Oocyte quality and viability in Nguni and Hereford cows exposed to a high protein diet

By

Robyn Hamman

Submitted in partial fulfilment of the requirements for the degree

MMedVet: Theriogenology

Department of Production Animal Studies
Faculty of Veterinary Science
University Of Pretoria
Onderstepoort

Supervisor

Professor Dietmar Holm

Co-supervisor

Professor Peter Thompson
Dr Annett Annandale

November 2015

Acknowledgements

I would like to express my absolute gratitude to the people and institutions which assisted in making this work possible. Without them I would never have succeeded:

To my promoter, **Professor Dietmar Holm**, for your support, guidance and unwavering patience. I learnt from you not only how to be a scientist but how to be a better clinician and person. For your positivity, and open door policy I will always appreciate you more than words can do justice.

Mr Mario Smuts, for the hours and hours spent in the field and IVF lab in order to make this project a success. Your extensive experience and perseverance through the challenges we faced was a corner stone in the production of this work.

Dr Takula Tshuma for sharing the workload and assisting in the care of the cattle and the oocyte pickups. I thoroughly enjoyed working with you.

Gauteng Department of Agriculture and Rural Development and **Mr Lomas Mavuluna** for funding and support of this project. It has been a great privilege to work with you.

Mr Pine Pienaar and **Mr David Hill** for loaning me the animals required for this trial. It is a large ask to allow a stranger to cart away your stud cows for 9 months and I am grateful for your trust and willingness to assist me

Professor Peter Thompson my co-promoter, for helping to make sense of my results and to interpret them accurately. I am unsure how you did it but I am in awe.

Bunn and Georgina Hamman, you are wonderful parents who have loved and supported me through all my endeavours. Thank you to listening to me while I shared my excitement of this work.

Summary

Protein is a fundamental part of nutrition in all animals but ruminants have the ability to metabolise and utilise non-protein nitrogen for this use. This allows for an economical alternative source of protein that does not compete with human resources. Previous evidence suggests that feeding high levels of protein to support increased demands for growth and production may have a negative impact on reproduction, since protein is rapidly converted to ammonia and further metabolised to urea which are both toxic to the reproductive system.

Investigations within the South African climate indicated that the Nguni cow maintained higher blood urea nitrogen (BUN) levels during periods of drought than other cattle breeds. The Nguni breed is well adapted to extreme situations but as its popularity grows and the breed is promoted for emerging farmers it becomes essential to know if it possesses adaptations which could predispose it to reproductive failure if supplemented in the same manner as other commercial breeds.

In this prospective experimental study, 22 multiparous cows of two breeds, the Hereford and the Nguni, were block randomised into a cross over design whereby all animals were fed a total mixed ration (TMR). The test group was provided with an increasing rumen degradable protein content in the form of feed grade urea. The control group was maintained on a urea free TMR. In the test group, the urea inclusion rate was increased weekly by 50 g to allow for rumen microflora adaptation and prevent acute urea toxicity. They reached a maximum inclusion rate of 200 g urea per cow per day. All animals had serum collected and oocytes aspirated by transvaginal ultrasound guided oocyte pick up (OPU) were counted and graded twice weekly. Following this, all usable oocytes were pooled per breed and treatment, and were subject to *in vitro* maturation, fertilisation and culture in order to assess the effect of elevated protein on oocyte competence and viability. Oocyte quality was defined as the number of oocytes with cumulus cells (Grade 1 – 3 oocytes) harvested per OPU, and oocyte viability was defined as the number of oocytes that cleaved by day 2 or that reached at least the morula stage by day 7 in the *in vitro* embryo system.

Multivariable analyses were performed on BUN, antral follicle count (AFC) and number of grade 1 – 3 oocytes harvested per OPU session, and on the oocyte viability outcomes in the vitro embryo system.

Nguni cows receiving ≥150 g dietary urea per day had lower mean BUN levels than Herefords (17.5 and 19.3 mg/dL respectively, $P = 0.02$). However dietary urea inclusion level, serum albumin level and sampling day were the only independent predictors of serum BUN. Nguni cows had lower mean antral follicle count (AFC) and number of oocytes harvested than Herefords (9.1 and 3.1, and 11.7 and 4.3 respectively, $P < 0.01$). The vet performing the aspiration, within day sampling order and BUN level >20 mg/dL were independently associated with the number of grade 1-3 oocytes after adjusting for AFC and the random effect of the individual animal. Increasing weighted mean serum albumin and decreasing weighted mean beta-hydroxybutyric acid (BHBA) were independently associated with the number of oocytes that cleaved (day 2) or that reached the morula stage (day 7) ($P = 0.01$ and $P = 0.08$ respectively).

It was concluded that increasing dietary urea, low BCS and increasing serum albumin, but not breed, were associated with increasing BUN levels in cows. It was further concluded that BUN >20 mg/dL adversely affected the occurrence of cumulus cells around oocytes and that serum albumin and BHBA were, but neither dietary urea level nor breed was independently associated with viability of bovine oocytes in this study.

Although the data were inconclusive about breed differences in protein metabolism, this study suggests that serum albumin has the potential to be used as predictor of the interaction between protein and energy metabolism and its effect on oocyte development.

Declaration

I, Robyn Hamman, do hereby declare that the research presented in this dissertation is my own work.

Neither the substance, nor any part of this dissertation has been submitted in the past, nor is it to be submitted to any other University.

This dissertation is presented in partial fulfilment of the requirements for the degree in MMedVet: Theriogenology.

I hereby grant the University of Pretoria free license to reproduce this dissertation in part or as a whole, for the purpose of research or continuing education.

