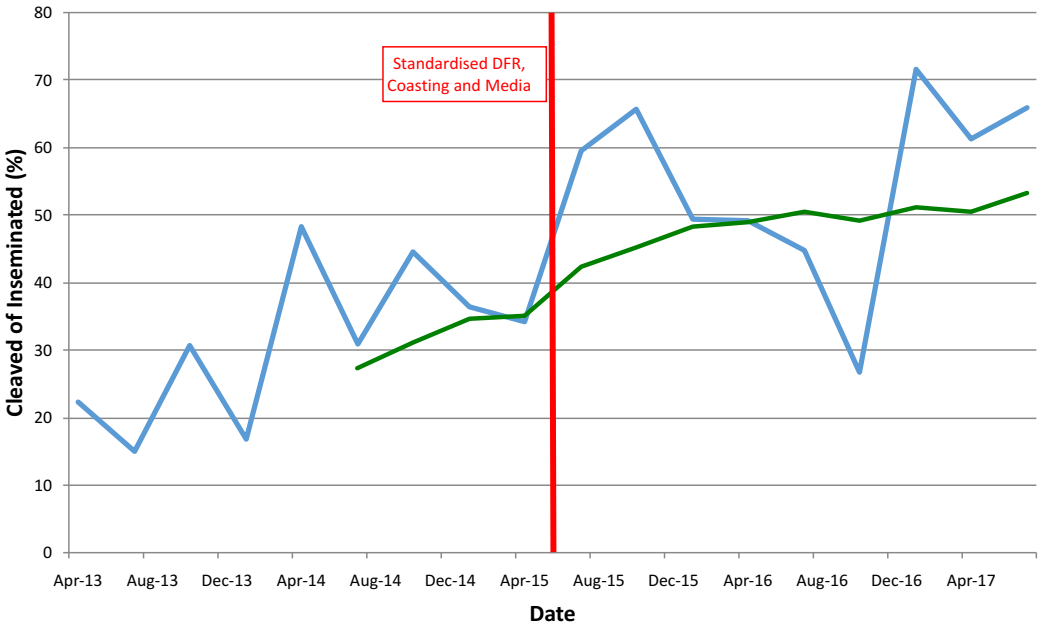


Figure 3.16. From Dataset 4 the proportion that cleaved of inseminated per calendar quarter is shown from quarter 6 of the project (solid blue line) with a rolling 6- period average trend line (solid green line). A vertical red line shows the point at 1st June 2015 from when standardised protocols for OPU, cow-side and laboratory processes were introduced.

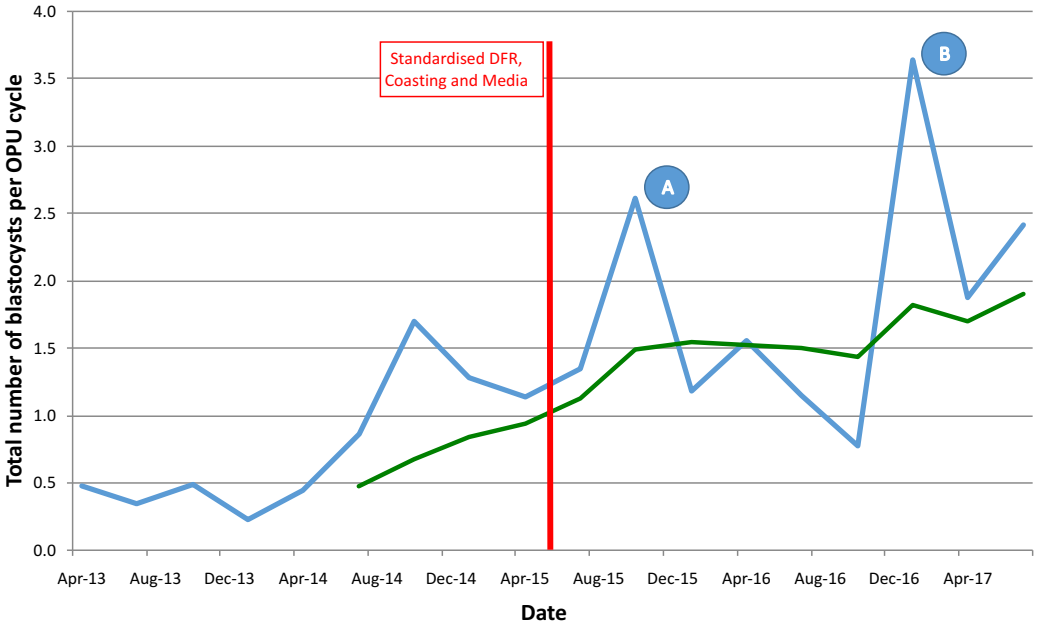


Figure 3.17. From Dataset 4 the number of blastocysts produced per OPU cycle per calendar quarter is shown from quarter 6 of project (solid blue line) with a rolling 6-period average trend line (solid green line). A vertical red line shows the point at 1st June 2015 from when standardised protocols for OPU, cow-side and laboratory processes were introduced. Two spikes of improved numbers are labelled with blue circles A and B.

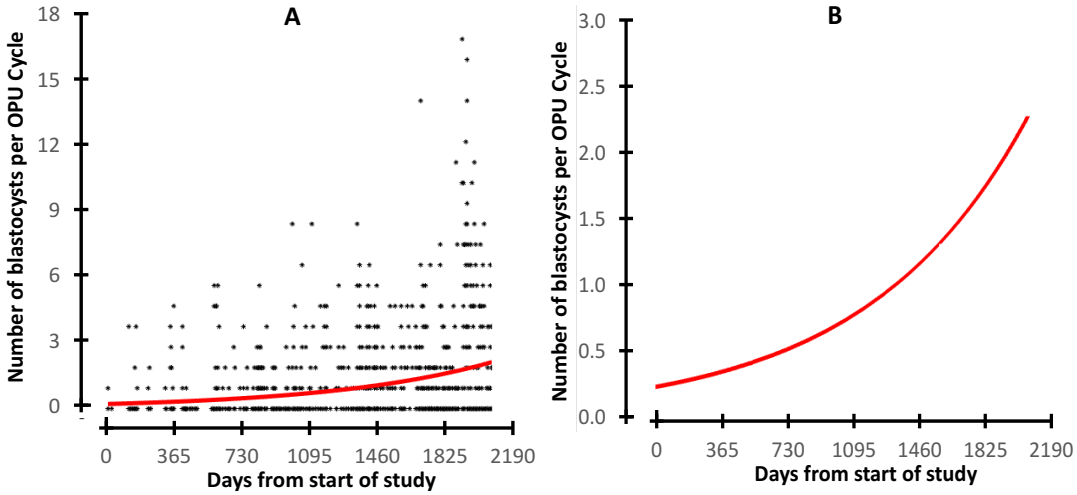


Figure 3.18. Relationship between blastocysts per OPU cycle and days from start of study (Dataset 4) showing an improvement over time (P<0.001). Adjusted scale in Figure B emphasises the increasing blastocyst yield.

There was an increase in OPU cycles undertaken by the OPU 1 team throughout the project, with around 40 per month being undertaken by the end of the project (Figure 3.15). The proportion of oocytes suitable for insemination that subsequently cleaved is shown in Figure 3.16, and it can be seen that there was a steady increase from approximately 20% to a six-period rolling average of over 50%. This increased further in the last 2 quarters of the dataset to over 60%. Similarly, it can be seen in Figures 3.17 and 3.18 that blastocyst production per OPU cycle increased from less than 0.5 blastocysts per cycle to greater than 2 per cycle over the time of the project. Interestingly, there are 2 spikes of improved blastocyst production (Figure 3.17) in Autumn 2015 and Winter 2016. These occurred when two large scale on-farm collection periods were undertaken, working regularly with a choice of normally fertile heifers.

Table 3.14. Summary results from mixed effects statistical models to quantify the impact of semen type (conventional or sexed) on oocytes per follicle, inseminated per follicle, oocytes inseminated, blastocysts of inseminated and blastocysts of cleaved in Dataset 4

Semen Type	Conventional	Sexed	(P)
Total No. Cycles	1207	89	
Response variate			
No. Oocytes Collected per Cycle	5.53	6.81	0.01
Oocytes per Follicle	0.60	0.70	0.004
No. Oocytes Inseminated per Cycle	5.00	6.30	0.004
Inseminated per Follicle	0.46	0.57	<0.001
Blastocysts of Inseminated	0.21	0.15	NS
Blastocysts of Cleaved	0.43	0.32	0.01

Data were log transformed prior to analysis with back transformed means shown.

Table 3.15. Summary results from mixed effects statistical models to quantify the impact of ovarian stimulation protocol on OPU (follicles and oocytes collected), IVP (oocytes inseminated, cleavage proportions and blastocyst production) and ET (pregnancies). Results of five different stimulation protocols within Dataset 4; non-stimulated with no DFR, non-stimulated with DFR, Folltropin® stimulated with no DFR, Folltropin® stimulated with DFR, and Pluset® stimulated with DFR.

OPU/IVP Protocol	Non-Stimulated		Stimulated			(P)
			Folltropin®		Pluset®	
	No DFR	DFR	No DFR	DFR	DFR	
Total No. Cycles	252	26	480	470	68	
Response Variate						
OPU						
No. Follicles Aspirated per Cycle	8.87^{a}	5.72^{b}	11.14^{c}	11.62^{cd}	14.43^{d}	<0.001
No. Oocytes Collected per Cycle	3.37^{a}	6.48^{b}	7.10^{b}	6.75^{b}	8.32^{b}	<0.001
Oocytes per Follicle	0.60	0.74	0.62	0.62	0.67	NS
IVP						
No. Oocytes Inseminated per Cycle	4.00^{a}	4.56^{ab}	5.82^{b}	5.77^{b}	9.09^{c}	<0.001
Inseminated per Follicle	0.43^{a}	0.53^{ab}	0.51^{b}	0.51^{b}	0.61^{b}	0.007
Inseminated per Oocyte	0.74^{a}	0.75^{a}	0.83^{ab}	0.85^{b}	0.91^{c}	<0.001
Cleaved of Inseminated	0.44^{a}	0.34^{a}	0.48^{ab}	0.50^{b}	0.62^{b}	<0.001
≥4 cells of Cleaved	0.46^{a}	0.32^{a}	0.61^{b}	0.66^{b}	0.76^{b}	NS(0.09)
Total No. Blastocysts per Cycle	0.74^{a}	0.41^{a}	1.25^{b}	1.03^{b}	1.95^{b}	<0.001
Blastocysts per Follicle	0.07^{a}	0.06^{ab}	0.12^{b}	0.09^{ab}	0.14^{ab}	<0.001
Blastocysts per Oocyte	0.13^{a}	0.07^{b}	0.17^{a}	0.15^{a}	0.22^{a}	<0.001
Blastocysts of Inseminated	0.18^{ab}	0.10^{a}	0.21^{b}	0.17^{ab}	0.24^{ab}	<0.001
Blastocysts of Cleaved	0.30	0.26	0.42	0.40	0.51	NS
ET						
No. Pregnancies per Transfer	0.24	0.26	0.34	0.38	0.37	NS

Data were log transformed prior to analysis with back transformed means shown. Letters in superscript indicate significant differences within a row (P<0.05).

As shown in Table 3.14, there were effects seen between conventional and sexed semen on the numbers of oocytes collected, oocytes per follicle, oocytes inseminated, and inseminated per follicle with the result being higher in each case for sexed semen. There were more follicles aspirated (P<0.001) from the groups that had received FSH stimulation compared to the group that had DFR only (which was a small subset with only 26 observations) or the group that underwent no preparation with DFR or stimulation prior to OPU (Table 3.15). There were fewer oocytes collected per cycle (P<0.001) from the untreated group (3.37) but no difference amongst the others, although the Pluset® with DFR group was numerically the highest (8.32).

More oocytes went into insemination within the Pluset® with DFR groups than within any other group (P<0.001), with the number of oocytes inseminated significantly lower in the no treatment group than any of the stimulated groups (Table 3.15). In addition, the Pluset® with DFR group had more oocytes selected for insemination per oocyte collected (P<0.001) while the Folltropin® with DFR had more than the groups that had no treatment or DFR only.

More oocytes cleaved of those inseminated from the Pluset® with DFR or Folltropin® with DFR groups (P<0.001) than those that had no FSH stimulation, with or without DFR (Table 3.15). Furthermore, although not statistically different at P=0.09, there was a trend towards stimulated groups having a greater proportion of cleaved oocytes at a stage of 4 cells or more, than the non-stimulated groups. The three stimulated groups produced more blastocysts (P<0.001) than the two non-stimulated groups, with the Pluset® with DFR group being highest at 1.95 blastocysts per cycle and the DFR only group lowest at 0.41. The group that was only subjected to DFR produced fewer blastocysts per oocyte (P<0.001) than any other group with no other differences. When the blastocyst production rate per OPU cycle for the two protocols that utilised DFR and an FSH treatment are plotted from the time of these standardised protocols (Figure 3.19) Pluset® with DFR performed better than Folltropin® with DFR (P=0.001). However, when plotted from the start of the study this difference was not significant, probably because of the wide variation in the

number of blastocysts produced and the lack of Pluset® with DFR data points early in the study.

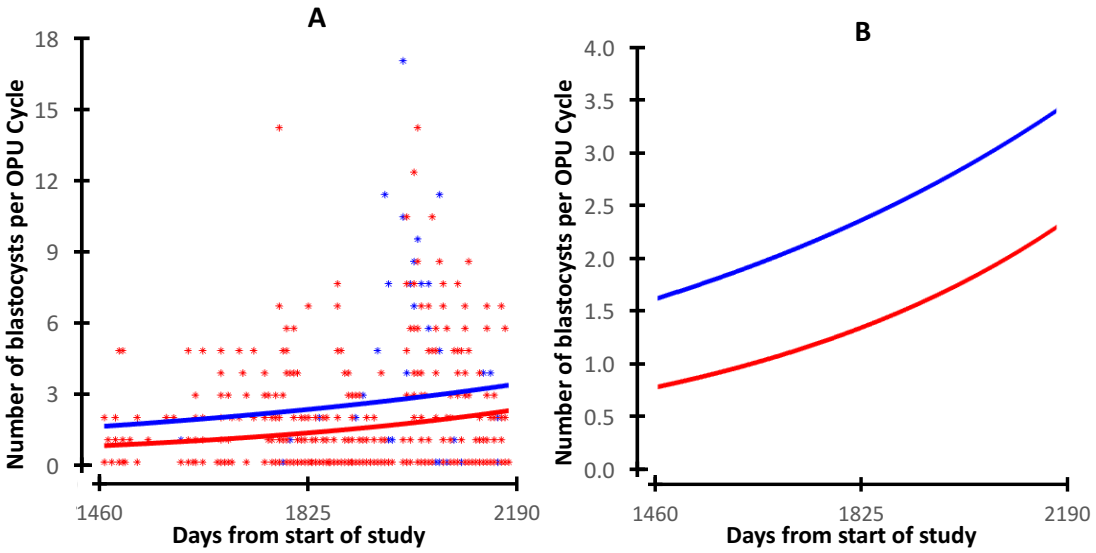


Figure 3.19. Relationship between blastocysts per OPU cycle and days from 1st June 2015 (Day 1460 of the study; Dataset 4). Blastocysts per OPU cycle were greater (P=0.001) for Pluset® with DFR (blue line) than Folltropin® with DFR (red line). Adjusted scale in Figure B emphasises treatment differences.

Although this data set was not large enough to prove statistical differences there is a numerical pattern which suggests that the blastocyst development of those that cleaved, and the pregnancies rate per transfer were better in stimulated than non-stimulated cycles (Table 3.15).

3.4. Discussion

The key findings from these overlapping datasets are that there are many factors which can affect the efficiency of a commercial OPU/IVP programme. Differences in semen between conventional and sexed were shown, as were effects of OPU teams and IVP laboratories. From Dataset 2, which incorporated all six OPU teams, the number of follicles was higher from stimulated animals, as was the morphological quality of the oocytes collected, as assessed by the proportion of oocytes suitable for insemination; this was highest from stimulated and coasted groups, with Pluset® (0.92), greater than Folltropin® (0.88), with those that were not-stimulated at 0.81

(P<0.001). After insemination, in Dataset 3 (the three most experienced OPU teams) there was a difference in the proportion of cleaved of inseminated, and the proportions of blastocysts per follicle, per oocyte collected and per oocyte inseminated with Pluset® outperforming Folltropin®. Ultimately in Dataset 3, there were more total embryos produced per cycle when using Pluset® stimulation and coasting than with Folltropin® stimulation and coasting. This was also numerically the case in Dataset 4 (1.95 versus 1.03) but this was not significant. There was a steady improvement in blastocyst production per OPU cycle from fewer than 0.5 blastocysts per cycle to greater than 2 per cycle.

Datasets 1 to 4 were all subsets of the complete database (as described in Table 3.1 and Figure 3.1) collected throughout the study, and were selected and analysed to investigate various aspects of the process, and to remove the larger known confounders. There is some overlap in these analyses.; each dataset is not truly independent of the others and inevitably common differences can be expected. For example, Dataset 3 is a subset itself of Dataset 2, while Dataset4 is throughout the study period is partly analysed in all 3 of the other datasets. Because of the nature of this project, the requirement to have OPU teams throughout the country and the need to work with the cattle available at times to suit the breeders, truly controlled prospective studies were not possible. However, this is a large dataset for a field study of this kind and there are 4149 lines of data recorded from 2138 individual OPU sessions, over more than 5 years.

3.4.1. Semen effects

When considering the effects of semen on the IVP process, the Datasets 2, 3 and 4 (Tables, 3.7, 3.10, and 3.14) all showed differences in the proportions of oocytes per follicle and oocytes inseminated per follicle. Clearly at this stage of the process the semen has not even been introduced so this must be a donor selection difference, possibly with sexed semen being more frequently used on younger or dairy breed donors. However, although an imbalanced set of data, the different blastocyst production rate (P=0.01) from those that cleaved was greater for conventional semen (0.43) than sexed semen (0.32) and this is potentially a true semen effect on

fertilisation rates. Blondin et al. (2009) found that blastocyst rates were poorer with sexed semen than conventional semen, with obvious bull differences, and while also recognising effects of the bull and of sorting techniques, Xu et al. (2006) found that blastocyst rates and pregnancy rates were similar between the two types of semen. More recently An et al. (2017) have shown that sexed semen fertilisation rates can match those of conventional semen by optimising with heparin and that fine adjustments in heparin concentration can further enhance individual bulls, a concentration of 40 μg/ml is generally acceptable. Watanabe et al. (2017) in a recently published, large retrospective study, of both *Bos indicus* and *Bos taurus* cattle concluded that the sire effect could mean the difference between "outstanding" and "very low" blastocyst rates.

3.4.2. Operator effects

There is evidence in DataSets 1, 2 and 3 that there is a confounding "human variable". In Dataset 2, where all six teams were compared, all the IVP was undertaken at the IVP 1 laboratory to minimise variability, yet there were differences across the OPU Teams which may reflect operator skill and/or oocyte selection and handling and a similar operator effect was reported by Merton et al. (2003). The increased number of oocytes collected by the OPU 2 team in all 3 datasets, is probably an operator effect, and this was also noted with the OPU 6 team when they joined in DataSet 2. It transpired that the vet at OPU 2 was collecting every follicle that was visible on the scanner (over 2mm) as opposed to only those over 5mm, and as the OPU 2 team vet trained the OPU 6 vets this effect has apparently been replicated. OPU 2 did not record the number of follicles aspirated before the standardised protocols were insisted upon from 1st June 2015, but oocytes per follicle is also a measure of operator skill and OPU 2 collected more oocytes per follicle as well as puncturing more follicles in Datasets 2 and 3. This must be interpreted with caution however, as it is likely that detailed follicle counts were not undertaken on every occasion with the result of an underestimation of follicle numbers giving a falsely high oocyte recovery rate. The recovery rate from small follicles is also higher (Seneda et al., 2001) and this is thought to be partially due to the way a large follicle collapses and traps the oocyte (Grand, F.X., 2017, Personal Communication). Chaubal et al. (2006) cites Muullart et al (1999)

as suggesting that abattoir derived follicles are easier to aspirate, as at post-mortem, changes in the follicle means that the COC loosens from its attachments and therefore is easier to collect intact, and it may be that something similar is happening in coasted follicles in which the cumulus is starting to expand, and atresia in the follicular cells is commencing. Although human antral follicles are smaller than stimulated bovine follicles, there is a suggestion that recovery may be easier from larger follicles using double lumen needles (Rose and Laky, 2013). Some human ovum aspiration systems now incorporate two-way flushing of the follicle with medium, which also results in fewer blood clots.

Lonergan et al. (1994) neatly showed that the proportion of blastocysts produced from follicles that were more than 6mm in diameter was almost twice that of follicles in the 2-6mm range. This was found in both unstimulated and FSH stimulated OPU cycles. Furthermore Machatkova et al. (2004) showed that the stage of follicular growth had an effect on oocyte numbers and quality with oocytes from small follicles being less competent during the growth phase. Total oocyte recovery is also a measure of operator skill assuming the quality of the donor is similar and the additional experience in OPU 1, OPU 2 and OPU 4 probably accounts for the higher numbers recovered. Selection of oocytes for maturation and subsequent insemination is undertaken by the cow-side embryologist, and there was a different technician with each team, so there is a risk of subjectivity. So, a high proportion of oocytes being selected morphologically for insemination may be an indication of the oocyte quality being good, or a less experienced embryologist giving everything “a chance”. This may be the reason that OPU 5 and OPU 6 had better proportions of oocytes being presented for insemination than the other teams who were all quite consistent. Tellingly, there was no difference across the 3 experienced teams in this parameter suggesting that with practice a consistent standard of selection was being applied. Evidence of effects earlier in the process is that fewer zygotes reached a 4 cell or greater stage from the OPU 3 team suggesting that the oocytes had already been compromised. This may have been due to less experienced OPU vets, although other factors such as the donors, or transport incubator temperature fluctuations could also be a reason.

3.4.3. Laboratory effects

The higher cleavage and 4-cell or greater rates seen with the OPU 2/IVP 2 combination in Dataset 1 may be a lab effect in that the culture system used at IVP 2 may have encouraged more oocytes to cleave. Although this is possible and different media specify different proportions of components (Fukui et al., 1991), there were so many other variables outwith the lab itself that this as being the reason is pure speculation. The donors at the time of the IVP 2 lab activity were not stimulated, and the population of oocytes would have been more heterogeneous than those from stimulated and coasted cycles. However, we do know as discussed in Section 3.4.4 that oocytes from smaller follicles are less competent, which may be the reason that the IVP 1 lab achieved better blastocyst rates of those that did cleave.

3.4.4. Stimulation and coasting effects

Throughout the study we were interested in the effects of FSH stimulation and coasting as first described by Blondin et al. (2002). And in each dataset analysis we have chosen to investigate the effects on the OPU, IVP and ultimately ET processes.

Superovulation of cattle was initially undertaken with pregnant mare serum gonadotrophin (PMSG) until this was largely superseded by pituitary extract containing FSH and LH (Elsden et al., 1978). As these superovulation products have, over recent years, been improved in purity and predictability, it would be expected any stimulation programme will increase the numbers of follicles and if oocyte recovery rates were comparable, higher oocytes numbers going into the IVP system as well (Bo and Mapletoft, 2014).

In Dataset 1, whilst working under several older protocols, stimulation and coasting seemed to have a beneficial effect on number of follicles, number of oocytes collected, number inseminated, the proportions inseminated per follicle aspirated and per oocyte, proportion of inseminated that cleaved, total blastocysts produced, blastocysts per follicle and the proportion of blastocysts produced from those that cleaved. This is potentially explained by the increased follicle size and homologous nature of the population of oocytes aspirated.

The difference between the OPU 1/IVP 1 and OPU 2/IVP 2 in the proportion of oocytes going onto insemination could be an operator effect as discussed in Section 3.4.2, but the effect is also seen between coasted and not coasted at OPU 1/IVP 1, which eliminates a technician or lab selection effect and suggests better quality oocytes were being produced from coasted cycles and were more suitable to go onto insemination. Similarly, when considering the proportion of those that went onto insemination of those that cleaved, the IVP 2 lab was either somehow selecting oocytes that were capable of cleaving or the processes that were being used in the lab encouraged more cleavage, while at the IVP 1 lab the effect again looks like a result of stimulation. Throughout Datasets 3 and 4 the number of follicles available for aspiration was higher from stimulated animals, as was the proportion of oocytes that went onto insemination, which would suggest that the oocytes produced from these cycles were more morphologically suitable to be selected.

Within Dataset 4, although only using data from one OPU team, which would have reduced the risk of confounding from some variables such as equipment, OPU operator technique, incubator travel time, and breed distribution, there were still many factors within the system that cannot be allowed for. But analysis of various preparatory protocols was undertaken and FSH stimulation in any protocol increased the number of follicles aspirated. This does not agree with the findings of Oliveira et al. (2016) who found no difference between FSH stimulated cycles and those with no hormonal control on the total number of blastocysts per OPU or the blastocyst rate. Nor do the findings concur with da Silva et al. (2017) who reported that using FSH (Folltropin®) in either 4 or 6 injection stimulation protocols did not increase the number of follicles in Holstein cows after 36 hours coasting, nor the number of viable oocytes, nor the number of transferable embryos. However, they did find a shift in the population from small follicles to medium. They also showed a very significant effect of the antral follicle population (AFP) with cows having an AFP of more than 52 at OPU yielding almost twice as many embryos (6 versus 3). This follicle population donor effect which is highly variable between individuals but highly repeatable within (Burns et al., 2005, Ireland et al., 2007, Monteiro et al., 2017) and the unpredictability of how an individual will respond to gonadotrophins (Mikkola and Taponen, 2017)

further complicate any conclusions. The studies are not identical however, as da Silva et al. (2017) classified small as <6mm and collected approximately 30-40 of this size at each session and so were aspirating much smaller follicles than in our study. They also used a prostaglandin injection at the start of the FSH protocol rather than DFR and acknowledged that 20% of the cows still had a corpus luteum which may have confounded their findings. It was not possible in the study reported here to compare coasting periods, however Nivet et al. (2012) have shown that there is a window of coasting after FSH withdrawal when blastocyst rates are optimised (44h – 68h) and suggested the best time was 54h +/- 7h. They also found that the oocyte competence was not linked in a linear fashion to follicle size and that the timing of the coasting period must be adjusted in order to collect the oocytes at the optimal point of follicular differentiation.

Many protocols have been tried over the years as reviewed by Bo and Mapletoft (2014), and with the ultimate objective being the highest number of competent embryos (which will result in a pregnancy) there is ongoing research to optimise superovulation (sometimes referred to as superstimulation) programmes that will improve follicle numbers and oocyte recovery, as well as oocyte and blastocyst competence.

3.4.5. Folltropin® and Pluset® compared

When analysing Dataset 1, the specific FSH/LH product used for stimulation was not specified, but in Datasets 2, 3 and 4 there was an investigation of the two products available commercially in the UK. The differences in these products are explained in Section 3.2.3.2, but primarily they contain different proportions of FSH and LH and the manufacturer's recommended dose rates are different. Furthermore, FSH has a half-life of 5 hours (Laster, 1972), with a more rapid initial clearance phase and a slower secondary phase, being fully cleared from the cow in 10-12 hours (Demoustier et al., 1988). This therefore requires repeat injections every 12 hours, which results in an uneven pharmacological concentration of FSH in the plasma throughout the cycle. Datasets 2 and 3 gave very similar results, as might have been expected with Dataset 3 being a reanalysis of only the 3 largest contributing OPU teams from Dataset 2. However, several confounding variables had been removed, as described in Section

3.3.3, so this added statistical confidence to the results. As predicted, with products containing FSH, there were more follicles available for aspiration from both Folltropin® and Pluset® stimulated cycles. Ideally the dose of FSH would have been standardised between the groups, but like other authors who have reported on these products (Mikkola and Taponen, 2017, Kelly et al., 1997) they were used as per data sheet recommendations. This results in Pluset® treated animals receiving a higher dose of FSH than Folltropin® and this is the likely reason they produced more follicles. The increased follicular response agrees with the findings of Kelly et al. (1997) who conducted a prospective study comparing the same two products and assessed the response by ultrasound scanning and then by retrieval of the oocytes and embryos at slaughter 7 days after insemination. They concluded that Pluset® induced a greater number of follicles than Folltropin®, but a greater proportion of the recovery were unfertilised or degenerate. They also estimated in the same study that Pluset ® was 5.5 times more potent that Folltropin®, but Lerner et al. (1986) concluded that the dose response to FSH was affected by age with higher doses of FSH having a positive response in older animals and a negative response in younger heifers. We were not able to analyse these subsets, and so cannot draw any firm conclusions as to the reason for this superior follicle stimulation effect. There may also be a donor selection effect throughout as it was not possible to undertake a controlled randomised prospective study of the two formulations. The increased number of follicles collected from Pluset® coasted cycles would partially explain the increased number of oocytes that were suitable for insemination. But there was also a greater proportion of aspirated follicles that produced an oocyte suitable for insemination from the stimulated and coasted cycles (either Folltropin® or Pluset®), compared with the non-stimulated cycles. This effect is further demonstrated when considering the proportion of oocytes suitable for insemination of the total oocytes collected with more oocytes being morphologically suitable to go forwards for insemination from Pluset® (0.92) than Folltropin® (0.88), than non-stimulated (0.81) in Dataset 2 ($P<0.001$). This finding was replicated in Datasets 3 and 4. This is likely to be a result of a more synchronous follicular wave and the lack of dominant follicle suppression of subordinate follicles in coasted cycles, which will have followed DFR. DFR has been shown to be as effective as oestradiol (no longer available in the UK) combined with

progesterone at synchronising follicular waves prior to stimulation with FSH (Baracaldo et al., 2000). The same authors showed that only the two largest follicles need be ablated although in our study we removed all follicles at DFR greater than 5mm in diameter. In the larger but more confounded Dataset 3, Folltropin® only performed better than non-stimulated cycles in the number of follicles aspirated, and in the proportion of oocytes that were inseminated. Although of those oocytes that were suitable for insemination in Dataset 3, there were proportionally more from Pluset® coasted cycles that cleaved, this did not follow through into more oocytes at a greater than 4-cell stage of development, suggesting that there was no further improvement in development rate.

In Dataset 3 there was also a difference in the proportions of blastocysts per follicle, per oocyte collected and per oocyte inseminated with Pluset® outperforming Folltropin®. In Dataset 4, although a similar pattern was seen, these differences were not significant, possibly because of a lower number of observations. Ultimately in Dataset 3, there were more total embryos produced per cycle when using Pluset® stimulation and coasting than with Folltropin® stimulation and coasting. This was also numerically the case in Dataset 4 (1.95 versus 1.03) but this was not significant. Recently Mikkola and Taponen (2017) reported on a large retrospective study (4000 animals over 16 years) and compared the same superovulation products prior to conventional multiple ovulation embryo transfer (MOET). They report more recoverable structures with Pluset® than Folltropin®, but no difference in the number of transferable embryos, or in pregnancy rates as measured by non-return (to service) rates. However, neither this study or the one by Kelly et al. (1997) mentioned above were able to assess the stages of embryo development, whereas in this study we were able to. It is worth noting that although the IVP 1 lab was using Pluset® stock in the maturation media, Santos et al. (2017) have shown that there is no difference between Pluset® and Folltropin® when used at 10 µm/ml in the maturation media.

As well as the FSH dose being different between products, some of these differences may be accounted for by the difference in LH component of the programs. Pluset® has an LH:FSH ratio of 1:1, whereas Folltropin® is declared as being “highly purified porcine pituitary extract with a low LH:FSH ratio”. In the natural oestrus cycle of *Bos taurus* cattle LH pulses are of high amplitude and low frequency (every 3-4 hours)

during the luteal phase before increasing in frequency and building to a surge during the final maturation and ovulation of the dominant follicle (DF) (Forde et al., 2011). This is associated with LH receptors in the theca cells of growing follicle becoming more responsive and LH receptors being acquired by the granulosa cells. So, towards the end of follicular development and maturation LH is important, and there is some debate as to whether endogenous supply of LH is adequate during a stimulation programme (Bo and Mapletoft, 2014, Blondin et al., 2002). Chaubal et al. (2007) demonstrated that improvements in morphological quality of oocytes were achieved when LH (as human chorionic gonadotrophin hCG)) was administered 6 hours prior to OPU and after FSH stimulation. Furthermore, when no progesterone supplementation was used during the stimulated cycles and the LH was given as an intravenous injection (to mimic the LH surge) the proportion of blastocysts that developed was also improved. However, this study was in short cycle programmes (OPU once per week) and with no coasting and so is not completely comparable. In a more similar OPU/IVP protocol to that used in this study and using Folltropin® as the FSH product, Blondin et al. (2002) found that injecting LH intravenously 6 hours before OPU increased the proportion of blastocysts per COC inseminated with a 33-hour coasting period, but not after a 48-hour coasting period. The authors postulated that this was because there may be insufficient endogenous LH for several "dominant follicle like" structures to be matured while still in the growing phase after 33 hours and that exogenous LH induced follicular maturation. But that after 48 hours the follicular phase had progressed towards follicular cell atresia and achievement of developmental competence, and so additional LH had no effect. Within this same study there was no effect of LH on cleavage or morulae production rates indicating that LH is a requirement later in the development process. Barros et al. (2013) in a recent review of his own work and others considers that FSH stimulation does not reduce oocyte competence but does affect gene expression in the granulosa and cumulus cells. There is upregulation of LHR expression in the granulosa cells which may be part of the exogenous LH effect. On the other hand, Chupin et al. (1984) reported an incrementally antagonistic effect of adding LH to an FSH programme prior to MOET, but details of the programmes used and the times are not available. This finding was supported by Mapletoft et al. (2002) who, in a MOET programme,

compared superovulation products containing 100%, 32%, 16% and 0% LH and found that the higher ratios of LH suppressed embryo production, and suggested that the maximum acceptable ratio of LH to FSH was 20:80. Using recombinant DNA technology, Looney (2012) produced bovine FSH (bFSH) which was therefore effectively pure (and certainly purer than porcine pituitary extracts) and found acceptable MOET embryo production could be achieved with no exogenous LH.

In the study reported in this thesis, because the FSH dose and LH:FSH ratios were so different, and as this was a retrospective study with several other confounding variables, it is difficult to disentangle the various effects. However, there is a clear suggestion that our findings support those of Chaubal et al. (2007) in that we saw an improved morphological cohort of oocytes, and more so with Pluset®, which contains more LH. We also agree with Blondin et al. (2002) in that we demonstrated an improved number of total blastocysts with Pluset® (and numerically with Folltropin®), which was not just because of the increased number of follicles aspirated (an FSH effect as discussed above), but also improved competence as demonstrated by higher proportions of follicles that were inseminated cleaving and reaching the transferable embryo stage.

Nevertheless, the key commercial outcome is pregnancies, and there is a numerical suggestion that Folltropin® cycles resulted in a better pregnancy rate per embryo transferred of 0.41, compared with 0.28 for Pluset® cycles or 0.25 from non-stimulated cycles. This might partially offset the greater number of embryos produced by Pluset® cycles.

3.4.6. Overall IVP and blastocyst success rates

OPU 2 in Dataset 3 as discussed in Section 3.4.2 produced more total oocytes and more oocytes per follicle than OPU 1. Yet better proportions of blastocysts per oocyte, blastocysts of inseminated and blastocysts of cleaved suggests more competence of those oocytes that did go into the system were achieved by the OPU 1 team compared with OPU 2. It was also shown in each dataset analysed that there is a benefit in pure numbers of follicles aspirated which gives a greater total number of oocytes aspirated, which in turn directly correlates with the number of oocytes suitable for insemination. However, this is not the only contributing factor, because with stimulation, as

discussed in Section 3.4.5., a greater proportion of follicles result in an oocyte suitable to enter the IVP process, and with Pluset® a greater proportion still of those inseminated per oocyte collected – as discussed in Section 3.4.5.