Signed,

Robyn Hamman

Date:

Table of Contents

Summary 2

Declaration 5

Abbreviations 8

List of Figures 9

List of Tables 9

Literature review 10

1.1 Protein metabolism in ruminants 10

1.2 Nguni breed 13

1.3 Hereford breed 14

1.4 *In Vitro* Fertilisation 14

1.5 Oocyte competence and quality 15

1.6 Effect of urea on reproduction 16

Hypothesis 22

Objective 22

Materials and Methods 23

1. Model system 23

2. Experimental design 23

3. Blood sampling 25

4. Oocyte pick up 25

5. Maturation, fertilisation and culture 27

6. Observations and analytical procedures 28

7. Data analysis 29

Results 31

Discussion 37

Conclusions 44

References.....45

Appendices.....52

Abbreviations

AFC	Antral follicle count
BUN	Blood urea nitrogen
RDP	Rumen degradable protein
RUP	Rumen undegradable protein
ME	Metabolisable energy
PUN	Plasma nitrogen levels
MUN	Milk urea nitrogen
CP	Crude protein
COC	Cumulus oocyte complex
OTAU	Onderstepoort Teaching Animal Unit
CO_2	Carbon dioxide
NUN	Experimental group receiving normal urea nitrogen inducing ration
HUN	Experimental group receiving high urea nitrogen inducing ration
NEFA	Non-esterified fatty acids
BHBA	Beta (β)-hydroxybutyric acid
IETS	International Embryo Transfer Society
OPU	Oocyte pick up

List of Figures

Figure 1 Protein metabolism in the ruminant (adapted from (Wattiaux. 2014).....11
Figure 2 Overview of experimental plan 24
Figure 3 Factors associated with AFC and oocyte quality and developmental competence as detected within this study.....42

List of Tables

Table 1: Criteria used for assessing oocyte quality (Gordon. 2003).....**Error! Bookmark not defined.**5
Table 2: Stage of development of embryos(Wright. 1998) ... **Error! Bookmark not defined.**7
Table 3: Serum chemistry and oocyte aspiration data by dietary urea inclusion rate and breed. 30
Table 4: Mixed effects multiple regression model of factors associated with blood urea nitrogen level (mg/dl) ……..30
Table 5: Mixed effects negative binomial regression model of factors associated with antral follicle count…….31
Table 6: Mixed effects negative binomial regression model of factors associated with the number of Grade 1–3 oocytes harvested……..32
Table 7: *In vitro* fertilisation and culture data of cohorts of oocytes pooled per dietary inclusion level and per breed……...……33
Table 8: Effect of weighted mean serum albumin and weighted mean BHBA on oocyte viability and competence……..33
Table 9: Poisson regression model of factors associated with the number of oocytes that cleaved and reached the morula stage……..34

Literature review

1.1Protein metabolism in ruminants

Ruminants metabolise protein in multiple ways. They have a similar pathway to other mammals whereby protein is ingested and moves through the rumen into the abomasum where it is digested and absorbed. This is called rumen undegradable protein (RUP). However the main process whereby ruminants gain protein is via their symbiotic relationship with micro-organisms within the rumen. Rumen degradable protein (RDP) is degraded in the presence of sufficient metabolisable energy (ME), and utilised by the microbial population leading to multiplication (Figure 1). These micro-organisms are then digested in the abomasum to retrieve the microbial amino acids which are absorbed in the small intestine for utilisation by the host. The nitrogen (N_2) containing by-product of rumen protein degradation and fermentation is ammonia. The ammonia is absorbed rapidly into the portal vessels where it is carried to the liver for conversion to urea. Urea is easily excreted by the body as a waste product in the urine and faeces (Andrews *et al*. 2004). Ruminants however are particularly efficient at nitrogen recycling. This is a process whereby 40 -80% of the urea synthesised in the liver is returned to the rumen (mostly via salivary excretion) to be reused by the microbial population for growth and multiplication and will then subsequently supply the ruminant with further amino acids (Figure 1) (Lapierre and Lobley. 2001).