In Dataset 1 this benefit of stimulation persists throughout the IVP process, with an enhanced proportion of those inseminated cleaving, and in turn a greater proportion of those cleaved becoming blastocysts. However, these effects of stimulated versus non-stimulated are not statistically proven in DataSets 2 and 3, although Pluset® does statistically outperform Folltropin® when considering the proportion cleaved of inseminated and the proportion of blastocyst of inseminated. In Dataset 4, when comparing stimulated cycles that had been subjected to a DFR, Pluset® with DFR statistically produced more oocytes suitable for insemination (0.91 $P<0.001$) than either Folltropin® with DFR (0.85) or Folltropin® only (0.83). All stimulated cycles in Dataset 4 produced more cleaved cells of oocytes inseminated than non-stimulated cycles and more of those were at a greater than 4-cell stage. These beneficial effects tend to lose statistical significance further through the IVP process, which is potentially an issue of insufficient data at this time, but nevertheless indicates that some of these beneficial effects act higher up the chain of OPU/IVP.

Limited data on the number of pregnancies per OPU cycle at the end of the project were available and they were almost certainly distorted by the source and types of embryos so far transferred. However, with a higher blastocyst rate for the stimulated cycles and a very similar pregnancy rate per transfer for these embryos so far, more pregnancies per OPU Cycle from the Pluset® with DFR would be expected and could be extrapolated to approximately 0.7 pregnancies per OPU cycle across the time of the project.

3.4.7. Dataset 4 – Improvements achieved

Ultimately, in a commercial environment, success will be driven by the number of viable embryos produced per OPU cycle that are capable of creating a pregnancy. It is worth noting that across all these groups there were problem breeders, and some teething difficulties and variations that may have reduced the overall numbers. Nevertheless, it can be seen that there has been a steady four-fold improvement in

blastocysts per cycle through the OPU 1/IVP 1 combination and this has been even greater when dealing with normally fertile animals. Furthermore, there is a suggestion, with the caveats already described, that in our hands, with the population we are currently working with, a DFR with Pluset® coasting and stimulation programme gives better blastocyst yields but we do not yet have sufficient data to state with confidence that this leads to more pregnancies.

3.5 Conclusion

The aim of this part of the study was to develop a robust commercial OPU/IVP system for UK conditions. At the start of this trial there was no other OPU/IVP being undertaken in the UK, and Europe as a whole was lagging far behind the USA and particularly South America. The *Bos indicus* breeds are recognised as being more productive in OPU/IVP systems, and oocytes are often collected with no additional stimulation. However, modifications involving stimulation have become more widely used in *Bos Taurus* cattle, and this study has shown a benefit under UK conditions. Further benefits of ovarian stimulation with coasting have been demonstrated. Up to the point of blastocyst production a protocol utilising Pluset® has been more successful. The possible reasons for this have been discussed.

Other authors have considered the most commercially viable option for an OPU/IVP programme (Blondin et al., 2015, Chaubal et al., 2006). The latter authors proposed that the best protocol on Angus-cross heifers was a weekly procedure of DFR, FSH stimulation and OPU. The FSH given was Folltropin® as a single low dose (compared with manufacturers recommendations), and was split between intramuscular and subcutaneous injection sites – designed to give a longer FSH effect. They did not report pregnancy rates, but based their conclusions on embryo yield. Given that in our study we opted for the stimulation and coasting protocols as discussed in 3.3.4. weekly collection was not possible.

There are accepted weaknesses with a retrospective study like this, with so many variables, especially early on. As this is a new technology to the UK, the animals initially being provided by farmers were often already reproductively compromised

(for example could not conceive or were not producing embryos by MOET), and results were poor. However, with the synonymous wider use of genomic selection and an acceptance of the techniques, normally fertile animals are being more frequently selected. Throughout the study there were also some indications of numerical differences, but these were not statistically significant and a larger data set would be required to ratify these trends, and some of the confounding variables require further analysis. Ultimately the outcome is to produce viable offspring and at the stage when this data was being analysed there was not enough confirmed pregnancy data to include. However, data collection is ongoing and further analyses will be undertaken in due course.

The data analysed in this chapter indicates that with stimulation and coasting there is an opportunity for both an increased number of oocytes to be collected per OPU cycle and an improvement in the quality of those oocytes, leading to an increased yield of competent embryos. Chapter 4 will consider this issue further.

Chapter 4: Cumulus oocyte complex (COC) quality as a predictor of blastocyst yield

4.1. Introduction

The impact that cumulus oocyte complex competence has on embryo quality and ultimately live offspring is complex, as reviewed by Gilbert et al. (2015). The oocyte, to be deemed competent, must be able to resume meiosis, be fertilised and commence cleavage, complete early development with genome activation, be able to establish a pregnancy and continue foetal growth through to live delivery of offspring. The importance of the communication between the oocyte and cumulus cells is well established, and it has been known for some time that a two-way flow of ions and small molecules, in association with paracrine signalling are fundamental. However, Russell et al. (2016) propose that there are several other transfer mechanisms between these cells, including RNA transfer, and the as yet poorly understood role of haemoglobin in oocytes and cumulus cells. So, on the basis of these processes, Duranthon and Renard (2001) argue that oocyte competence is a "convenient but biologically fuzzy concept", and that it should only really apply to the point of becoming a blastocyst, as other factors are at play. These include the interaction between oocyte gene expression and the maternal endometrium (which in turn affect the elongation of the pre-implantation embryo), implantation, and the establishment of pregnancy. An aspiration, both in human and bovine advanced breeding systems is to have a more reliable set of criteria with which to accurately predict IVP outcomes (Gilbert et al., 2015), and the earlier within the OPU/IVP process, the better. In the field of bovine OPU/IVP more aspirations per donor are undertaken (fortnightly), and more patients per hour (three to four), than in the human theatre, so it is important to have a robust, reliable and rapid early indicator of likely transferable embryo yields so cow-side (such as vacuum pressures) and laboratory techniques (such as pooling of oocytes from multiple donors) can be adapted to maximise production. It is also very useful to be able to predict how many donors for fresh transfers are likely to be required the following week. Although morphological assessment is partially subjective, at its simplest it does consider the number of cumulus layers and degree

of expansion, the granularity and opacity of the cytoplasm, and the regularity of the nucleus. Some authors have rejected the use of such visual examination on the basis that follicular dynamics are more important (Vassena et al., 2003). In a stimulated and coasted protocol, all COCs are at a similar stage of maturity and therefore can reasonably be compared side by side. Having established in Chapter 3 some clear differences in the number and competencies of COCs further analysis was undertaken to investigate the impact of COC quality, as assessed morphologically, on blastocyst yield.

4.2. Materials and methods

Data were analysed throughout the study from the OPU 1 OPU team where all the oocytes were processed by the IVP 1 lab to minimise variability (Dataset 4, as described in Figure 3.14 and Table 3.13). The cow-side and laboratory protocols for each cycle were as described in Section 3.2.3 and 3.2.4. Cumulus oocyte complexes were counted, and individually graded on a 4-point scale, from 1 (best) to 4 (poorest) as described in Section 3.2.3.3.

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4.2.1. Data analyses

Data were analysed using REML Generalised Linear Mixed Models within Genstat (Genstat 18th ed, VSNi, Rothamsted, UK). Analyses were restricted to Dataset 4 and initially considered effects on COC quality from stimulated cycles and non-stimulated cycles. Embryo development (proportions) assumed binomial-error distributions with logit-link functions. Terms fitted to the fixed model were 'Stimulated Folltropin' vs 'Stimulated Pluset' vs 'Non-stimulated'. Terms fitted to the random model were 'Donor ID' (to account for multiple cycles and oocytes per donor). Embryo development data are presented as back transformed means. Differences between individual means established from predicted least-significant differences (LSDs). A composite score of COC quality and number (sCOC) was then generated (see Table 4.2) and fitted as a fixed term in a Generalized Linear Mixed Model analysis that also included 'Semen Type' (Sexed vs Conventional) as a fixed term.

4.3. Results

Table 4.1. Summary results from mixed effects statistical models to quantify the impact of ovarian stimulation protocols using different products (Folltropin® and Pluset®) on OPU (cumulus oocyte complex (COC) quality), and IVP (on cleavage proportions and blastocyst production) within Dataset 4.

OPU/IVP Protocol	Stimulated		Non-Stimulated	(P)
	Folltropin®	Pluset®		
Total No. Cycles	950	68	278	
Response Variate				
		OPU		
Grade 1 COCs of Total COCs	0.23[a]	0.25[a]	0.09[b]	0.025
Grade 2 COCs of Total COCs	0.25	0.25	0.22	NS
Grade 3 COCs of Total COCs	0.31	0.32	0.25	NS
Grade 4 COCs of Total COCs	0.19[a]	0.14[a]	0.42[b]	<0.001
		IVP		
Cleaved of Total COCs	0.52[a]	0.65[a]	0.21[b]	<0.001
Cleaved of Inseminated	0.63[a]	0.73[a]	0.39[b]	0.014
≥4 cells of Total COCs	0.33[a]	0.47[a]	0.11[b]	0.001
≥4 cells of Inseminated	0.39[a]	0.53[a]	0.20[b]	0.014
≥4 cells of Cleaved	0.63	0.72	0.45	NS
Blastocysts of Total COCs	0.20[a]	0.33[b]	0.06[c]	<0.001
Blastocysts of Inseminated	0.24[a]	0.37[a]	0.10[b]	0.006
Blastocysts of Cleaved	0.41	0.53	0.27	NS

Data were log transformed prior to analysis with back transformed means shown. Letters in superscript indicate significant differences within a row (P<0.05).

The stimulated cycles produced proportionately more Grade 1 COCs (P=0.025) and proportionately fewer Grade 4 COCs (P<0.001), while there was no difference in the proportions of Grade 2 or Grade 3 COCs (Table 4.1). There was no difference between Folltropin® and Pluset® in the proportion of COC grades recovered. The proportion of inseminated oocytes that cleaved, and had greater than 4-cells at assessment, together with blastocysts of inseminated, were also greater for stimulated than non-stimulated cycles. For each of these measures Pluset® gave better results than Folltropin®, but this was not statistically significant. However, blastocysts as a proportion of total COCs, was greater (P<0.001) in the Pluset® group (0.33) than for both other categories with Folltropin® (0.20) higher than non-stimulated (0.06).

The difference in oocyte quality then led to the development of a simple composite score (sCOC) based on the number and quality of COCs. Grade 1 COCs were given a score of 4, Grade 2 a score of 3, Grade 3 a score of 2, and Grade 4 and discarded a score of 1. The mean sCOC score was then multiplied by the total number of sCOCs going into maturation to give the total mean of the sCOCs. Because the range of total mean scores was large and non-linear (between 1 and 61 COCs with total mean sCOC scores from 0.25 to 1967), they were log transformed (Table 4.2).

Table 4.2. Calculation of the Log Total Mean sCOC showing each step.

COC Grade	1	2	3	4	Discarded
Quality Score	4	3	2	1	1
COC Number	a	b	c	d	e
sCOC	4a	3b	2c	d	e
Mean sCOC	$\left(\frac{4a+3b+2c+d+e}{4}\right)$				
Total Mean sCOC	$\left(\frac{4a+3b+2c+d+e}{4}\right) \text{ x } (a+b+c+d)$				
Log Total Mean sCOC	$\log\left(\left(\frac{4a+3b+2c+d+e}{4}\right) \text{ x } (a+b+c+d)\right)$				

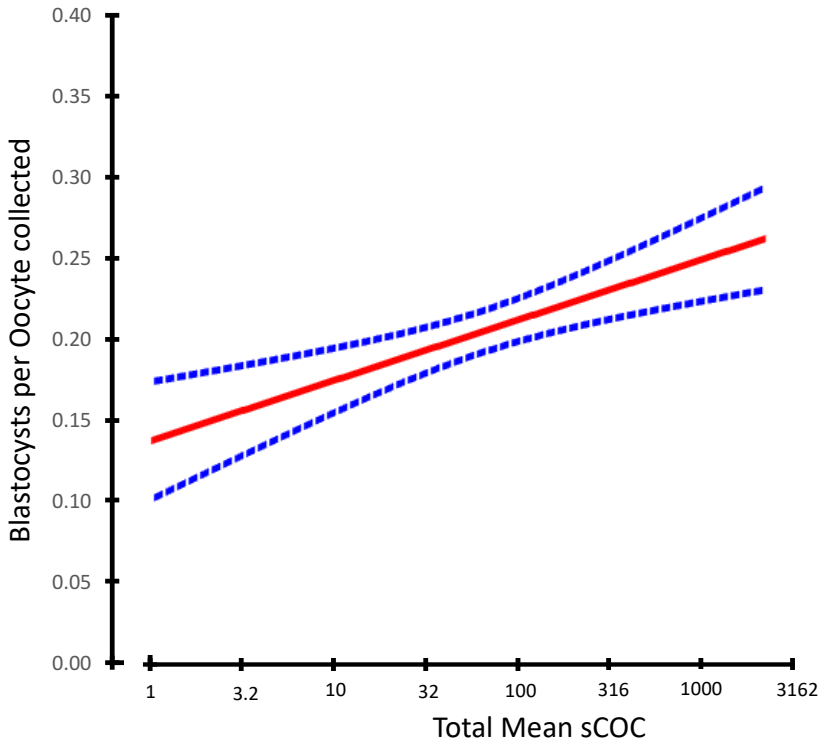

Figure 4.1. Blastocysts per oocyte collected versus Total Mean sCOC (red line) showing predicted 95% confidence limits in blue. X-axis is back transformed to original scale

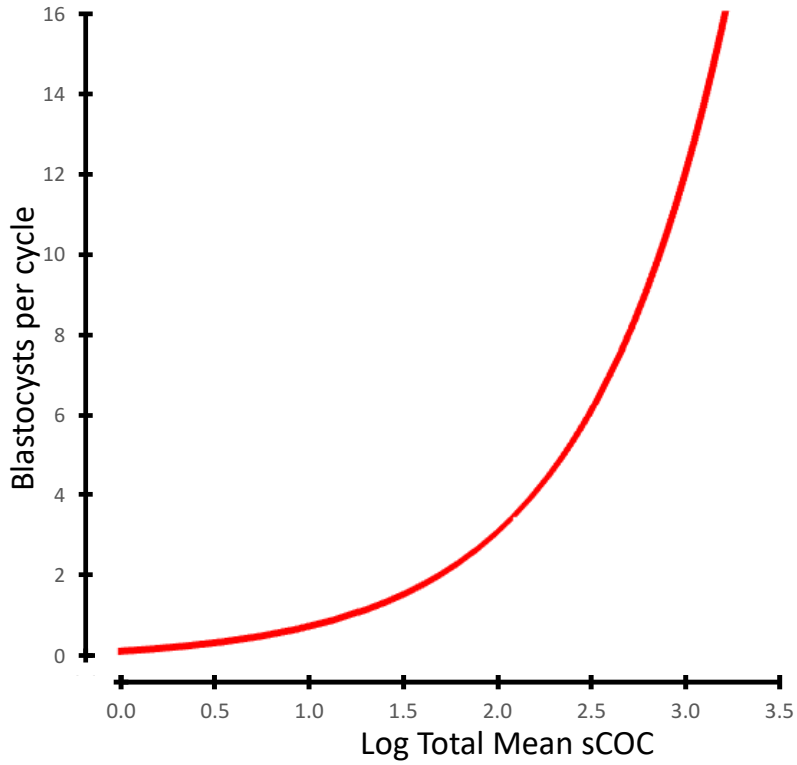

Figure 4.2. Blastocysts per cycle versus Log Total Mean sCOC

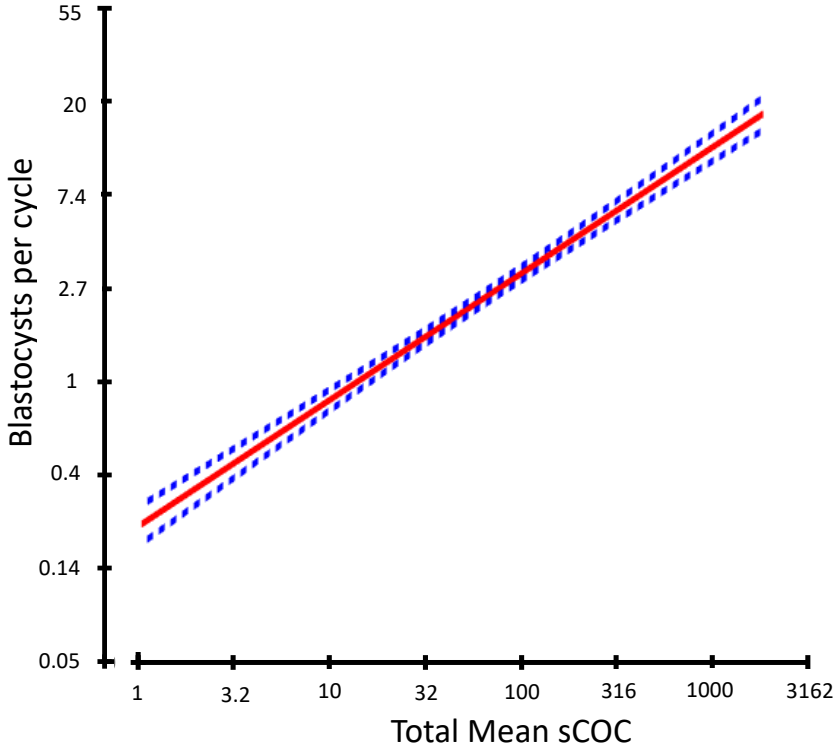

Figure 4.3. Blastocysts per cycle versus Total Mean sCOC (red line) showing predicted 95% confidence limits in blue. Y-axis back transformed (from Log_e) to original scale and X-axis back transformed (from Log_{10}) to original scale.

The proportion of blastocysts produced per oocyte collected (Figure 4.1) and the total number of blastocysts produced per cycle (Figures 4.2 and 4.3) were each positively related ($P<0.001$) to log Total Mean sCOC.

4.4. Discussion

The findings from this chapter extend those of Chapter 3 in that they demonstrate a positive effect of ovarian stimulation on COC quality which, in turn, is positively associated with subsequent embryo development.

4.4.1. Ovarian stimulation and coasting

Ovarian stimulation increased the proportion of Grade 1 COCs and reduced the proportion of Grade 4 COCs. Furthermore, up to the transferable blastocyst stage,

which is the furthest we can currently analyse in this dataset until we have more pregnancy data, animals on a Pluset® stimulation program tend to outperform those on a Folltropin® program. This improved COC competence may be associated with the higher cleavage proportions for Pluset® in association with the higher proportion of Grade 1 COCs, but possibly more importantly the reduced proportion of Grade 4 COCs. The probable reasons for this difference are discussed in Section 3.4.5. It was also shown that a simple composite score, which includes the number of oocytes and gives a nominal value to individual oocyte quality, is highly associated with blastocysts per oocyte per cycle and to the total number of embryos produced per cycle.