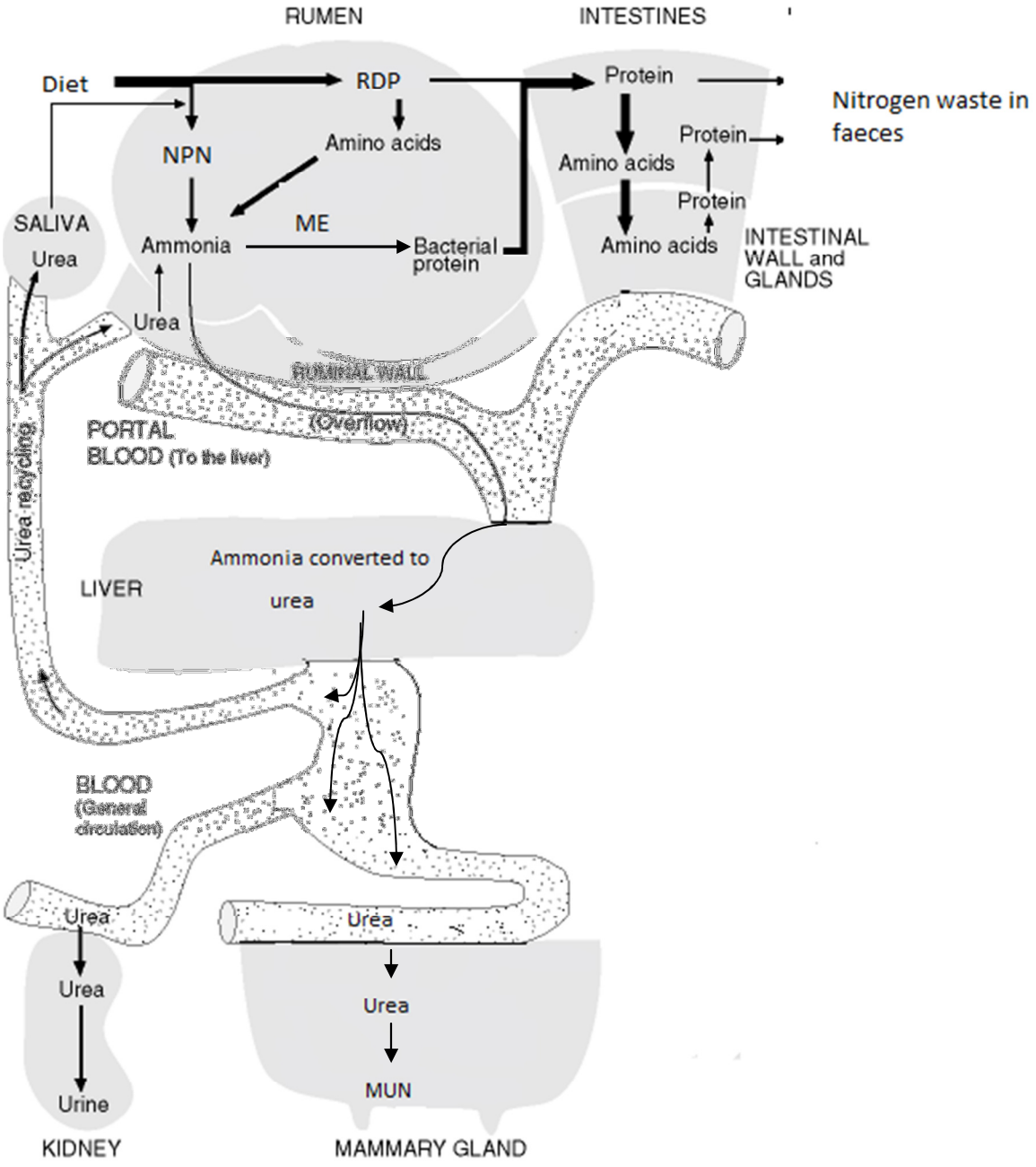


Figure 1 Protein metabolism in the ruminant (adapted from (Wattiaux. 2014)

Urea in its physical form is a white crystalline solid. It was first produced in 1773 by Rouelle le Cadet by evaporating urine and collecting the urea crystals that remained (Kurzer. 1956). The landmark discovery was however made by F Whöler in 1828 when he discovered that urea could be manufactured using ammonia and cyanogens (Whöler F. 1828). This was the beginning of mass production of an economical nitrogen source. Urea has a high N_2 content of 466 g/kg which is equivalent to 2913 g/kg crude protein (CP) accessible by

ruminants as a source of rapidly available RDP and as such is a very popular commercial feed additive (Butler. 1998).

Due to the fact that urea is highly water soluble it is able to cross membranes with ease and can be measured in all bodily fluids such as plasma, serum, whole blood, milk, follicular fluid or uterine secretions (Butler. 1998; Kohn *et al*. 2005). Hammon *et al* (2005) found that as plasma nitrogen (PUN) levels increased so too did the ammonia and urea concentration within the follicular and uterine fluid. The exception was that during oestrous there was no significant increase in ammonia concentration within the uterine fluid.

Blood urea nitrogen (BUN) directly reflects the balance between RDP and ME that arrives in the rumen (see fig 1) (Andrews *et al*. 2004). Utilisation of urea by the ruminant is initiated by microbial hydrolyses via the enzyme urease. Only specific bacteria contain this enzyme (eg *Succinivibrio dextrinosolvens, Prevotella ruminicola* and *Ruminicoccus bromii*). When urea concentrations in the diet are increased, it is therefore important to allow the rumen microorganisms capable of utilising urea to proliferate (Helmer and Bartley. 1971). Urea toxicity occurs when large amounts of urea are fed without an adaptation period. The result is excessive ammonia overwhelming the liver leading to acute death.

The building blocks of protein synthesis are amino acids sourced from microbial protein and the remainder coming from RUP. The liver is the organ responsible for the synthesis of proteins such as albumin and α globulin and β globulins. The ratio of albumin to globulin is fairly constant within cattle (0.84–0.94). Albumin is an integral component of colloid osmotic pressure as well as transporting a wide range of molecules and ions within the circulatory system. Albumin has been classified as a negative acute phase protein, with levels decreasing in response to inflammation or tissue injury (Alavi-Shoushtari *et al*. 2006) . Many different proteins make up the globulin fraction however a large proportion of them are made up by immunoglobulins.

Protein regulation is highly complex. Although Koeln *et al* (1993) demonstrated that albumin concentration remains highly stable over periods of fasting in sheep (Koeln et al. 1993), Strydom *et al* (*2008*) showed that albumin was the best predictor of malnourishment in cattle in South Africa. It has also been shown by Connell et al. (1997) that the transition from fasting to feeding results in a sharp increase in albumin synthesis. Hypoproteinemia is most commonly associated with hypoalbuminemia in adult cattle (Russell and Roussel. 2007). Albumin loss can occur with any protein losing pathology of the gastro intestinal tract

or kidney, including parasite infestation (Abbott *et al.* 1985). Severe hepatic disease can result in decreased albumin levels as it is incapable of meeting the demand for production. Hyperproteinemia as a result of hyperalbuminemia can only be caused by dehydration. If an animal is exposed to excess protein in their diet which exceeds their requirements then the excess will be excreted in urine and faeces in the form of urea. In the case of dehydration both the albumin and globulin components will increase in concentration (Russell and Roussel. 2007). Hyperproteinemia in the absence of dehydration is caused by hyperglobulinemia. In dairy cows it has been shown that globulin levels substantially increase with age (Roussel *et al*. 1982). The most common causes of hyperglobulinemia are chronic antigen stimulation and hepatic disease (Roussel *et al*. 1982).