4.4.2. Oocyte quantity

The data in Chapter 3 showed that the total number of follicles and oocytes collected were affected by stimulation ($P<0.001$) (Tables 3.10 and 3.13). (Watanabe et al., 2017) concluded from a large retrospective study in Brazil of both *Bos Taurus* and *Bos indicus* cattle that as more oocytes are aspirated, more blastocysts are produced. In the *Bos taurus* sub-group this was associated with a reduction in blastocyst rate as the number of oocytes increased and a trend towards an increased probability of pregnancy. This study did not report on the quality of the oocytes recovered nor explain the OPU/IVP programmes used. Similarly, Monteiro et al. (2017), again in *Bos indicus*, and from OPU sessions performed at random stages in the oestrus cycle, rated donors as "High COCs" if over 15 COCs were collected at the first session of OPU and "Low COCs" if less than 15. It was shown that there was a high repeatability of the number of COCs collected (0.81), blastocysts per OPU cycle (0.79) and blastocyst rate (0.69) over 12 consecutive OPU/IVP cycles. So, it seems that for any one individual there is a remarkable consistency of embryo production potential.

4.4.3. Oocyte quality

Gilbert et al. (2015) defined the three key stages involved with successful blastocyst production (both *in vitro* and *in vivo*) as being oocyte maturation, the fertilisation process and the effectiveness of embryo culture. Not specifically mentioned in that paper but very influential is the quality of the semen used, which can affect the kinetics of pronuclear formation, cleavage and blastocyst yields, and possibly the sex

ratio of offspring (Alomar et al., 2008). Several criteria other than microscopic morphological assessment have been developed to identify the likely competency of bovine oocytes. For example, Yang and Rajamahendran (2002) have proposed that the ratio of the Bcl-2 and Bax proteins, which are associated with apoptosis may be a good indicator of likely oocyte survival. Boni et al. (2002) showed that higher Ca^{2+} current across the plasma membrane of the immature oocyte was associated with better cleavage and blastocyst yields, while Ca^{2+} stores were also higher in the more developmentally competent *in vitro* matured oocytes. But the same authors also showed that the morphological assessment of the COC was directly associated with developmental potential, with the B grade COCs being highest, these being derived from partially atretic follicles. This concurred with the conclusions of Hazeleger et al. (1995) and Blondin and Sirard (1995) that oocytes only acquire competence later in the follicular phase, often after the first signs of cell atresia are seen and at which stage, having been exposed for longer to the follicular micro-environment, are more differentiated. However, all these experiments were done on dissected follicles from abattoir ovaries, whereas the COCs in the experiment described in this chapter were collected from stimulated and coasted cycles, designed in part to increase oocyte competence and intra-follicular differentiation.

It is well understood that the relationship between follicular somatic cells and the gamete is critical, and one of the reasons for this, that is becoming more clearly understood, is around gene expression and RNA transfer (Bunel et al., 2014, Blondin et al., 2012, Macaulay et al., 2016). Blondin et al. (2012) consider some of the gene expression studies that have been undertaken so far in granulosa cells, cumulus cells, and blastocysts, and the tasks associated with these genes. Gilbert et al. (2015) agree that blastocyst production per se is not suitable to assess in vitro systems, and cites human studies (Van Blerkom, 2009) in supporting the concept that healthy mitochondria are key to oocyte competence. However, Plourde et al. (2012) showed that the source of the oocytes, either from an abattoir or via OPU, had more effect than the gene expression of the collected COCs, albeit the gene expression was different in the oocytes from each source. Other techniques have been proposed and are likely to be developed, such as microfluidic chips systems to analyse, by colour

differences, zona pellucida and cytoplasmic features (Kempisty et al., 2011), but these are as yet unproven. In the human, where individual follicular fluid can be more practically collected and analysed, several predicting markers have been identified, including amino acids, particularly alanine and glycine (Sinclair et al., 2008). However, in the bovine commercial environment it is difficult to see how these techniques could be practically applied.

4.4.4. Oocyte Assessment

Although morphological assessment is subjective, in the commercial environment it remains the technique most commonly used, and will be until another technique such as those described above, is validated as a rapid and non-invasive predictor of likely COC competence. Morphology is usually assessed alongside follicle size, and since Lonergan et al. (1994) showed that oocytes were most competent from follicles which were at least 6 mm in size, these have been described as "dominant-type" follicles. Several other authors have confirmed that oocytes from follicles less than 3 mm have very little developmental potential (Blondin et al., 2012, Pavlok et al., 1992, Machatkova et al., 2004). Hence in this study no follicles smaller than 5 mm in diameter were aspirated.

The composite score selected was a nominal score based on COC quality. With a better understanding of the likely outcomes from each grade, a differential scoring system might be developed, placing greater weight, on (for example) Grade 1 COCs, or even a negative score for poor quality Grade 4 COCs. Although this type of outcome study was undertaken by Blondin and Sirard (1995) it was on abattoir derived material and with an outdated IVP system. The author is unaware of similar work having been published on stimulated and coasted cycles utilising modern IVP protocols. If it was to be undertaken, each individual oocyte would require tracking, and so a well-of-the-well (WOW) or time lapse photography technique would be required. However, it is logical to develop a composite score, as it has been shown that different grades of COC support each other during culture. Kelly et al. (2007) graded oocytes on a 3-point system, purely on the number of cumulus layers with Grade A having 5 or more, Grade B having 3 or 4 and Grade C, 3 or less. Those oocytes that were denuded, had

expanded cumulus or discoloured cytoplasm were excluded. Of the selected monocultures, Grade A COCs, had higher cleavage and blastocyst yields, and a higher number of nuclei in the resulting embryo than Grade B COCs, and in turn Grade C COCs. However, an unselected population of mixed COCs grades cultured together performed very similarly to the Grade A monoculture. More enlightening was that the unselected group, performed better than the combined performance of the three monocultures, indicating that there were cross support mechanisms - presumably paracrine - between the different grades of COCs.

4.5. Conclusion

Cumulus oocyte complex grades are an important assessment tool, are quick and non-invasive and, if undertaken rigorously, are a useful indicator of subsequent embryo development. It appears that a composite score, allowing for quantity and quality of oocytes from any one collection, can be highly predictive of the proportion of oocytes that become blastocysts and of total blastocyst number. There is merit in refining this composite score to give differential weighting to the different COC grades, especially Grade 1 and Grade 4 COCs, and possibly other factors such as follicle size. However, there are many other factors that impact blastocyst development, and importantly the likelihood of an embryo implanting and establishing a pregnancy that will proceed to term with healthy offspring. Many of these factors have a genomic or epigenetic component and a further understanding of how these impact is critical. Further work is required to refine this composite scoring system, but this is currently a useful tool, and has the potential to be used within small scale prospective studies of interventions that may have an impact on the OPU/IVP system. This will be considered further in Chapter 5.

Chapter 5: Differences between CIDR® and PRID® Delta progesterone releasing devices within OPU/IVP programmes in peri-pubertal heifers

5.1. Introduction

As described in Section 3.2.3.1., progesterone (P4) releasing intravaginal devices are frequently used to control the oestrus cycle in cows undergoing OPU/IVP. They are intended to mimic the P4 profile that would be expected mid oestrus cycle, and to therefore allow a positive ovarian response to exogenous FSH. There are currently two such devices available commercially in the UK, but throughout the early part of this study the device used was not recorded, although different OPU teams tended to use the same device throughout. There was also a previous PRID® design which was a coil and was available early in the study but is no longer on the market in the UK. There has been little research on the direct effects of progesterone on oocyte quality in the bovine, but it is known that progesterone is one of the mediators of cumulus expansion and COCs secrete progesterone into the surrounding culture media (Nuttinck et al., 2008). Progesterone also has a significantly positive effect on developing bovine embryos in vitro although the exact mechanism is not understood (Ferguson et al., 2012). Along with the known impact that progesterone has on the frequency and amplitude of LH pulses (Crowe 2013), it seems possible that these devices could influence the results recorded throughout the datasets in this study and this therefore required investigation. These intravaginal progesterone releasing devices are primarily designed and used for synchronising cycling animals or induction of oestrus in non-cycling animals prior to breeding. In the studies reported here, these devices were on occasion used in younger (peri-pubertal) and therefore smaller heifers, than the usual clinical candidates. So, although these devices were being used within their stated product licenses, it was important to assess how comfortable these devices were for the heifers and if any vaginal inflammation was caused. This trial was designed to test the suitability and efficacy of the PRID® Delta® (Ceva Santé, Libourne, France) versus the CIDR® (Zoetis, New Jersey, USA) progesterone releasing intravaginal devices for use in OPU/IVP cycles in young peri-pubertal heifers.

5.2. Materials and Methods

Commercial OPU collections were being undertaken on a farm fortnightly on high genetic pedigree heifers, and the OPU team was using CIDR® intravaginal progesterone releasing devices as a standard procedure. However other OPU teams were reporting that PRID® Delta devices were also being used successfully. It was therefore agreed that these commercial and licensed devices would be compared in a controlled way on animals already committed to the OPU/IVP programme by the breeder to provide embryos for his own replacement stock and to sell commercially. As this was not deemed to be an intervention, both devices are commercially available and licensed for use in animals of this age, and the analysis of the study was retrospective, ethical approval was not deemed necessary.

Twelve of these peri-pubertal Holstein heifers (9 to 11 months of age) weighing approximately 300kg on average were selected from the same batch of home-bred heifer replacements from this single farm and randomly allocated to 1 of 2 groups in a crossover designed trial. All cattle were housed together and fed the same diet. The study was undertaken by the OPU 1 team and the IVP 1 lab, between September and November 2016.

The intra-vaginal progesterone (P4) releasing devices (Figure 5.1) were;

i. PRID® Delta (Ceva Santé, Libourne, France); a triangular shaped device, consisting of polyethylene spin and EVA (Ethyl Vinyl Acetate), impregnated with 1.55g of progesterone, and with a string tail for removal.

ii. CIDR® (Zoetis, New Jersey, USA) a T-shaped device consisting of plastic and silicone elastomer, impregnated with 1.38g of progesterone, and with a plastic tail for removal.

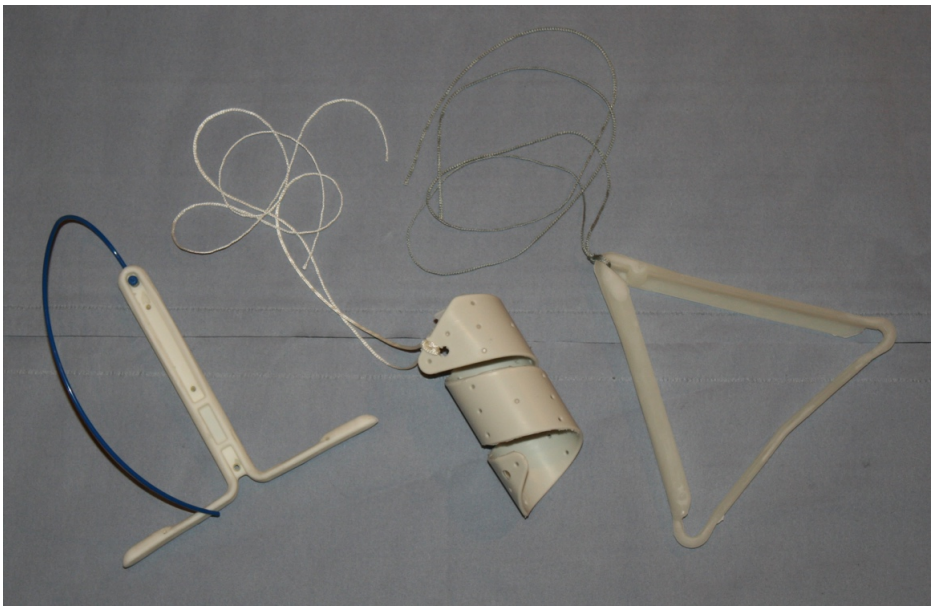

Figure 5.1. Three types of intravaginal progesterone releasing device; from left to right, CIDR®, PRID® (no longer available and not used in the current study) and PRID® Delta.

The cow-side and laboratory protocols for each cycle were as per the standardised system described in Section 3.2.3 and 3.2.4, with the additional specification that only follicles greater than 5mm in diameter were aspirated.

There were 2 OPU/IVP cycles per period; the protocol is described below and is shown schematically in Figure 5.2. The tails were removed from the devices which were inserted using the commercial applicators supplied, and lubricated with an obstetric gel (Vet-Lubigel®, Millpledge Pharmaceuticals, Clarborough, UK).

Cycle 1

i. DFR performed and CIDR® or PRID® inserted (Day 0)

ii. FSH (Folltropin®) stimulation

- 6 injections of 1.5ml (52.5iu) from 56 hours, 12 hours apart

iii. FSH withdrawn and coasted for 26 hours

iv. OPU performed and CIDR® or PRID® replaced (Day 6)

Cycle 2

i. DFR performed and CIDR® or PRID® replaced (Day 14)

ii. FSH (Folltropin®) stimulation

- 6 injections of 1.5ml (52.5iu) from 56 hours, 12 hours apart

iii. FSH withdrawn and coasted for 26 hours

iv. OPU performed and CIDR® or PRID® removed (Day 20)

v. Rest for 2 weeks

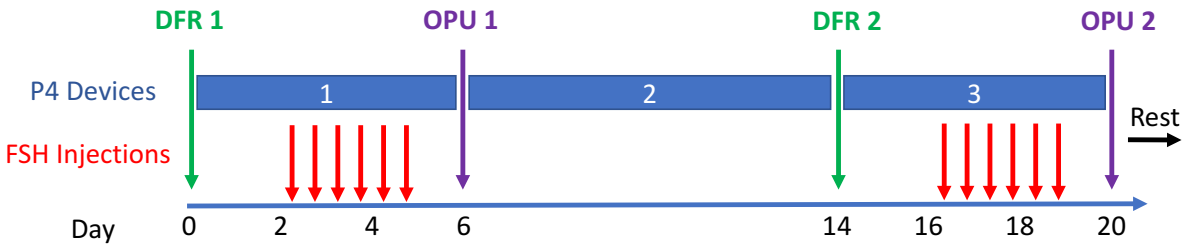

Figure 5.2. Schematic showing one period of the trial (2 cycles) with DFR (green arrows) and OPU sessions (purple arrows), FSH injections (red arrows), and the periods of insertion of each progesterone (P4) releasing device (blue bars).

At the start of the trial, prior to devices being inserted, and immediately prior to device removal at each OPU session, heifers were visually scored for device comfort. This was undertaken by the same vet with the heifers unrestrained and undisturbed in their pen, on a 4-point scale, as described below;

i. Grade: 0 = Tail down, back straight, bright demeanour

ii. Grade: 1 = Tail up, back straight, bright demeanour

iii. Grade: 2 = Tail up, back arched, bright demeanour

iv. Grade: 3 = Tail up, back arched, dull or unwell

At the commencement of each intervention (DFR or OPU) gross external dirt was removed from the area around the vulva with dry paper towel and the intravaginal device removed. The vagina was examined with gloved hands (to prevent any cross-

contamination), and using a speculum and light source scored using a 4-grade vaginal inflammation assessment system that had been adapted from trial work involving examination of cattle with Mycoplasma infection by Grand F.X. (2017, Personal Communication)

i. Grade: 0 = normal mucous membrane without any discharge
ii. Grade: 1 = pustulae or petechiation of the mucous membrane without any discharge
iii. Grade 2: = pustulae or petechiation, and erythema of the mucous membrane without any discharge
iv. Grade 3: = pustulae or petechiation, with erythema of the mucous membrane and purulent discharge

At each DFR and the first OPU a new device of the same type was inserted following washing of the vagina with 10ml of 1% Virkon S solution and flushing with sterile saline. After the two-week rest period, the process was repeated for another period of two cycles with each group of heifers being treated with the opposite progesterone releasing device. All the follicle, oocyte, maturation, fertilisation and embryo production parameters were recorded and entered onto the database and analysed as described in Section 3.2.6.

5.2.1. Statistical analyses

Data were analysed using REML Generalised Linear Mixed Models within Genstat (Genstat 18th ed, VSNi, Rothamsted, UK). Embryo development (proportions) assumed binomial-error distributions with logit-link functions. Count data (e.g follicle number) assumed Poisson-error distributions with log-link functions. The experimental design consisted of two progesterone (P4)-releasing devices (PRID vs CIDR) applied in two successive cycles (1 vs 2) of OPU within period. At the end of Period 1 animals were allocated to the alternative P4-releasing device for two further cycles of OPU during Period 2. Consequently, terms fitted to the fixed model were 'P4 device' (PRID vs CIDR), 'Cycle No.' (1 vs 2) and interactions between these terms. Terms fitted to the random model were 'Heifer ID', 'Period' and 'Heifer ID.P4Device'. This last term accounted for the fact that heifers swapped P4-releasing-device treatments between

Periods 1 and 2. Vaginal inflammation data are presented as a series of boxplots, indicating median and interquartile ranges. Embryo development data are presented as back transformed means.

5.3. Results

In the first cycle one heifer from the PRID® group presented at OPU without a device present in the vagina. This was replaced, but at the DFR for the second cycle the device had again been lost. No oocytes from 4 follicles were recovered from this animal. Another heifer lost her intravaginal device (CIDR®) prior to DFR on the second cycle, and again although it was replaced, it was missing at the second OPU. At this collection 5 oocytes from 13 follicles were recovered. After these no further devices were lost.

One heifer had a comfort score of 1 during the first cycle of period 1, and a second heifer had a comfort score of 2 during both cycles of Period 2. Both these heifers had CIDR® devices inserted at the time. These findings were not significant across the group.