1.2Nguni breed

Nguni cattle originated in East Africa and made their way south, arriving in Zambia by 300AD (Epstein. 1971). They form part of the collective group of Sanga cattle and have been classified into the scientific subspecies of *Bos taurus africanus* (Strydom *et al*. 2001). They are therefore well adapted to African conditions with good resistance to ectoparasites (Spickett *et al*. 1989). Osler *et al* (1993) found that the Nguni had a greater ability to maintain condition over winter and it was suspected that this may be due to the ability to maintain a high BUN due to a very efficient N_2 recycling pathway. This was further confirmed in a review by Schoeman (1989), citing that Nguni cattle had significantly higher BUN levels when compared to Hereford and other beef cattle managed under the same environmental conditions (Scholtz and Lombard. 1984). They suggested that this could be due to different grazing habits or altered excretion from the kidney. Ndlovu *et al*. (2009) performed a comparative study of Nguni, Angus and Bonsmara steers on high quality grazing. They found that the animals of the Nguni breed had higher glucose and cholesterol levels than the other breeds but that there was no significant difference in total serum protein, urea, albumin, globulin, creatinine and other non-protein metabolites between breeds. A possible explanation for this contradiction may be the proposed grazing habits of the different breeds. Radloff *et al*. (2013) found that Nguni cattle were highly adaptable to their environment and while previously thought to be primary grazers, they were found to gain up to 85% of their nutritional intake from browse when living in heavily wooded environments. Nguni cows that were maintained in Namibia showed a superior calving percentage over nine consecutive years when compared to Hereford cows within the same production systems (Schoeman. 1989). The Nguni also had a higher proportion of cows that calved again in the following

season than Hereford cattle (92.9% vs. 70.4%). Collins-Lusweti (2000) performed a retrospective observational study on three breeds of cattle maintained under the same extensive conditions over a period of three years. Nguni cattle were found to have a significantly higher calving percentage (87%) when compared to the Afrikaner and Bonsmara breeds (69% and 70% respectively) when evaluated during drought conditions. Schoeman (1989) noted that the Nguni had a shorter inter calving period when compared to other beef breeds including Herefords. Schoeman (1989) also published results from the Omatjenne Research Station showing that Nguni cattle had low neonatal calf losses and no dystocias recorded over a 6 year period. The Nguni has a feed conversion ratio, within an extensive farming system, that was found to compare favourably with other beef breeds (Scholtz and Lombard. 1984).

At the turn of the 21st century, this breed re-emerged in South Africa due to their suitability for the emerging farmer sector which required a low input, relatively high output animal (Bester *et al*. 2003).

1.3Hereford breed

The Hereford is a *Bos taurus taurus* beef breed of cattle that dates back to the 1600's in Britain. It was developed from Red Welsh cattle and due to their adaptability they have been exported to many countries throughout the world including South Africa when two bulls were imported in 1890. The Hereford in the Southern African environment was reviewed by (Schoeman. 1989). It was found that while the Hereford had good growth and feed conversion it performed poorer than the Nguni in all reproductive parameters. The breed has been used extensively for research worldwide (Ray et al. 1989; Guilbault et al. 1991; Morris *et al*. 1994).

1.4*In Vitro* Fertilisation

In vitro fertilisation (IVF) involves the aspiration of an oocyte from a follicle before meiosis has been completed and introducing it into an artificial environment. Here the oocyte is matured, fertilised and supported to develop into an embryo capable of being transferred back into a recipient animal for further growth and development.

In vitro fertilisation (IVF) was first accomplished in cattle in 1981 by placing a very early embryo into the oviduct of a recipient cow (Hasler. 2014). From here onwards the processes of sperm capacitation and *in vitro* maturation were refined; however, it was not

until 1988 when transvaginal, ultrasound guided oocyte pick up became available that IVF moved from being purely a research tool to a commercial one. Oocytes could now be harvested from live animals in a reliable and efficient manner (Hasler. 2014). In South America IVF has almost entirely replaced traditional embryo transfer with more than 318 000 IVF produced embryos being transferred in Brazil in 2011 (Hasler. 2014). *In vitro* fertilisation has become an invaluable tool in the replication of genetics as well as an essential research tool.

1.5Oocyte competence and quality

The quality of the oocytes aspirated for IVF has a profound impact on the developmental potential of the embryo. Oocyte competence can be defined as the ability of an oocyte to develop through the key steps required for successful embryo production:

i. The ability to resume meiosis is an intrinsic ability of mammalian oocytes to spontaneously resume meiosis once removed from their follicles. This is determined microscopically by the visualisation of the first polar body (Sirard *et al*. 1998).

ii. The ability of the oocyte to cleave following fertilisation is an essential predictor of oocyte competence. Oocytes have the intrinsic ability to cleave in the absence of fertilisation by a simple stimulus such as an electrical current or ethanol (parthenogenesis). If cleavage does not occur this can be an indicator that the oocyte did not have the ability to undergo cellular division. It cannot however be ruled out that a defective spermatozoan was responsible for not providing an adequate stimulus for oocyte cleavage to occur.

iii. The ability for an oocyte to develop into a blastocyst is a key step in successful embryo production however, it is a controversial predictor of oocyte competence. This is due to the fact many external factors play a significant role in the successful development of an oocyte into a blastocyst. Oocyte competence is only one of them. Factors such as laboratory environment, culture process and male gamete all influence the successful outcome (Sirard *et al*. 2006).

iv. The ability of an oocyte to successfully induce a pregnancy is once more an essential outcome of an IVF system. Oocyte competence is critical but so is the

quality of the blastocyst, as they are not all created equal as well as all the physiological parameters of the recipient animal (Sirard *et al*. 2006).

v. The ultimate outcome of a competent oocyte is the ability to develop to term in good health. There is already evidence that the IVF process can impact the future health of offspring as has been shown in Large Calf Syndrome. Although this syndrome is known to be as a result of the culture processes and not the oocyte competence, focus is now being applied to the possibility of epigenetic alterations that may occur in oocytes which would affect the long term viability of subsequent offspring (Sirard *et al*. 2006).

Oocyte competence and quality are often referred to as a single unit, however competence is based on a series of subsequent levels an oocyte must achieve in order to prove it is viable. While this is a far more effective method by which to grade oocytes, it can obviously only be done retrospectively. Oocyte quality is aimed at predicting which oocyte has the potential to develop through all the key steps by analysing it prior to culture. Microscopy is the most common method to assess oocyte quality. Components of the oocyte such as the quantity and distribution of cumulus surrounding the zona pellucida, as well as the uniformity and appearance of the nuclear chromatin are assessed and based on these findings the oocyte is graded. However microscopic grading of oocytes has proven inefficient in achieving a predictable level of correlation between oocyte quality and oocyte competence with many oocytes appearing healthy under the microscope but incapable of supporting embryo development (Gordon. 2003; Sirard *et al*. 2006).

1.6Effect of urea on reproduction

Increased CP in the diet, leading to elevated BUN has been shown repeatedly to have a detrimental effect on fertility (Elrod B. 1993; Ferguson et al. 1993; McCormick et al. 1999; Rhoads *et al*. 2004). Ammonia concentration also rises in the blood and other fluid environments with increased CP intake or nutritional imbalances (Elrod B. 1993).

Milk urea nitrogen (MUN) is a good predictor of urea nitrogen concentrations within the body (Rodriguez *et al*. 1997; Butler. 2000; Kohn *et al*. 2005). A large study, using over a thousand lactating Holstein cows looked at the correlation between MUN and reproductive failure (Melendez *et al*. 2000). They found that an animal with a high MUN had an 18 times higher risk of being non pregnant than an animal with a normal MUN. Ferguson *et al* (1993)

investigated the relationship between conception rate and serum urea nitrogen in 332 Holstein cows in milk. They interpreted the results using Bayes theorem (dichotomization vs. continuous) and found that while the likelihood ratio test indicated that conception rate would decrease as serum urea nitrogen >14.9 mg/ dL the dichotomised test revealed that this effect only occurred when serum urea nitrogen was > 20 mg/ dL. These findings were replicated in a study by (Boland *et al*. 2001) where PUN and MUN concentrations exceeding 19 mg/ dL were associated with reduced fertility.

In an early investigation into the reproductive consequence of elevated dietary protein, Blanchard *et al* (1990) fed 38 dairy cows in early lactation isocaloric and isonitrogenous diets (16% CP) containing either 73% or 64% of CP in the form of RDP. They discovered that the percentage of ova recovered post superovulation and fertilisation was higher in the lower RDP group and the number of embryos that were of transferable quality was significantly lower in the high RDP group. In a study involving sheep fed variable quantities of urea (0 g/d, 15 g/d or 30 g/d) in an isocaloric diet following which the ewes were synchronised, inseminated and then embryos flushed, they found that ewes receiving high protein supplementation in the form of urea (30 g/d), had a marked decrease in embryo viability (McEvoy *et al*. 1997). He also discovered that those embryos which survived the high BUN had an elevated metabolism and utilised more glucose for growth than the control embryos.

In contrast to these early findings Rhoads *et al* (2006) established in his trial that when dairy cows were fed diets causing either a moderate or high PUN that the embryos collected from these cows after superovulation where similar in number, stage and quality. However when these embryos were transferred into heifer recipients, those embryos from the moderate PUN group yielded higher pregnancy rates when compared to the high PUN group. The effect of the nutritional status of the recipient heifers with regards to PUN levels played no significant role in conception rate and therefore it was deduced that the oocyte or embryo is affected prior to recovery at 7 days post insemination.

The exact mechanism of action and site at which urea has its effect on the reproductive system and its processes has been extensively researched yet is still not completely understood.

Follicle

The environment within the follicle was investigated by Leroy *et al* (2004). They found that metabolites including urea and ammonia within the follicular fluid are correlated to that of the serum and these products are therefore in direct contact with the developing oocyte and granulosa cells.

When bovine granulosa cells were collected from small and medium follicles and exposed to varying concentrations of ammonium chloride in an *in vitro* study, it was established that the growth and metabolism of the granulosa cells was inhibited as ammonia concentrations were increased (Rooke *et al*. 2004). They also found that this effect was not immediately reversible and that the granulosa cells were inferior at supporting oocyte growth post exposure.

Oocyte

De Wit *et al* (2001) experimented on the effect of urea on bovine cumulus-oocyte-complexes (COCs). They aspirated COCs from abattoir collected ovaries and exposed half to physiologically elevated urea of 6mM (16.8 mg/dl) levels in maturation media while the rest were maintained as controls. They noted that a higher proportion of oocytes within the urea elevated media arrested in metaphase I and telophase. In their second experiment they looked at embryo development in the presence of elevated urea in the fertilisation and culture media (16.8 mg/dl) and noted that significantly less oocytes were fertilised and cleaved in the high urea concentration batch but that the proportion of embryo's formed post cleavage was the same between the test and control group. This led to the conclusion that the negative effect of urea was during oocyte maturation and fertilisation. These findings were replicated in a study by Sinclair *et al* (2000). They fed heifers' high ammonia and low ammonia generating diets. This was accomplished by feeding a synchronous energy to protein diet producing the low ammonia generating diet, alternatively they were fed a diet with energy to nitrogen mismatch and this resulted in the high ammonia producing diet. They discovered that there was a significant decrease in cleavage and blastocyst formation in the animals fed the high ammonia producing ration.