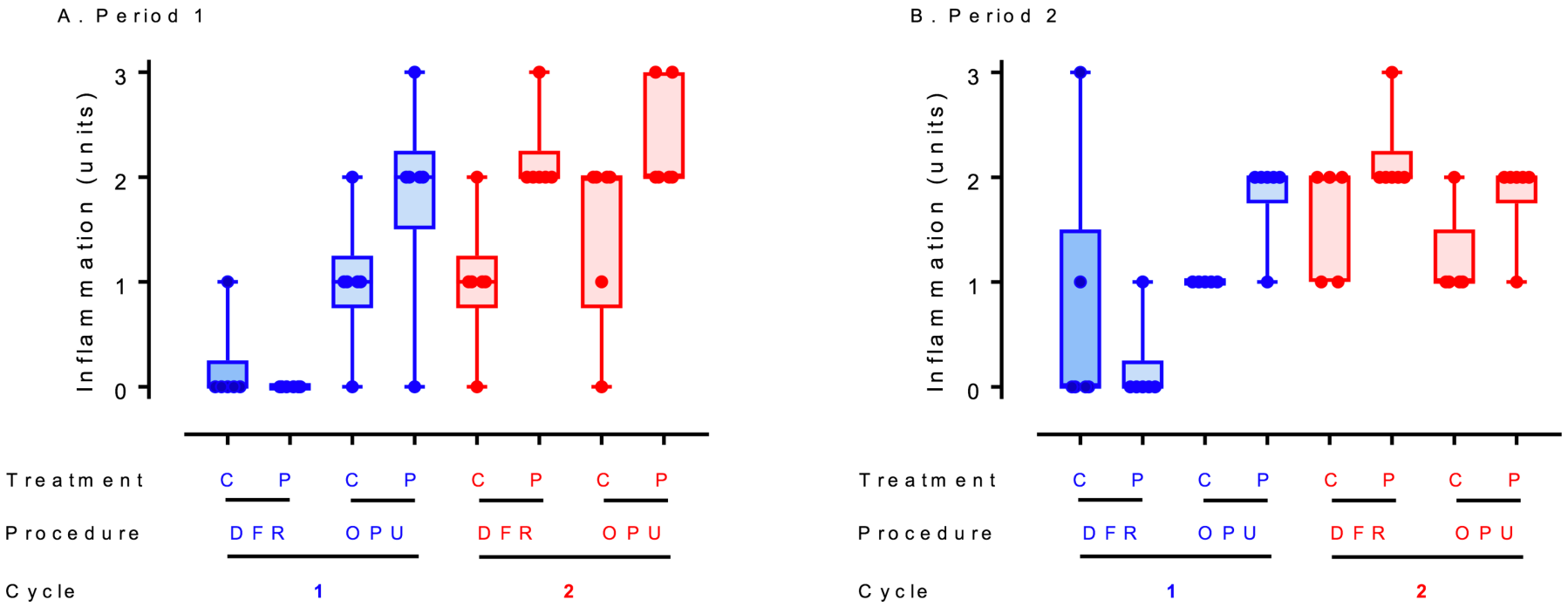

Figure 5.3. Box and whisker plots of vaginal inflammation grade for CIDR® (C) and PRID® (P) treatments over two successive cycles (1 and 2) of dominant follicle removal (DFR) and transvaginal follicular aspiration (OPU) during Periods 1 (A) and 2 (B). Whiskers depict minimum and maximum values, and data points are shown. Vaginal inflammation grades were greater (P<0.001) for PRID® than CIDR® treatment groups, and during Cycle 2 than during Cycle 1.

At the start of the trial (Period 1, Cycle 1) one heifer had a vaginal inflammation Grade 1 prior to any device being inserted, all others were scored 0. After the devices had been in for 6 days, at the time of the first OPU, all but 2 animals showed some degree of vaginal inflammation (Figure 5.3). A similar pattern of inflammation was seen at the time of the second DFR with no heifers in the PRID® group less than 2. There were higher median grades (P=0.005) for heifers in the PRID® than in the CIDR® group. Vaginal inflammation grades were also higher (P<0.001) during Cycle 2 than Cycle 1. One heifer was withdrawn from the trial after the first period (when she had been in the PRID® treatment group) as she had developed a localised peritonitis and some early adhesions around the ovary. This is an uncommon occurrence and although the condition was successfully treated, it is interesting to note that she was the only heifer to have a vaginal score of 3 at the first OPU, which fell to a score of 2 at the second DFR, before rising again to 3 for the subsequent OPU. Only one other animal had a vaginal score as high as 3 at the second OPU. At the DFR at the start of the first period of the second cycle she still had a vaginal score of 3 at which point she was withdrawn.

The proportion that cleaved of inseminated (Figure 5.4F) in those animals in the PRID® treated groups was higher (P<0.05). There is a numerical suggestion that the PRID® treated heifers produced more blastocysts per cycle than CIDR® treated heifers, but this was not statistically significant (P=0.15) (Figure 5.5). Similarly, when the sCOC score described in Chapter 4 was applied to the oocytes collected from this trial, there was an indication that the log Total Mean sCOC results were higher per cycle for the PRID® group than for the CIDR® group, but again this was not statistically significant (P=0.13).

Although these heifers did not appear ill immediately before, during or after the trial, other animals on this farm were found to be positive for Schmallenberg virus (SBV) in the weeks that followed, including 3 similar aged heifers that were undergoing OPU/IVP at the same time.

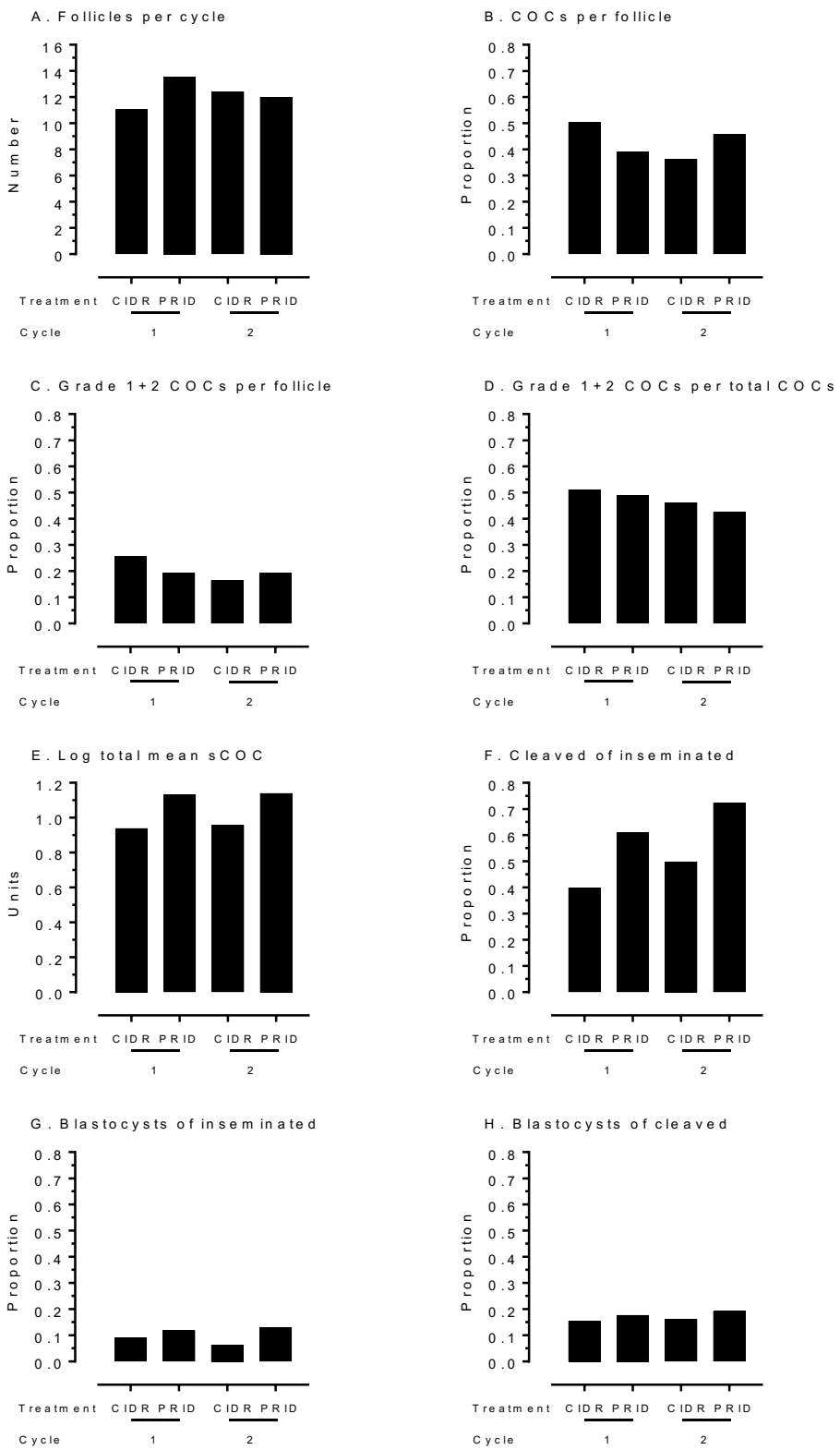

Figure 5.4. Effect of progesterone releasing device (CIDR® vs PRID®) on recovery success of cumulus-oocyte complexes (COCs) and embryo development over two successive cycles of ovum pick-up (OPU) in two groups of six heifers. Data are back-transformed means following analyses using Generalized-Linear Mixed Models. Only proportion cleaved of inseminated oocytes (F) was greater ($P<0.05$) for PRID® than CIDR® treated donors.

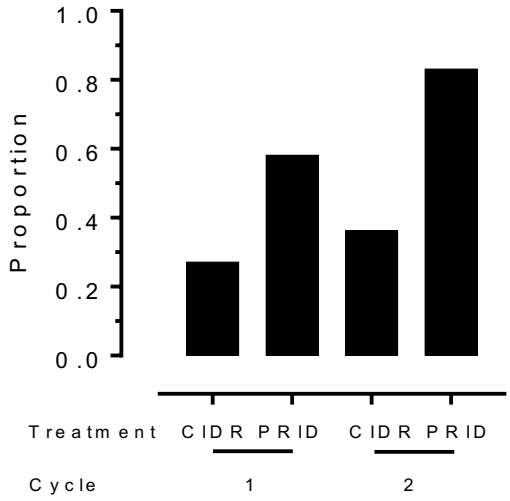

Figure 5.5. Effect of progesterone releasing device (CIDR® vs PRID®) on blastocysts per cycle over two successive cycles of ovum pick-up (OPU). Data are back-transformed means following analyses using Generalized-Linear Mixed Models. No significant effect of treatment (P = 0.149) or cycle (P = 0.509).

5.4. Discussion

The most significant findings to emerge from this trial were that although only two animals showed any overt physical signs of discomfort, from vaginoscopic examinations there were signs of irritation caused by both devices, with higher vaginal grades, and therefore more inflammation with PRID® than CIDR®. There was evidence of a "carry-over" effect of vaginal inflammation from one period to the next, indicating that a 2-week rest is insufficient for the vaginal mucosa to recover completely. The only statistically significant finding from the OPU/IVP parameters measured was that the proportion that cleaved of oocytes inseminated was higher for PRID® than CIDR®. There was a numerical but not significant indication from the data that there was a higher proportion of blastocysts per cycle and a higher log total mean COC score per cycle with PRID® than CIDR® treatments.

5.4.1. Vaginal inflammation

It was shown that both progesterone releasing devices caused some vaginal inflammation. This concurs with previous findings. Chenault et al. (2003) used vaginal mucous discharge on the CIDR® device at removal (scored 0 to 5) as a proxy measure for vaginitis. They found that 65% of the animals had scores of 3 or 4 (classified as mild

irritation) and 2% had a score of 5 (classified as severe irritation). Similarly, a trial by Villarroel et al. (2004), using PRID®s as a P4 support after repeat breeder cows had already been inseminated, found that 81 out of 143 cows in the PRID® treated group had vaginitis as diagnosed by vaginal discharge, although this did not affect subsequent pregnancy rates. The appearance of the vaginal lining and inflammation was not reported in these studies, but in another experiment using PRID®s Walsh et al. (2008) reported a mild purulent discharge on the PRID® device at removal as well as for a non-progesterone containing placebo device, with no vaginal mucosa damage. No evidence of inflammation was found as measured by haptoglobin (which is an acute-phase protein) but a reduction in circulating white cells was noted which could not be explained. However, an antibacterial lubricant was used on the intra-uterine devices in that study which may in itself have had an effect. All these studies were undertaken in calved cows which are likely to have larger, more flaccid vaginas than peri-pubertal heifers.

More comparable with the current study was that undertaken by Fischer-Tenhagen et al. (2012) who assessed vaginitis by vaginoscope as well as bacteriology associated with CIDR® implants in heifers. They used the score described by Sheldon et al. (2006) but designed to measure post-partum uterine disease, and found that every heifer had a vaginal discharge score at CIDR® removal of 2 or 3 (on a 4-point scale of 0-3), and 32 out of 33 heifers had pyogenic bacteria present in the vaginal discharge. This had improved by the time of artificial insemination (AI) 2-3 days later (at observed oestrus) with the vaginal discharge score reduced to 0 or 1 in 96% of heifers. Also, 17 out of 33 still had pyogenic bacteria detectable. This improvement was not seen in our study, but oestrus is known to be a very effective mechanism to clear infections in the bovine (Bretzlaff, 1987, Sheldon, 1999), which may account for the improvement seen in the report of Fischer-Tenhagen et al. (2012). It was also noted by these authors that those animals in which the devices had their plastic tail removed prior to insertion had reduced vaginal discharge scores, which suggests that the tails were acting as wicks. This tail is used to withdraw the device, but can also be of interest to curious pen-mates who on occasion will manage to remove a device, so the technique of 'tail removal' is common and was undertaken in this study.

There is a possibility that the persistant high vagina inflammation score in one heifer was associated with the localised peritonitis and adhesions she developed, as infection may have been introduced with the aspiration needle through the inflammed vaginal wall. However, there is no evidence that this was the case, as no bacteriology or further investigation was undertaken.

5.4.2. Oocyte recovery and embryo development

Progesterone (P4) releasing devices are used in OPU/IVP programmes to synchronise and control the oestrus cycle, and in this experiment were first inserted after all folllicles greater than 5 mm had been aspirated (DFR). Absorption of P4 through the vaginal wall then raises and maintains circulating levels. Thus, via negative feedback on the hypothalamo-pituitary axis, P4 modulates LH pulsatility to a low fequency, low amplitude pattern, which is inadequate for ovulation (Crowe, 2013). van Werven et al. (2013) found that circulating levels of P4 were higher in animals treated with a PRID® Delta compared with a CIDR® especially for the first 4 days after insertion. However, although increased P4 levels are associated with improved embryo survivability in vivo (associated with more rapid elongation of the post-hatch embryo, and higher interferon-tau levels), the effects have been previously thought to be mainly on the uterine endometrium, and not on the embryo itself (Crowe, 2013). Carter et al. (2010) showed that increasing progesterone by insertion of a PRID® did not increase the proportion of blastocysts in vivo, but did identify a different gene expression pattern in the blastocyst that was significantly interacting with the dam's endometrium. Rivera et al. (2011) stimulated lactating cows with FSH and induced them to ovulate, flushing the *in vivo* produced embryos at 7 days. Those induced at the first follicular wave, when endogenous P4 priming was low, produced poorer quality embryos than those induced to ovulate at the first wave, but with exogenous P4 support (2 CIDR®s to mimic physiological levels from a natural CL), or those induced to ovulate at the second follicular wave having been exposed to higher levels of endogenous P4. Subsequent pregnancy rates per transfer were not affected. Although the authors were surprised not to have been able to show changes in LH pulsatility when circulating P4 levels were low, they could not rule this out as a reason. They further suggested, citing the work of Cerri et al., (2008), that reduced

concentrations of insulin-like growth factor 1 (IGF1) associated with low P4 may have had a detrimental effect on the blastocyst.

5.4.3. OPU/IVP yields

There was nothing remarkable about the number of follicles per cycle in this study, but oocyte recovery rate per follicle per cycle was low throughout at less than 0.5 (Figure 5.4B). As discussed in Section 3.4.2 this could be an operator effect but, from Table 3.15, it can be seen that this OPU team would generally expect to recover approximately 0.65 oocytes per follicle from stimulated cycles, and the vet who undertook these collections was very experienced. Similarly, the proportion of blastocysts of inseminated (5.4G), and blastocysts of cleaved (Table 5.4H), were lower than this team would have expected from animals on this DFR, Folltropin® stimulation, and coasting protocol (Table 3.15 for comparative data). The proportion that cleaved of inseminated (Figure 5.4F) was closer to expected results than the other parameters, but the blastocyst number per cycle was disappointing at less than one overall (Figure 5.5). This is especially notable, as by the stage of the overall project when this experiment was carried out, the average blastocyst per cycle rate when using Folltropin® was expected to be around two (Figure 3.19). Furthermore, these were fertile heifers and the data in Figure 3.19 is representative of all donors including lower-yielding problem-breeder cattle.

Schmallenberg virus is an Orthobunyavirus and is transmitted by biting (culicoides) midges, which can cause disease in cattle, as well as sheep and goats. Spread of the disease is linked to midge poplulations and therefore prevaillence peaks in late summer and early autumn (NADIS, 2017). The disease is associated with aborted or stillborn calves and if born alive, limb , brain and spinal cord damage is common. In the adult, acute disease is reported as causing fever, inappetance, production losses and scour. The negative effect on fertility is mainly because of abortion and stillbirth rate increases, but it has been recognised that there is also a reduced conception rate as measured by the increased number of second and third inseminations required on infected farms (Veldhuis et al., 2014). Also, within farm, differences in the number of inseminations required before and during an SBV outbreak have been recorded

although these were not signifcantly different to control farms (Lechner et al., 2017). The mechanism of action is not known, and it may be an effect of the transient fever associatd with SBV. There is no data available on the effect of pyrexia on cattle OPU/IVP outcomes, but fever has been associated with a lower number of pre-ovulatory follicles and lower serum oestradiol per follicle in women on an IVF programme (Awwad et al., 2012). In many instances this disease is only diagnosed by positive serology for SBV antibody indicating exposure, as was the case in a cohort of animals on the same farm in the early winter following this experiment (Paragon Veterinary Group, 2017, Personal Communication).

5.5. Conclusion

It was disappointing that the heifers recruited into this trial, although apparently perfectly healthy, and being subjected to a rigorous and standardised OPU/IVP programme, performed very poorly as a group. Performance was particularly poor during the second period, and this could have been due to circulating Schmallenberg virus at the time, which we were unaware of at the time and which now cannot be assessed. The low number of blastocysts overall will have reduced the statistical power of the study.

The devices are different in design, with the triangular PRID® and the T-shaped CIDR® being made of different materials as described in Section 5.2. These devices will therefore have a different contact pattern with the vaginal wall and will potentially release P4 at different rates. It is also possible that the degree of inflammation of the vaginal wall may affect the absorption pattern of P4 across the mucous membrane, but plasma P4 concentrations were not determined in the current study.

Given the poor embryo production performance of the heifers in this study, there is merit in repeating it, especially with the additional experience the veterinary team now has with managing the FSH stimulation and coasting programmes, and with the improved performance using the current OPU/IVP platform. However, if the results of the recovery of cumulus-oocyte complexes (COCs) and embryo development that were achieved in this trial were to be repeated and found to be statistically significant, practitioners would also have to consider the evidence provided here of the increased

vaginal inflammation with the PRID® device, and any risks that may carry, against an improved blastocyst production performance.

Chapter 6: Discussion

The modified aims of the study, as described in Chapter 1 were to:

i. Assess the attitudes and approach to trace element diagnosis and treatment in the UK
ii. Optimise and standardise OPU/IVP under UK conditions
iii. Establish a robust OPU/IVP system and identify key markers in the system
iv. Investigate FSH/LH stimulation and coasting protocols under UK conditions
v. As proof of concept use a crossover trial utilising OPU/IVP to compare interventions that may affect oocyte quality

6.1. Mineral survey and implications

The mineral survey undertaken found that there was very little consensus amongst professional animal nutrition advisers in the UK, on which mineral imbalances are common or rare, or on the most suitable approaches to dealing with imbalances. There appears to be a wide variation in the understanding of mineral interactions, although some further research has been undertaken since the survey, notably that of Sinclair and Atkins, (2015) and Sinclair et al. (2017). There has been some additional clarity also around the concept of liver loading as opposed to storage, particularly of copper and selenium. In the biological sense, storage assumes a mechanism to control release whereas accumulation is more passive and potentially the precursor to toxicity (Kendall et al., 2015). There is a small but real risk of copper being a risk to human health via toxicity, as discussed in Chapter 2. But possibly more importantly are the recent concerns around the use of copper in agriculture and the known link between bacterial exposure to metals (particularly copper and zinc) and antimicrobial resistance (Poole, 2017, Wales and Davies, 2015, Romero et al., 2017). Long low exposure to a biocide, such as copper or zinc, may create a selection pressure such that a resistant population of microbes develop over time. Or there may be a genetic component with linked genes coding for both antimicrobial and biocide resistance.

(Wales and Davies, 2015). This concern has largely been focussed on the pig industry (Zou et al., 2017) and has resulted in the European Food Safety Authority (EFSA) reducing the permitted level of copper in pig feed (Aquilina et al., 2016), but copper containing footbaths on cattle farms have also been implicated. So, with the increasing focus on 'One Health' it is imperative that the mechanisms of copper responsive conditions are both understood and communicated to the advisory professionals to minimise the unnecessary use of copper supplementation, with it subsequently being excreted into the environment. The results of this survey suggest a need for further research into the true prevalence and importance of trace element imbalances in the UK, their various interactions and the most appropriate methods of treatment. In particular the true copper-molybdenum interaction needs to be further elucidated.

6.2. Establishing OPU/IVP in the UK

It has been possible to establish a reliable and efficient ovum-pickup and in vitro embryo production system under UK conditions, and improvements have continued. The limitations of a retrospective analysis have been discussed in Chapter 3, yet data was analysed up to July 2017 and is ongoing. It is expected that as pregnancy data in particular continues to be collected it will be possible to reach further conclusions. It has been shown that there are a number of interactions throughout the process including influences from the sire and dam, operators and equipment, and laboratory processes.

6.3. Follicle number

It has been demonstrated that both quantity and quality of oocytes are important, and that these can be influenced by factors such as operator skill, laboratory process, hormonal preparation of the donor and semen used, as discussed in Chapter 3. Assessing the potential of the donor is a prerequisite for successful commercial *in vitro* embryo production. Scanning the ovaries by ultrasound gives a good indication of the antral follicle population (Silva-Santos et al., 2014, Watanabe et al., 2017, Singh et al., 2004), however it is time consuming. An alternative is to use anti-Mullerian hormone (AMH) as a predictor of reproductive potential (Stojsin-Carter et al., 2016, Batista et

al., 2016, Ireland et al., 2011). Although AMH does not improve the potential of the COC to develop to a transferable blastocyst (Guerreiro et al., 2014), it is correlated with the follicle population, the oocytes collected and the blastocyst yield.

6.4. Oocyte quality

It has been shown in this thesis that although the number of follicles aspirated are important, because this results in more cumulus oocytes complexes, the competence of the COCs is crucial. There have been many proposed methods of assessing oocyte quality or competence, as reviewed by Bukowska et al. (2012). In the commercial environment, morphological assessment is still commonly used, but is subjective, even with the very best microscopes, so a rapid, objective, economical, parametric test would be welcome. Advanced digital imaging and scanning techniques are currently being investigated and it may be that microfluidic technologies (Lab-on-Chip®) will offer another solution ((Bukowska et al., 2012, Wheeler and Rubessa, 2017).

The success of an OPU/IVP programme and probability of a live calf can be affected by any of the processes between aspiration of the oocyte and parturition, but this study has confirmed that the preparation of the donor and managing the follicular microenvironment up to the point of collection is a critical first step.

6.5. Hormonal control and stimulation

Once selected, an important aspect of the donor preparation is the use of a progesterone device to mimic the mid-cycle ovarian environment. It was demonstrated that there is a balance between a higher degree of vaginal inflammation with a PRID® device versus a CIDR® device but that there was also an improved proportion of oocytes cleaving of those inseminated. There were also numerical indications of differences, but due to a disappointing low oocyte recovery rate and yield of blastocysts from all of the heifers during this trial, the number of data points were lower than expected. It would be worthwhile repeating this study, either as a standalone dataset, or in addition to the data already collected. It was of interest from a clinical point of view that 2 weeks after complete withdrawal of the devices

when the second period commenced, the vaginal inflammation scores were higher than at the start of the study suggesting an incomplete resolution of the vaginal mucosa. Another solution which has also been suggested would be to use sheep CIDR® devices for smaller heifers, which are the same design but much smaller in size, and contain only 0.3g of progesterone (compared with 1.38g in the cow CIDR®). These could be tied together and inserted vaginally, and could have the benefit of being more comfortable, but also the dose could be adjusted readily by adjusting the number inserted.

The findings, that under UK conditions with *Bos taurus* cattle, an FSH stimulation and coasting protocol is the most effective donor preparation programme support the work of Blondin et al. (2002), Nivet et al. (2012) and others. In this study, up to the point of blastocyst production, Pluset® performed better than Folltropin® driven by an increased number of more competent oocytes being produced. Pregnancy data will be analysed as it becomes available, and further analysis of variables such as age and breed will be undertaken on the existing database. It is expected a further prospective study will be proposed, controlling for age, breed, genetic potential and management as well as for semen to further elucidate these differences. If as is suspected the presence of LH earlier in follicular development is detrimental, yet an exogenous "surge" to assist maturation is beneficial, then stimulation programmes will need to be developed that more closely mimic the natural cycle. Gonadotrophins produced by recombinant DNA techniques may give better control over ovarian stimulation and particularly of the timing of the LH introduction, than current naturally derived gonadotrophin products. Porcine pituitary derived products have been shown to have variability in LH:FSH ratios (Murphy et al., 1984, Lindsell et al., 1986). While the products are now highly purified, for example the producers of Folltropin® (Vetoquinol SA, Lure cedex, France) claim the product is stable and uniform and cite (Armstrong and Opavsky, 1986), there is inevitably a risk of impurities and of potency variations that would not occur with recombinant DNA derived products. These might also be designed to be slow release, minimising the fluctuation of FSH levels currently created by 4 to 6, 12-hourly injections. This also has welfare benefits for the animal and is logistically easier to manage in practice (Looney and Pryor, 2012).

6.6. Composite score for oocyte recovery and predicting outcomes

Having established that COC quality is important, Chapter 4 went on to consider this in more detail and showed the effect that coasting and stimulation had on the distribution of oocyte grades at aspiration. The composite score proposed, which combined quality and quantity, was found to be related to the proportion of oocytes that became blastocysts and to total blastocyst number. The next stage would be to assess the correlation, if any, with pregnancy outcomes as it is not known at this stage how competent the blastocysts were. That is, could they establish and maintain a pregnancy to term? Work is potentially needed to refine the score, possibly incorporating different weightings, or incorporating other criteria such as, for example, granulosa cell appearance, or age and breed of dam. A composite score would be highly valuable in a commercial environment, in that it gives an early indicator of likely success which could inform the teams. For example, a lower score might encourage a decision to "pool" COCs with those of another donor to increase the support during maturation, insemination and culture. Or it may help in the management of available recipients if, for example, only a few embryos were expected then the cost of synchronising a batch of heifers could be avoided. It also has potential to eliminate poor donors at an early stage, as it is known that there is a wide variability in donor potential (Pontes et al., 2011, Ireland et al., 2011) as well as a variable response to gonadotrophins (Mikkola and Taponen, 2017). It has been known for some time that some of these traits are hereditable. For example, Merton et al. (2009) showed that the number of COCs collected at OPU had a heritability of 0.25, and the number of transferable embryos a heritability of 0.16. More recently as the bovine genome is better understood, there are opportunities for improved selection, from understanding genetic inheritance, epigenetic influences and the use of genomic screening as a common breeding tool (Weigel et al., 2017). Cornelissen et al. (2017) developed single-step genomic evaluation for the number of oocytes and the number of embryos from a MOET program, though not yet OPU/IVP embryo yield. These breeding values will enhance the ability to select the best donors in future which will facilitate efficiencies within the system both genetically and financially.

At the outset, the study design also proposed to test the concept that OPU/IVP could be used as a platform to investigate interventions that may have an effect on the ovary or on oocyte number, quality or competence. This idea was based on the following known drawbacks of a conventional full-scale field study;

i. As discussed in 1.3.1, to investigate relatively small differences needs very large numbers of animals in as controlled a study as possible.
ii. It can be challenging to find large groups of cattle of a similar age, breed, and genetic potential.
iii. It can be challenging to manage these animals in a controlled environment – it is unlikely they would be in one field, shed or possibly not even on the same farm
iv. There is a delay of approximately 5 weeks before a pregnancy can be confirmed or otherwise.
v. There will be a proportion of these animals that do not become pregnant and are either used again with the associated confounding effect, or are lost from the study.

Alternatively, it would be possible to utilise a reliable OPU/IVP platform to test the intervention as a precursor to a large field trial;

i. A much smaller number of donors is required, which can be as similar as possible, including genetically
ii. They can be kept in identical conditions under the same management regime
iii. This thesis has shown that it is possible to predict with some accuracy the proportion that cleave of inseminated and the blastocysts per cycle for a certain oocyte quality and quantity.
iv. The process can be repeated and as discussed there is a high repeatability within animals in their antral follicle population, and number of COCs aspirated and the blastocyst rate.
v. The trial would be crossed over and repeat as described in Chapter 5, with the heifers as their own controls.

A study of this nature would therefore increase precision through repeated measurements in a highly controlled design, and the number and quality of the COCs collected would be assessed. However, in a mono-ovular species such as the cow the quality of the oocyte from the 'dominant' pre-ovulatory follicle will always be superior to that of oocytes from the subordinate follicles. And although with ovarian stimulation and aspiration follicles are being encouraged to develop that otherwise would not have, by utilising the coasting programmes as described in Chapter 3, effectively a population of "dominant-type" follicles is being produced. These would act as a proxy for the fertility potential of that animal, subject to the intervention. If somehow the inherent ability of a morphologically good embryo to produce a live calf has been affected by the intervention, the pregnancy rates would not be as expected and the reliability of the prediction would be reduced. Ultimately it is likely that the sponsor of any study would specify a viable offspring as the outcome variable, and as the experimental unit is the donor, and with pregnancy data being binomially distributed there would potentially still be requirement for a large number of embryos to be transferred to establish those.

The value of this type of study would depend on where the perceived influence of the intervention might be, and how accurate techniques can become to assess the competence of the blastocyst produce and its likelihood of producing a pregnancy.
There is still much work to be done to establish the confidence limits for some of the assumptions that have been made, but the author believes that there is merit in further assessment, by running larger, carefully controlled prospective studies, and in particular investigating the predictive value of a composite COC score on the ultimate outcome which is a healthy calf.

6.7. Minerals and OPU/IVP

Chapter 1 considered impacts that key minerals might have on bovine fertility. Although there have been reviews of the role of micro nutrition on bovine reproduction (Smith and Akinbamijo, 2000, Hostetler et al., 2003, Velazquez, 2011), there is very little published literature on the effect of minerals on follicle populations,

COC production or competence, or blastocyst yield. Studies that have been undertaken using MOET programmes have often found no differences from the addition of micronutrients, possibly because the animals on trial were already adequately nourished (Lamb et al., 2008, Chorfi et al., 2007, Cerri et al., 2009). Similarly, it is important that there are no other rate-limiting factors, for example both Shaw et al. (1995), who supplemented with Vitamin A, and Sales et al. (2008) who supplemented with beta-carotene and tocopherol (Vitamin E) reported low numbers of MOET recovered embryos, which raises the question about other influences.

Selenium has been shown to be important for spermatogenesis and sperm function, particularly at the point of fertilisation as discussed in Section 1.2.2. Currently, many of the benefits of selenium in association with Vitamin E are believed to be around their mitigation of oxidative stress. Sperm require a level of reactive oxygen species for normal function (ROS) but are damaged by excessive levels. In the human, there are some indications that selenium is protecting against oxidative stress in the female, and that the pre-ovulatory follicles produce ROS which may play an important role in oocyte maturation and ovulation (Mirone et al., 2013). Combinations of insulin–transferrin–selenium (ITS) are routinely added to *in vitro* maturation media, again with an anti-oxidant role. Most recently this has been shown to improve bovine embryo quality and quantity when used in conjunction with L-ascorbic acid (Guimaraes et al., 2016), including in prepubertal calves (Cordova et al., 2010).

In Section 1.2.3 the impact of iodine was reviewed – the most significant effects within cattle reproduction are currently thought to be associated with stillbirth, weak calves, and retained placentae. There are few recent papers that refer to the effects of iodine on conception or effects on oestrus although there are reports based on clinical anecdote, but without significant findings, for example Anderson et al. (2007). However, it is likely that iodine has an influence through its action in the thyroid gland. In *Bos indicus* cattle, artificially induced hypothyroidism improved the ovarian response to FSH stimulation, although the number of transferable embryos was not improved (Bernal et al., 1999). Several other authors have reported an indirect effect by demonstrating the effect of thyroid hormone (TH) on the oocyte and cumulus cells

(Costa et al., 2013) and potentially in association with other metabolic hormones at the level of the follicle itself (Dupont et al., 2016). Ashkar et al. (2010) demonstrated that by adding both T3 and T4 to the IVP media used, there was an improved yield of blastocysts, which had a higher cell number and reduced rates of apoptosis. These authors further showed that the reported effects are driven by an upregulation of gene expression in the early embryo (Ashkar et al., 2016).

The current understanding of the role of copper in reproduction is reviewed in Section 1.2.4. In Chapter 3 there is a discussion around the role of LH:FSH ratios at different points in follicular development and maturity. Phillippo et al. (1987a) first demonstrated that molybdenum supplementation (known then as secondary copper efficiency) impacted LH pulsatility. This mechanism was further elucidated, showing that as well as an impact on ovarian steroidogenesis, thiomolybdates can disrupt the normal ovulatory follicle growth patterns. *In vitro* both FSH-induced differentiation of bovine granulosa cells (Kendall et al., 2003) and LH-induced differentiation of bovine theca cells (Kendall et al., 2006) can be blocked by tetrathiomolybdates. In a small trial on 12 sheep, Kendall 2005, further demonstrated that an intra-venous infusion of thiomolybdates reduced pre-ovulatory LH levels, but that there were also indications of a reduction in pre-ovulatory FSH, oestradiol peak, and post ovulatory progesterone. This clearly signposts some additional research, to investigate if thiomolybdate affects bovine ovarian cycles in a similar way with a statistically large enough cohort, particularly looking at FSH and LH concentrations which we have demonstrated are key to oocyte competence.

As with selenium, copper containing enzymes play an important role as antioxidants and Combelles et al. (2010) have shown the 3 isotypes of superoxide dismutase in differing concentrations in bovine antral follicles. It is reasonable to assume that thiomolybdates would impact on this copper containing enzyme, potentially increasing the oxidative stress on the follicular cells. The importance of copper in the maturation stage has been neatly demonstrated by Rosa et al. (2016) who reported that by adding different concentrations of copper to *in vitro* maturation media, it was possible to decrease DNA damage and apoptosis in COCs. Furthermore, although the

supplemental copper did not affect cumulus expansion, it did improve blastocyst yields. A relatively new concept in this field is that of toxic nanoparticles which theoretically could impact the development of embryos. Nanoparticles are defined as particles between 1 and 100 nanometres (nm) in size, and can occur naturally having been used for centuries in glazes etc. However, a recent increase in synthesised or engineered nanoparticles has led to concerns about environmental contamination and toxicity (Maurer-Jones et al., 2013). Copper nanoparticles have electrical, catalytic, fungicidal and bactericidal properties (Ramyadevi et al., 2012), and can cross biological barriers (Singh et al., 2009). Copper nanoparticles have been shown to impact on the reproductive system (Roychoudhury et al., 2016), albeit not yet in mammals (Chen et al., 2006).

Many of the papers that discuss the impacts of micro nutrients, including minerals call for urgent additional research (Mirone et al., 2013, Velazquez, 2011, Combelles et al., 2010). Few authors dispute the importance of these elements but the exact mechanisms must be better understood. Although several of the mechanisms here are described within an *in vitro* situation, with media additives for example, it seems reasonable that there are effects earlier in the recruitment, development, and maturation phases of the follicle, which may lead to a greater number or more competent COCs. The “u-shaped” response to many nutritional factors is particularly relevant to mineral research in that function can be disturbed by overly low, or overly high inclusion, so both ends of a range must be investigated. There is usually a normal range with homeostatic mechanisms maintaining physiological concentrations. This concept of nutritional hormesis has been described by Hayes (2007).