A separate study in which oocytes were matured in an elevated urea containing media (0, 14, 21 or 28 mg/dl), found no variation between the proportion of oocytes cleaved when exposed to any concentration of urea. However there were a reduced number of blastocysts

formed by d8 when exposed to 21 mg/dl urea concentration (Ocon and Hansen. 2003). In a second experiment zygotes were collected 9 hours post insemination and exposed to varying urea concentrations (0, 14, 21, 28 mg/dl). Blastocyst development was only affected once urea concentration reached 28 mg/dl (Ocon and Hansen. 2003). The last part of this study was to expose zygotes to media supplemented with variable levels of dimethadione to decrease the pH (from 7.2 to 6.6, 6.4, and 6.3). It was found that blastocyst development was inhibited at all pH variations indicating that the reproductive system is highly sensitive to decreasing pH which is seen with high BUN (Ocon and Hansen. 2003).

Oviduct

(Kenny *et al*. 2001) looked at the effect of administering urea and ammonium chloride to heifers and then monitoring the effect on oviductal fluid. They found that the oviductal environment was synchronous with the vascular compartment. Therefore when urea was administered both BUN and oviductal urea were elevated (maximum concentration of 39 mg/dl reached) and the same occurred for ammonia. However other metabolites or hormone concentrations within the oviduct were not affected.

Uterus and endometrium

Rhoads *et al* (2004) investigated the effect of acute changes in PUN on the uterine environment by infusing urea into the jugular vein and monitoring the changes to uterine conditions. They found that as PUN rose so too did the urea concentration within the uterus and there was a simultaneous drop in uterine pH. They found no change in progesterone or protein levels in the uterus.

Gunaretnam *et al* (2013) exposed endometrial explants to variable urea concentrations (0, 11, 22, 33 and 44 mg/dl) and looked at how it affected endometrial mRNA gene expression. They hypothesised that due to the endometrium being responsible for the production of substances essential for embryonic growth and development such as growth factors, cytokines, hormones and transport proteins, any factors which affect this would be detrimental to embryonic development. It was found that under moderate urea concentrations (11 mg/dl) there was a positive effect on gene expression but as soon as urea levels were elevated to high physiological concentrations (33 mg/dl) then there was a negative effect on gene expression within the endometrium indicating this may be a site of reproductive failure.

Adaptability to increased urea nitrogen levels

The potential for animals to adapt to high PUN levels have been investigated (Laven *et al*. 2004). Forty mature post-partum dairy cows were fed a high production ration with half being supplemented additional urea. They fed this diet for 10 weeks and found no variation in follicular development or embryo growth between the groups. They also noted that there was no difference in progesterone and glucose concentration despite the consistently elevated PUN (7.2mM ± 0.12 equivalent of 20 mg/dl) (Laven *et al*. 2004). This study indicates that cows may be able to adapt to high circulating urea levels if given sufficient time.

In another study (Dawuda *et al*. 2002) established that the duration of feeding of RDP prior to ovulation and fertilisation effected the degree of urea toxicity to the oocyte. Animals that were fed a high protein diet starting 10 days prior to ovulation and insemination had no effect on the quantity and quality of the embryos collected post superovulation and embryo retrieval, whereas the group that was fed a high protein diet starting just prior to ovulation had significantly reduced number of embryos collected.

Interaction of Crude protein and metabolisable energy

The impact of urea on decreased reproductive performance is seen most commonly in dairy cattle. As milk production increases there is a worldwide trend for reproduction to decrease. This is due to the increased nutritional requirements of these animals in order to sustain high milk production. Often high producing dairy cows suffer from a negative energy balance which has led to the debate that it is in fact a mismatch between CP and ME, instead of merely a protein excess that delays the reproductive processes (Butler. 2000). This is due to the fact that the microbial population is unable to utilise the RDP if there is insufficient ME available, leading to excess ammonia crossing from the rumen into the vasculature to be converted to urea by the liver.

In a trial involving ewes whereby they fed diets consisting of urea supplementation or not and variable energy inclusions (0.5 or 2.0 times maintenance energy requirements) they found that the animals fed high energy and urea were most significantly affected with regards to cleavage rate and blastocyst rate in both in vivo and *in vitro* studies. This is in contrast to findings within dairy cattle where insufficient ME in the diet appears to be more significant (Papadopoulos *et al*. 2001).

Armstrong *et al*. (2001) divided heifers into groups based on high and low ME and high and low CP. Animals that were fed both high ME and high CP had a faster rate of dominant follicle development and produced less small follicles. The conception rate of this group post insemination was lower than that of the low ME and CP group. This result was believed to be due to elevated levels of Insulin-like growth factor present in this group. The group of heifers that performed best reproductively were the low ME and low CP group and ME was the independent factor associated with poor conception.

Energy balance can be assessed by monitoring β-hydroxybutyric acid (BHBA) and non-esterified fatty acids (NEFA). Negative energy balance will result in elevation of both of these parameters (Andrews *et al*. 2004). In the presence of insufficient dietary energy, the body uses fat reserves as a substitute. This results in the production of NEFA which can be taken up by the liver and used as a replacement energy source. The liver however becomes over whelmed by large quantities resulting in secondary complications such as hepatic lipidosis. With an elevation of NEFA being utilised by the liver, together with increased carnitine palmitoyltransferase-1 activity, energy generation is via ketogenesis with the resultant BHBA production.

While extensive research into the effect of high NPN on reproduction has been carried out in dairy cattle, there have been limited studies involving beef cattle. The mechanism by which reproduction is negatively impacted is still unresolved.

Hypothesis

Blood urea nitrogen level and oocyte quantity, quality and developmental competence in Nguni and Hereford cows are not affected by urea inclusion level in the diet.

Objective

The objective of this study was to compare the effect of a high urea nitrogen inducing ration on BUN and oocytes retrieved from Nguni and Hereford cows using oocyte pick-up and *in vitro* embryo production.