Velazquez (2011) describes a potential experimental methodology whereby using MOET, uterine luminal fluid could be sampled immediately prior to flushing of the embryos. These would then be biopsied and subjected to gene expression profiling, to assess the influences of nutritional modification. He identifies the same challenges as this thesis has, particularly around controlling the experimental animals, their management, and the stimulation process. However, this is based on a MOET model,

whereby even further value would be added now that we have identified some of the key drivers within an OPU/IVP system in the UK.

6.8. Conclusion

In conclusion, this thesis intended to develop an OPU/IVP platform and use it to consider how minerals can affect oocyte and embryo quality. It was explained in the context statement at the start of this thesis why a mineral trial was not ultimately possible in Chapter 5. In the longer term, it would be very useful for commercial advanced breeding companies (both MOET and OPU/IVP) to understand the many influences and interactions better to ensure that they can offer the best advice to clients who are presenting donors/recipients for OPU-IVP-ET, and optimise the outputs for the breeders with a view to amplifying the desirable genetic traits.

6.9. Associated Further Work

As part of the second project described in the introduction to this thesis, entitled *"Optimising the Delivery of Superior Genetics through Advanced Genomic Selection of Bovine Embryos"*, the same team reported some interesting results using this OPU/IVP platform which merit further investigation. The principal was proved that combining advanced embryo breeding technologies with embryo biopsy and genomic screening (so called pre-implantation genetic diagnosis, (PGD)), along with karyomapping may be useful to deliver superior genetics more efficiently to the breeding herd. From our study, 77 embryos were biopsied and successfully generated SNP chip results, of which 37 were karyomapped, with approximately 50% having a chromosome abnormality, many of these being aneuploid. Chromosomal abnormalities were also identified by Hyttel et al. (2000) who found that 72% of IVP embryos were mixoploid, (contained both diploid and polyploid cells), compared with 25% of *in vivo* produced embryos, and concluded that these embryos must be compensating somehow as the pregnancy rates were not as low as these data would suggest. Griffin and Ogur (2018 – In Press) discuss how important this pre-implantation tool might be to minimize the chances of genetically abnormal embryos. They propose that preimplantation genetic testing for aneuploidy (PGT-A) may be a useful screen for embryos prior to implantation to improve pregnancy rates. As pregnancy rates in cattle remain low

(Kerby, 2013), it may be that aneuploidy and chromosomal abnormalities are part of the problem. Further biopsy studies should be undertaken to confirm these initial aneuploidy findings and rule out whether these were a sampling artefact due to the biopsy only being from the trophectoderm. These cells are not representative of the whole embryo, and potentially a greater proportion of them will have chromosomal abnormalities that would naturally become redundant through apoptosis. However as in humans where this research is current (Babariya et al., 2017) it is important to assess the rate of segmental chromosome abnormalities as a high rate is likely to reduce the success of OPU/IVP programmes.

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Appendix 1: Mineral Survey Questionnaire

The questionnaire was conducted via an online tool – www.surveymonkey.com.

WIN A £50 MARKS and SPENCERS VOUCHER!!

Thank you for your help in completing this important websurvey which is designed to identify current views and perceptions of vets, nutritionists and advisers about the trace mineral status of cattle herds in the UK. We are interested in the overall importance with which mineral status is viewed, the perceived deficiencies or excesses (toxicities) of various minerals, and the various therapies or policies that might be employed to combat any problem that is identified.

Please note that this relates to CATTLE only

Although some of the questions might appear to have a long list of possible responses, each section is in a similar format, and so this survey should take no more than 20 minutes to complete – so please persevere and a £50 M&S voucher will be awarded to one lucky winner!

Please note that all answers are confidential and will not be reported individually. However, if you choose to leave an e-mail address, I will send you a copy of the survey conclusions.

1. How important do you think minerals are in cattle nutrition? Estimate the importance on a 10-point scale where 1 is not important at all and 10 is absolutely critically important.
(click on a button to select your choice)

	1 (not important)	2	3	4	5	6	7	8	9	10 (extremely important)
Importance of Minerals	○	○	○	○	○	○	○	○	○	○

* 2. How often do you identify mineral imbalances?
(please select the most appropriate answer)

- ○ Daily
- ○ Several times per week
- ○ Several times per month
- ○ Several times per year
- ○ Very occasionally
- ○ Never

3. At the most basic level, which minerals do you identify as being a problem in cattle and how often do you identify these?

	Never	Very occasionally	Several times per year	Several times per month	Several times per week	Daily
Copper Deficiency	○	○	○	○	○	○
Copper Toxicity	○	○	○	○	○	○
Selenium Deficiency	○	○	○	○	○	○
Selenium Toxicity	○	○	○	○	○	○
Iodine Deficiency	○	○	○	○	○	○
Iodine Toxicity	○	○	○	○	○	○
Cobalt Deficiency	○	○	○	○	○	○
Cobalt Toxicity	○	○	○	○	○	○
Zinc Deficiency	○	○	○	○	○	○
Zinc Toxicity	○	○	○	○	○	○
Molybdenum Deficiency	○	○	○	○	○	○
Molydenum Toxicity	○	○	○	○	○	○
Other	○	○	○	○	○	○

Other (please specify)

4. When investigating a perceived mineral problem identify which of these techniques you would use (tick ALL that apply)

	Laboratory Feed Analysis	Computer Based Feed Analysis (using standard values)	Blood Sampling	Try supplementation or diet modification and see what happens	Consult an "expert" or other authority	Other (specify below)
Zinc	☐	☐	☐	☐	☐	☐
Copper	☐	☐	☐	☐	☐	☐
Selenium	☐	☐	☐	☐	☐	☐
Molybdenum	☐	☐	☐	☐	☐	☐
Cobalt	☐	☐	☐	☐	☐	☐
Iodine	☐	☐	☐	☐	☐	☐

Other (if you use a different technique, please briefly describe it)

5. When investigating a perceived mineral problem identify which of these is your PREFERRED technique (tick ONE that applies)

	Laboratory Feed Analysis	Computer Based Feed Analysis (using standard values)	Blood Sampling	Try supplementation or diet modification and see what happens	Consult an "expert" or other authority	Other (specify below)
Zinc	○	○	○	○	○	○
Copper	○	○	○	○	○	○
Selenium	○	○	○	○	○	○
Molybdenum	○	○	○	○	○	○
Cobalt	○	○	○	○	○	○
Iodine	○	○	○	○	○	○

Other (if you use a different technique, please briefly describe it)

For the purposes of this survey;
1. It is assumed that two types of deficiency exist in practice; "primary", resulting from the simple deficiency of a mineral in the diet, and "secondary", resulting from the depression of utilisation through the antagonistic effects of other substances, and
2. "Copper deficiency" can be taken to mean any copper responsive problem

* 6. Which of these statements BEST suits your understanding of "copper deficiency" (copper responsive problems) in the UK.

- ○ Copper deficiency is extremely rare in the UK, either primary or secondary
- ○ Primary copper deficiency is extremely rare in the UK – it is usually antagonism from other substances that causes a secondary deficiency
- ○ Primary copper deficiency is extremely rare in the UK – it is usually molybdenum interaction that causes a problem
- ○ Primary copper deficiency is common in the UK, and is exacerbated by the presence of other substances which can antagonise the copper
- ○ Primary copper deficiency is common in the UK and is not associated with antagonism from other substances
- ○ I am not sure, but I recognise that cattle respond to copper supplementation

If necessary, qualify your answer or comment

7. What conditions do you associate with “copper deficiency” and how often do you identify each condition– tick all that apply

	Never	Very occasionally	Occasionally	Frequently
Weak calves	○	○	○	○
Stunted growth	○	○	○	○
Delayed puberty	○	○	○	○
Lethargy	○	○	○	○
Fertility Effects	○	○	○	○
Production Effects	○	○	○	○
Immune System Effects	○	○	○	○
Lameness and/or hoof abnormalities	○	○	○	○
Mastitis and/or increased Somatic Cell Counts	○	○	○	○
Hair and/or coat changes	○	○	○	○
Other	○	○	○	○

If you selected "Other" please specify

8. If you recognise fertility effects are associated with “copper deficiency” which of these do you identify – tick ALL that apply

- ☐ Poor pregnancy rates
- ☐ Abortions
- ☐ Stillbirths
- ☐ Anoestrus
- ☐ Poor/Lack of expression of oestrus
- ☐ Cystic ovaries
- ☐ Early embryonic death/Resorption
- ☐ Late embryonic death/Abortion
- ☐ Retained placenta
- ☐ Endometritis (whites)
- ☐ Other

If you selected "Other" please specify

9. If you identify “copper deficiency”, how often do you recommend each of the following approaches, either singly or in combination?

	Never	Very occasionally	Occasionally	Frequently
Alter the ration only, no supplementation	○	○	○	○
In-feed supplementation	○	○	○	○
In-water supplementation	○	○	○	○
Copper injections	○	○	○	○
Copper needles	○	○	○	○
Mineral drench containing copper	○	○	○	○
Matrix boluses which contain copper	○	○	○	○
Glass boluses which contain copper	○	○	○	○

Other (please specify)

10. And, if you routinely recommend a combination of approaches, please specify which you would commonly use together
(eg feed supplementation, injections)

11. How common do you believe “copper toxicity” is in the UK?

- ○ Very rare
- ○ Quite rare
- ○ Quite common
- ○ Very common

12. In order of importance, rank these possible causes of "copper toxicity" in the UK

	1 (most common)	2	3	4	5	6	7 (least common)
Excess supply specified in the ration	○	○	○	○	○	○	○
Excess supply above level specified in the ration	○	○	○	○	○	○	○
Accidental oversupply	○	○	○	○	○	○	○
Inappropriate formulations of supplementation being used	○	○	○	○	○	○	○
Combinations of supplementations being used	○	○	○	○	○	○	○
Advice based on inappropriate test results	○	○	○	○	○	○	○
Other	○	○	○	○	○	○	○

Other (please specify)

For the purposes of this survey;
1. It is assumed that two types of deficiency exist in practice; "primary", resulting from the simple deficiency of a mineral in the diet, and "secondary", resulting from the depression of utilisation through the antagonistic effects of other substances
2. "Selenium deficiency" can be taken to mean a deficiency of selenium, of Vitamin E, or both

* 13. Which of these statements BEST suits your understanding of “selenium deficiency” in the UK

- ○ Selenium deficiency is extremely rare in the UK, either primary or secondary
- ○ Primary selenium deficiency is extremely rare in the UK – it is usually antagonism from other substances that causes a secondary deficiency
- ○ Primary selenium deficiency is common in the UK, and is exacerbated by the presence of other substances which can antagonise the selenium
- ○ Primary selenium deficiency is common in the UK and is not associated with antagonism from other substances
- ○ I am not sure, but I recognise that cattle respond to selenium supplementation

If necessary, qualify your answer or comment

* 14. Selenium deficiency and Vitamin E deficiency are said to be linked – which statement most accurately reflects your view

- I only ever recognise Selenium deficiency
- I only ever recognise Vitamin E deficiency
- I recognise that these deficiencies are usually linked, and recommend supplementation with selenium
- I recognise that these deficiencies are usually linked, and recommend supplementation with Vitamin E
- I recognise that these deficiencies are usually linked, and recommend supplementation with selenium and Vitamin E
- I dont have a view on this

15. What conditions do you associate with “selenium deficiency” and how often do you identify each condition– tick all that apply

	Never	Very occasionally	Occasionally	Frequently
Weak calves				
Stunted growth				
Delayed puberty				
Lethargy				
Fertility Effects				
Production Effects				
Immune System Effects				
Lameness and/or hoof abnormalities				
Mastitis and/or increased Somatic Cell Counts				
Hair and/or coat changes				
Other				

If you selected "Other" please specify

16. If you recognise fertility effects are associated with “selenium deficiency” which of these do you identify – tick ALL that apply

- [ ] Poor pregnancy rates
- [ ] Abortions
- [ ] Stillbirths
- [ ] Anoestrus
- [ ] Poor/Lack of expression of oestrus
- [ ] Cystic ovaries
- [ ] Early embryonic death/Resorption
- [ ] Late embryonic death/Abortion
- [ ] Retained placenta
- [ ] Endometritis (whites)
- [ ] Other

If you selected "Other" please specify

17. If you identify “selenium deficiency”, how often do you recommend each of the following approaches, either singly or in combination?

	Never	Very occasionally	Occasionally	Frequently
Alter the ration only, no supplementation				
In-feed Vitamin E supplementation only				
In-feed selenium supplementation only				
In-feed Vitamin E and selenium supplementation				
In-water supplementation of Vitamin E and/or Selenium				
Selenium injections				
Vitamin E injections				
Mineral drench containing Vitamin E and/or Selenium				
Matrix boluses which contain selenium				
Glass boluses which contain selenium				
Other				

If you selected "Other" please specify

18. And, if you routinely recommend a combination of approaches, please specify which you would commonly use together
(eg feed supplementation, injections)

19. How common do you believe “selenium toxicity” is in the UK?

- Very rare
- Quite rare
- Quite common
- Very common

20. In order of importance, rank these possible causes of "selenium toxicity" in the UK

	1 (most common)	2	3	4	5	6	7 (least common)
Excess supply specified in the ration							
Excess supply above level specified in the ration							
Accidental oversupply							
Inappropriate formulations of supplementation being used							
Combinations of supplementations being used							
Advice based on inappropriate test results							
Other							

Other (please specify)

For the purposes of this survey, it is assumed that two types of deficiency exist in practice; "primary", resulting from the simple deficiency of a mineral in the diet, and "secondary", resulting from the depression of utilisation through the antagonistic effects of other substances

* 21. Which of these statements BEST suits your understanding of “iodine deficiency” in the UK

- Iodine deficiency is extremely rare in the UK, either primary or secondary
- Primary iodine deficiency is extremely rare in the UK – it is usually antagonism from other substances that causes a secondary deficiency
- Primary iodine deficiency is common in the UK, and is exacerbated by the presence of other substances which can antagonise the iodine
- Primary iodine deficiency is common in the UK and is not associated with antagonism from other substances
- I am not sure, but I recognise that cattle respond to iodine supplementation

If necessary, qualify your answer or comment

22. What conditions do you associate with “iodine deficiency” and how often do you identify each condition– tick all that apply

	Never	Very occasionally	Occasionally	Frequently
Weak calves				
Stunted growth				
Delayed puberty				
Lethargy				
Fertility Effects				
Production Effects				
Immune System Effects				
Lameness and/or hoof abnormalities				
Mastitis and/or increased Somatic Cell Counts				
Hair and/or coat changes				
Other				

If you selected "Other" please specify

23. If you recognise fertility effects are associated with “iodine deficiency” which of these do you identify – tick ALL that apply

- [ ] Poor pregnancy rates
- [ ] Abortions
- [ ] Stillbirths
- [ ] Anoestrus
- [ ] Poor/Lack of expression of oestrus
- [ ] Cystic ovaries
- [ ] Early embryonic death/Resorption
- [ ] Late embryonic death/Abortion
- [ ] Retained placenta
- [ ] Endometritis (whites)
- [ ] Other

If you selected "Other" please specify

24. If you identify “iodine deficiency”, how often do you recommend each of the following approaches, either singly or in combination?

	Never	Very occasionally	Occasionally	Frequently
Alter the ration only, no supplementation				
In-feed supplementation				
In-water supplementation				
Iodine injections				
Topical iodine application				
Mineral drenches which contain iodine				
Matrix boluses which contain iodine				
Glass boluses which contain iodine				

Other (please specify)

25. And, if you routinely recommend a combination of approaches, please specify which you would commonly use together
(eg feed supplementation, injections)

For the purposes of this survey, it is assumed that two types of deficiency exist in practice; "primary", resulting from the simple deficiency of a mineral in the diet, and "secondary", resulting from the depression of utilisation through the antagonistic effects of other substances

* 26. Which of these statements BEST suits your understanding of “zinc deficiency” in the UK

- Zinc deficiency is extremely rare in the UK, either primary or secondary
- Primary zinc deficiency is extremely rare in the UK – it is usually antagonism from other substances that causes a secondary deficiency
- Primary zinc deficiency is common in the UK, and is exacerbated by the presence of other substances which can antagonise the zinc
- Primary zinc deficiency is common in the UK and is not associated with antagonism from other substances
- I am not sure, but I recognise that cattle respond to zinc supplementation

If necessary, qualify your answer or comment

27. What conditions do you associate with “zinc deficiency” and how often do you identify each condition– tick all that apply

	Never	Very occasionally	Occasionally	Frequently
Weak calves				
Stunted growth				
Delayed puberty				
Lethargy				
Fertility Effects				
Production Effects				
Immune System Effects				
Lameness and/or hoof abnormalities				
Mastitis and/or increased Somatic Cell Counts				
Hair and/or coat changes				
Other				

If you selected "Other" please specify

28. If you recognise fertility effects are associated with “zinc deficiency” which of these do you identify – tick ALL that apply

- [ ] Poor pregnancy rates
- [ ] Abortions
- [ ] Stillbirths
- [ ] Anoestrus
- [ ] Poor/Lack of expression of oestrus
- [ ] Cystic ovaries
- [ ] Early embryonic death/Resorption
- [ ] Late embryonic death/Abortion
- [ ] Retained placenta
- [ ] Endometritis (whites)
- [ ] Other

If you selected "Other" please specify

29. If you identify “zinc deficiency”, how often do you recommend each of the following approaches, either singly or in combination?

	Never	Very occasionally	Occasionally	Frequently
Alter the ration only, no supplementation				
In-feed supplementation				
In-water supplementation				
Mineral drenches which contain zinc				
Matrix boluses which contain zinc				
Topical zinc application				

Other (please specify)

30. And, if you routinely recommend a combination of approaches, please specify which you would commonly use together
(eg feed supplementation, injections)

For the purposes of this survey, organic would include chelates and proteinates

31. If you recommend in-feed supplementation, which of the following for each trace mineral do you advise

	Always organic	Always inorganic	A mixture of organic and inorganic	Dont specify
Zinc				
Copper				
Selenium				
Molybdenum				
Cobalt				
Iodine				

32. If you recommend in-feed copper supplementation, how often do you advise the following

	Never	Occasionally	Usually	Always
Copper sulphate				
Copper oxide				
Copper carbonate				
Organic copper				
A combination of the above				

And finally some information about yourself please

* 33. Which of the following most closely describes your role/the type of work you do

- Vet in Practice
- Vet in Academia
- Vet in Industry
- Company Nutritionist
- Independant Nutritionist
- Research Nutritionist
- Agricultural Consultant
- Feed Consultant
- Feed Sales
- Mineral Sales

Other (please specify)

* 34. Which area are you based in?