Additional objectives were to determine which other factors (apart from breed and treatment) were associated with BUN levels, AFC, number, quality and developmental competence of oocytes.

Materials and Methods

1. Model system

Randomised, prospective, cross-over experimental study.

2. Experimental design

Animals

10 Hereford and 12 Nguni cows were enrolled based on the criteria that they were multiparous, not pregnant and not lactating. All cattle were housed at the Onderstepoort Teaching Animal Unit (OTAU) for the entire duration of the study. All animals were uniquely identified by ear tag.

On arrival all animals underwent a clinical and breeding soundness exam to confirm ovarian function defined by the presence of corpora lutea and/or follicles. Antral follicle count of individual animals was determined using transvaginal ultrasound (Mindray, micro convex 5MHz transducer, Taiwan). Antral follicles were defined as all follicles ≥ 3mm. Measurements were performed using the electronic calliper system of the ultrasound unit. An initial ultrasound guided transvaginal OPU was performed and oocyte numbers recorded.

Based on this information, animals were ranked within each breed by oocyte count, and then equally divided between two treatment groups using block randomisation in pairs. The two groups of cows were allowed 4 weeks for adaptation to the basic total mixed ration (TMR) and to establish social stability within the grouping. The basic TMR (balanced using the University of Minnesota Beef cow ration balancer XLS (retrieved from http:// www.extension.umn.edu/.../beef/docs/UM-Beef-Cow-Ration-Balancer.xls) consisted of a combination of milled eragrostis hay, wheat straw, molasses meal, maize meal, salt, brewers grain, rock phosphate and a vitamin premix. This diet was formulated to meet the maintenance requirements of a mature cow (7-9% Protein; 60-90 MJ nett energy for maintenance). Animals were kept in camps thereby restricting their access to alternative grazing or browse. Following the adaptation period, each group was assigned either a high urea inducing diet (HUN) or a normal urea inducing diet (NUN). The HUN group was fed the basic TMR with the addition of feed grade urea starting at an inclusion rate of 50 g/cow/day and increasing weekly (by 50 g increments) to reach a maximum inclusion rate of 200

g/cow/day for a week. The NUN group was maintained on the basic TMR ration. Feed quantity (dry matter) was calculated at 1.8% of body weight. The cows were fed twice daily and any remaining feed was removed from the feed troughs. Cows remained on these rations for 30 days (first repeat), after which the groups were crossed over to the opposite treatment (second repeat).

Cows were sampled twice weekly for the last 22 days (7 sampling days) of the first repeat and for the last 19 days (6 sampling days) of the second repeat of the trial. Cow side recordings included: Animal ID, rectal temperature, body condition score (BCS), body weight (BW), ovarian dimensions, operator, AFC, number of follicles aspirated and the quantity and quality of the oocytes harvested.

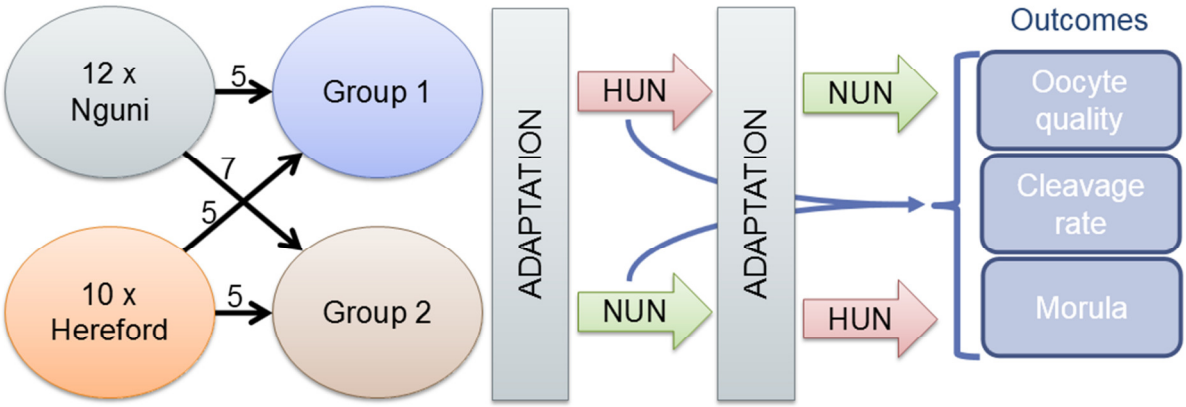


Figure 2 Overview of experimental plan

Sample size

Sample size calculation was based on cleavage rate, representing the most detectable measure of oocyte quality prior to the effects of the IVF system.

We assumed a baseline cleavage rate of 50% based on previous experience in the laboratory.

We assumed a level of significance of $\alpha = 0.05$ and a power of 80%. It was assumed that we wanted to be able to detect a difference in cleavage rate of 10% away from the control (in other words 40% vs 50%).

Formula by (Thrusfield. 2005)

$$n = ((p_1q_1 + p_2q_2)K)/(p_1-p_2)^2$$

where:

n = number required in each cohort

p_1 = anticipated incidence in unexposed animals

$q_1 = (1-p_1)$

p_2 = anticipated incidence in exposed animals

$q_2 = (1-p_2)$

K = 7.84 (for significance level of $\alpha = 0.05$ and power of 80%)

$n(Nguni) = ((0.5x0.5 + 0.4x0.6)K) / (0.1)^2$

= 384 oocytes per treatment group per breed

We estimated to collect 10 oocytes per cow per collection, therefore we needed 38 collections; we had 10 or 12 cows in a group so each cow had to be collected 4 times to achieve the desired sample size.