- England North West
- England North East
- England Yorkshire and Humber
- England West Midlands
- England East Midlands
- England Eastern
- England South East
- England South West
- Scotland North
- Scotland Central
- Scotland South
- Wales North
- Wales South
- Northern Ireland

Other (please specify)

* 35. Are you

- Male
- Female

* 36. How old are you?

- Under 25
- 26-35
- 36-45
- 46-55
- 56-65
- Over 65

37. This section is optional, but if you would like to be entered into the prize draw, and have a copy of the survey conclusions sent to you, then please at least leave an e-mail contact address.

Name:
Company:
Address 1:
Address 2:
City/Town:
County:
Post Code:
Country:
Email Address:
Phone Number:

Your help is very much appreciated - thank you for your time

David Black

38. Please enter any further comments that you would like to make below

Appendix 2: Poster presentation based on Chapter 2

Presented at the World Buiatrics Congress in Santiago, Chile, 2010

The Attitudes and Approach to Trace Element Diagnosis and Treatment in the UK

Black, D.H.[1] and Kendall, N.R.[2]

1,2. Paragon Veterinary Group, Carlisle House, Dalston, Carlisle. CA5 7JF

2. School of Veterinary Medicine and Science, University of Nottingham, Loughborough. LE12 5RD

Introduction

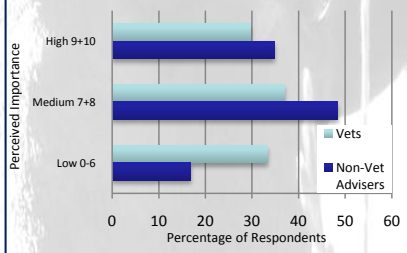
- Many veterinarians, nutritionists and farmers cite the importance of mineral balance in cattle, in particular with relation to fertility, yet there is still much debate about the significance, mechanisms, assessment and treatments of these conditions.
- **An online survey was carried out in the UK, of veterinary surgeons, nutritionists and other advisers to ascertain how important they felt various mineral deficiencies and toxicities were in relation to bovine health and fertility, and how well they understood the various interactions. The survey also assessed how they identified, confirmed and treated these mineral imbalances.**

Materials and Methods

- The survey was designed and conducted using a web-based tool (SurveyMonkey®)
- For the purposes of this survey, respondents were given the following definitions;
  - "Primary", resulting from the simple deficiency of a mineral in the diet, and
  - "Secondary", resulting from the depression of utilisation through the antagonistic effects of other substances,
  - "Copper deficiency" can be taken to mean any copper responsive problem
  - "Selenium deficiency" can be taken to mean a deficiency of selenium, of Vitamin E, or both.

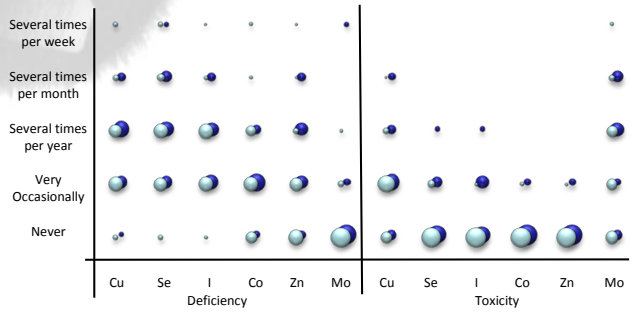
Results

Perceived Importance of Minerals in Cattle Nutrition in UK



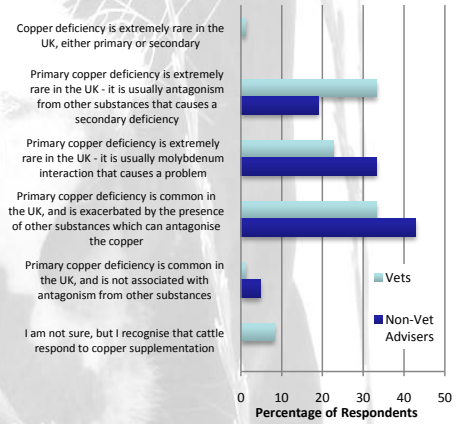
Of 173 respondents, 78% were vets in practice. Only 30% of vets recorded the overall importance of minerals as high and only 35% of non-vets.

Relative Frequency of Identification of Conditions by Vets and Non-Vet Advisers



Most frequently identified deficiencies across all professionals were selenium, copper and iodine (and zinc by non-vets), while the most commonly identified toxicity was molybdenum (with only some recognition of copper toxicity).

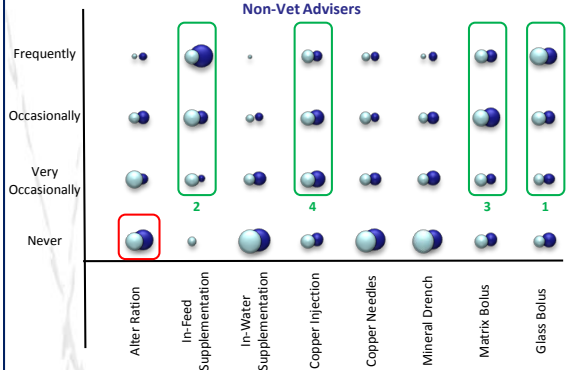
Understanding of Copper Deficiency by Vets and Non-Vet Advisers



When asked about their understanding of the prevalence and mechanisms of the various deficiencies (in this example copper) the range of responses demonstrated the wide variation in the understanding of the various deficiencies both between and within the groups of professional advisers.

Bubbles are proportional in size to the percentage of respondents selecting each option for each mineral or treatment option.

Relative Frequency of Treatment Options for "Copper Deficiency" by Vets and Non-Vet Advisers



Of the treatments available for copper responsive conditions, all were used at least "occasionally"; the most frequently selected treatments by all advisers in order of preference were glass boluses, in-feed supplementation, matrix boluses, and copper injections. Altering the ration was never selected by the majority of respondents in each professional group.

Conclusions

- **Little consensus and no clear agreement on imbalances being common or rare**
- **Mineral deficiency mechanisms poorly understood and copper toxicity poorly recognised as a problem**
- **Surprisingly alteration of ration is less commonly advised**
- **Further research and understanding is required especially regarding copper and molybdenum**

Appendix 3: Innovate UK Full Project Design

The study described in this thesis was partially supported by an Innovate UK (initially Technology Strategy Board (TSB)) match-funded grant and so was designed and conducted in accordance with those regulations and in accordance with the Second Level Project Plan. The project was titled "Applying Advanced Breeding Technology to Amplify and Distribute Bovine Genetics to Increase Production and Sustainability"

The project was split into "work packages" which are summarised below. Those sections particularly relevant to this thesis are described in more detail within Chapter 3.

Work Package 1: Project Management

This was undertaken by the author, as Project Lead and with the assistance of a Project Manager. This package was largely around governance, budgetary management, communications, risk management and reporting to the TSB Monitoring Officer.

Work Package 2: Development of an efficient, robust & repeatable IVP system for bovine embryos

Objectives:

i. To evaluate and compare selected culture media, including commercially available bovine specific complete media and similar formulations available for human IVP with a view to establishing a robust and repeatable protocol that results in the production of viable blastocysts at a satisfactory rate.

ii. To transfer the IVP blastocysts produced by the selected protocol into recipients and create pregnancies which ultimately result in the birth of healthy calves at a commercially acceptable rate.

These comparisons were undertaken in the IVP laboratory and the initial simplified protocol and subsequent amendments are described in Chapter 3, Section 3.2.4.

The following work packages were undertaken in conjunction with project partners at Cogent Breeding Ltd;

Work Package 3: Protocol for processing and transportation of sexed semen for bovine IVF

Objective:

i. To deliver a robust protocol for processing and transportation of sorted semen between laboratories for efficient production of IVF embryos

Work Package 4: Protocol for processing and transportation of reverse sorted semen for bovine IVF

Objective:

i. To deliver a robust protocol for processing and transportation of reverse sorted semen between laboratories for efficient production of IVF embryos To optimise the emerging IVF protocol to allow the use of frozen semen that has been sorted (sexed) after thawing.

These packages were to trial various sorting parameters and extenders suitable for IVF embryo production with standard sexed semen, and with "reverse sorted" sexed semen; this is semen that has been conventionally cryopreserved and is then thawed and sexed. Data from these work packages are not reported in this thesis, however there were ongoing issues with bacterial infection in the reverse sorted sexed semen which were attributed to the egg-yolk extender and were never fully resolved. As time progressed through the study, it became clear that there was less imperative for this technology to be utilised.

Work Package 5: Integration of sexed and reverse sorted sperm into the IVP protocol

Objective:

i. To optimise the emerging IVF protocol to allow the use of frozen semen that has been sorted (sexed) after thawing.

Work Package 6: Evaluate the pregnancy rates of IVP embryos which have been transferred fresh

Objective:

i. To achieve a pregnancy rate in the region of 25% positive, for OPU collected and IVP produced embryos which had been non-surgically transferred in to suitable recipients.

This work involved sourcing and synchronising suitable recipients, and transferring non-surgically 50 fresh IVP produced embryos with recording and analysis of results

The following work package was largely undertaken by project partners at The University of Nottingham;

Work Package 7: To develop a robust vitrification /direct transfer protocol for bovine IVP embryos

Objectives:

i. To evaluate and compare current protocols for vitrification and recovery of bovine blastocysts based on survival and hatching post thaw.

ii. To evaluate these vitrification/thawing procedures for ease of use at the farm level.

iii. To modify current protocols as required to establish a one step, farm friendly, warming and transfer procedure for bovine vitrified blastocysts.

iv. To establish conception rate and calving efficiency for selected procedure in comparison to conventional one step controlled freezing and thawing procedures.

The work was directed at comparing the survival and hatching of blastocysts produced from abattoir derived oocytes (IVP) after vitrification using the then current protocols and comparing them with conventional slow freezing/rapid thawing methods. The intention was then to use IVP blastocysts to modify and develop thawing procedures to produce an effective one step, farm friendly procedure. Then to determine

conception and pregnancy rates for vitrified thawed bovine blastocysts produced from OPU derived oocytes using optimal maturation and culture conditions.

Work Package 7A: Evaluate the selected vitrification method in field/commercial conditions.

Objectives:

i. Achieve a pregnancy rate in the region of 15% positive, for OPU collected and IVP produced embryos which had been vitrified, warmed and subsequently non-surgically transferred in to suitable recipients.

Work Package 8:

Establish a network of OPU regionally based XLVet collection teams to provide a national service for UK farmers

Objectives:

i. To optimise an OPU and cow-side protocol – for more detail see Chapter 3, Section 3.2.3

ii. To train OPU teams to provide a consistent supply of viable Cumulus Oocyte Complexes (COCs) to the regional IVP labs.

Work Package 9:

Establish a network of regionally based XLVet IVP embryo transfer teams to provide a national service for UK farmers

Objectives:

i. To train and provide 12 reliable and competent embryo transfer teams.

Work Package WP 10: Data Collection

Objectives:

i. To develop and refine a data collection system and parameters to be recorded

ii. To develop a database to collate and interrogate the data

This is described in detail in Chapter 3, Section 3.2.6.

Work Package WP11: Intervention Crossover Trial

Objectives:

i. To develop and run a small-scale intervention cross-over trial

The aim of this section was to setup a trial of 2 x 6 heifers and use OPU/IVP techniques to measure the effectiveness of a fertility intervention – this was initially proposed to be a mineral based supplement trial, but was ultimately a comparison of two intra-uterine progesterone releasing devices.

This is described in detail in Chapter 5

Work Package Milestones

Six key milestones were agreed for the whole TSB/Innovate UK project and completion dates set (Table A1, Figure A1)

Table A1. Milestones agreed for the TSB/Innovate UK Project.

Ref	Title	Due Date
M1	Minimal acceptable and repeatable blastocyst and pregnancy rates for fresh transfers using sexed semen.	31-08-12
M2	Optimal and repeatable blastocyst and pregnancy rates for fresh transfers using sexed semen.	31-05-13
M3	Minimal acceptable and repeatable blastocyst and pregnancy rates for vitrified transfers using reverse sorted semen.	01-02-14
M4	Optimal and repeatable blastocyst and pregnancy rates for vitrified transfers using reverse sorted semen.	30-11-14
M5	Completion of training OPU and Embryo Transfer Teams and establishment of network.	30-11-15
M6	Completion of data analysis and initial crossover trials	30-11-16

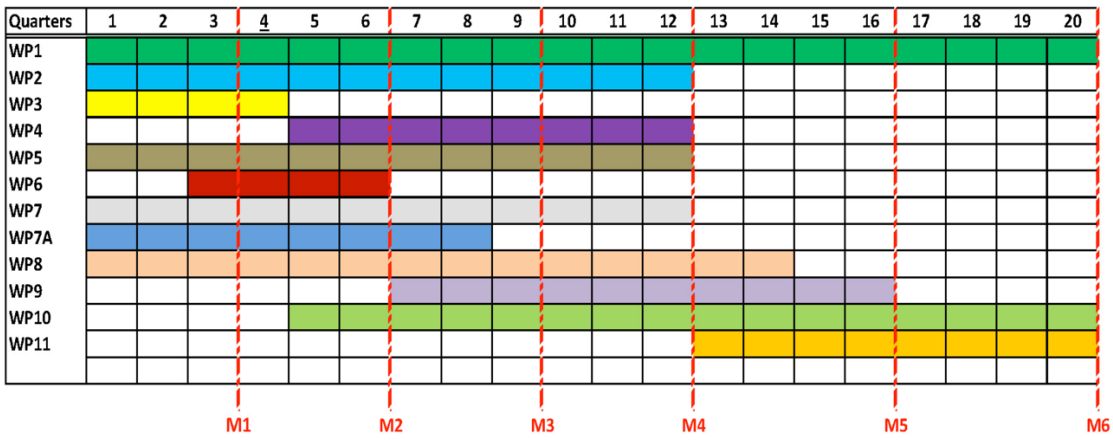
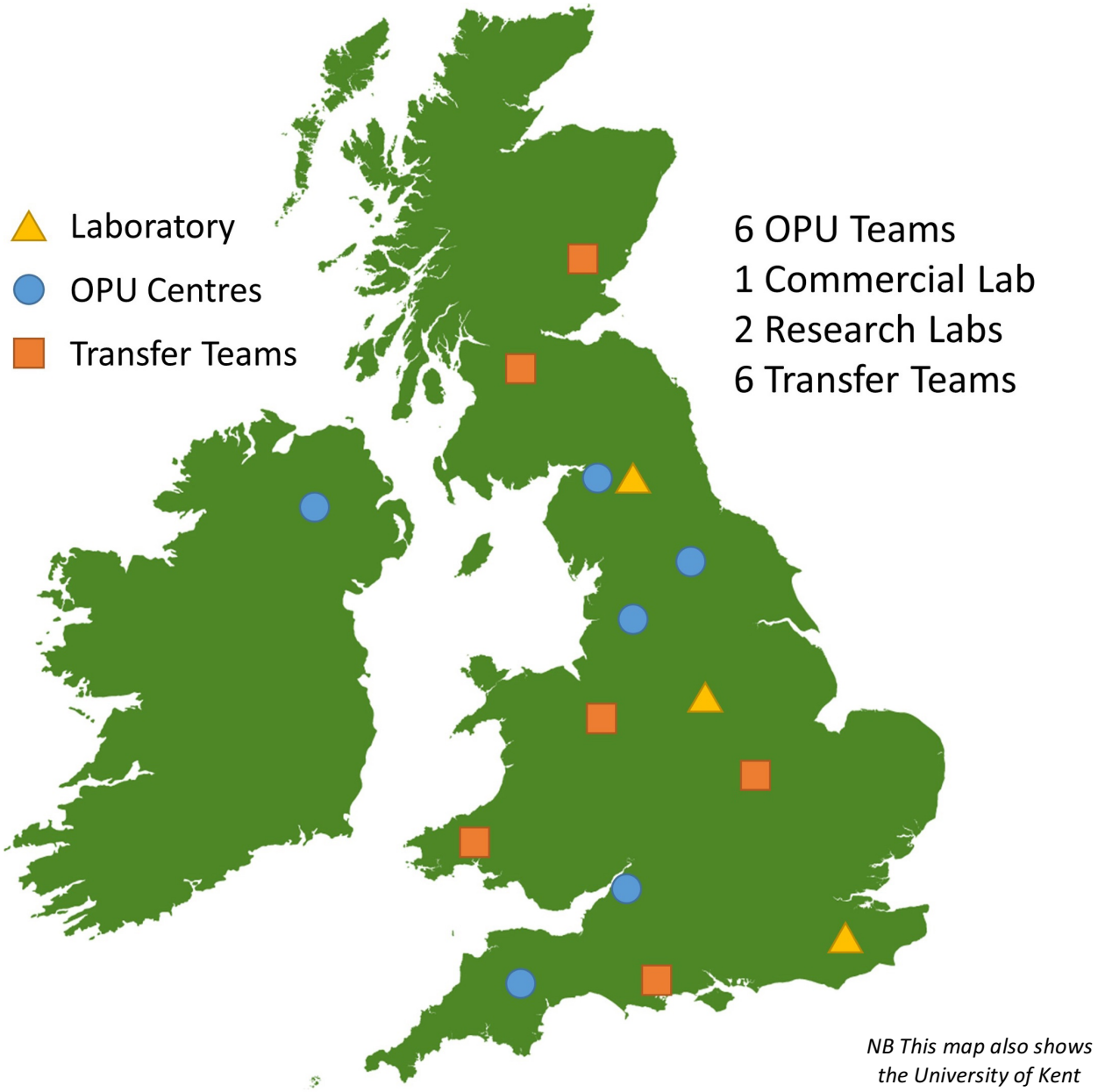


Figure A1. Gant Chart of showing milestones (M) and relationships with work packages (WP) for the TSB/Innovate UK Project

Appendix 4: Map of OPU and transfer teams, and IVP laboratories



NB This map also shows the University of Kent who are now collaborating on an extension to this project