3. Blood sampling

A venous blood sample was collected from the median coccygeal vein. The blood was allowed to clot and then centrifuged at 300 x g for 8 minutes. The serum was separated and immediately frozen in individually marked aliquots at - 18°C. These stored samples were all run as a single cohort for blood urea nitrogen (BUN), BHBA, total serum proteins and albumin determination.

4. Oocyte pick up

Cows were restrained in a crush and neck clamp. Epidural anaesthesia was performed using 5ml of 2% lignocaine (B.Braun Melsungen, Germany) administered with an 18G needle placed between the 1st and 2nd coccygeal vertebrae. The rectum was evacuated of faeces. Once there was loss of tail tone, the tail was tied out of the way and the vulva and perineum washed using water and a disinfectant (F10 SC®) and dried using disposable paper. The aspiration probe (Watanabe Technologia Applicada, Brazil), consisting of a sector transducer and needle guidance system was connected via a vacuum line to a 50ml polypropylene centrifuge tube containing PBS (Dubelco, Onderstepoort Biological Products,

South Africa) with 5% Foetal bovine serum (FBS) (Life Technologies, South Africa) and maintained at 38°C . The probe was inserted into the vagina, first ensuring the aspiration needle was drawn far back into the protective casing. At this stage the free gloved hand retracted the uterus and manipulated the ovaries trans-rectally so as to fix the ovaries against the head of the transducer where the ovarian structure could be fully visualised by ultrasound. Once follicles were identified, the ovary was positioned facilitating the puncturing of the follicle with a 19 ga, 1.5 inch needle attached to the vacuum line through the vaginal wall. All follicles with a diameter ≥2mm were aspirated..

Oocyte grading

Oocytes were rinsed in a bovine embryo filter and placed in a 4-well dish (Nunc®) where they were counted and graded directly following aspiration using the system shown in Table 1. All oocytes graded 1 to 4 were selected for maturation in 4 cohorts based on breed and treatment (Nguni NUN, Nguni HUN, Hereford NUN, Hereford HUN). All degenerate oocytes were disposed of at this stage.

Table 1: Criteria used for assessing oocyte quality (Gordon. 2003)

Grade	Criteria
1	Compact multi-layered cumulus, homogenous ooplasm, total COC[1] light & transparent
2	Compact multi-layered cumulus (at least 2 complete layers), homogenous ooplasm but with a coarse appearance and dark zone at periphery of the oocyte. Total COC darker & less transparent
3	Less dense cumulus, irregular ooplasm with dark clusters; total COC darker than 1 or 2
4	Denuded oocytes or oocytes with expanded cumulus, cumulus cells scattered in dark clumps in jelly matrix, ooplasm irregular; total COC dark & irregular
5	Degenerate oocyte

[1] Cumulus oocyte complex

5. Maturation, fertilisation and culture

Media were prepared using the Faculty of Veterinary Science (University of Pretoria) IVF protocol (Jooste. 2005). Aspirated oocytes were pooled by treatment and breed for *in vitro* maturation, fertilisation (IVF) and culture (IVC).

All COCs were washed twice in Hepes (Sigma-Aldrich co. SA) buffered solution and then transferred into maturation media for 24 h in a 5% CO_2 incubator at 38.5°C. Oocytes were mechanically denuded of some of the cumulus by gentle pipetting prior to fertilisation. The cumulus remained in the maturation media and was incubated for 24 h to form the co-culture monolayer.

Frozen semen from a single bull was thawed in a water bath at 37°C for 60 seconds and deposited on top of a BoviPure® gradient (Nidacon Laboratories AB, Göthenburg, Sweden) and centrifuged for 20 minutes at 300 x g. The supernatant was removed and the pellet was re-suspended in sperm prep media and centrifuged again for 10 minutes at 300 x g. The pellet was re-suspended and 10µl of the final concentration of 1×10^6 spermatozoa/ml was added to each 40-µl fertilisation droplet under mineral oil containing matured oocytes and incubated for 18 to 20 h.

Following fertilisation, putative zygotes were denuded by vortex for 1 minute to remove remaining cumulus and spermatozoa. They were then placed into early synthetic oviduct fluid (ESOF) for 4 days. The ESOF was then replaced by late SOF (LSOF) within the same co-culture. Stage of development and embryo quality was recorded on days 2 and 7 and embryos were destroyed by desiccation..

Grading of embryos

The number of oocytes that cleaved was recorded at day 2 post fertilisation.

Embryo quality and progress was described and recorded on day 7 post fertilisation. The embryos were graded according to the International Embryo Transfer Society (IETS) recommendations (Wright. 1998).

Table 2: Stage of development of embryos (Wright. 1998)

No.	Stage of development
1	Unfertilized
2	2- to 12-cell
3	Early Morula
4	Morula
5	Early Blastocyst
6	Blastocyst
7	Expanded Blastocyst
8	Hatched Blastocyst
9	Expanded Hatched Blastocyst

6. Observations and analytical procedures

Serum sampling and weighing

Approximately 24hours prior to each oocyte pick up session, the following data were recorded:

Body weight (kg)

Body condition score (9-point scale)

Rectal temperature

Serum was collected and immediately frozen for later analysis of BUN, BHBA, TP and Albumin

Oocyte pick-up

At the time of OPU the following data were recorded:

Time and date

Cow identification

Breed

Veterinarian performing the aspiration

Body condition score and rectal temperature

Flow rate within aspiration line (ml/min)

Right and left ovarian dimensions (mm)

Presence and diameter (mm) of a corpus luteum (CL) on each ovary

Number of antral follicles present per ovary categorised into small (2 - 4mm), medium (5 – 8mm) and large (>8mm) follicles

Diameter of the largest follicle (mm)

Number of follicles aspirated per ovary

7. Data analysis

Data were entered into two spreadsheets: one data set for all individual cow data up to just prior to pooling of COCs, and a second data set for pooled embryo data. In cases where body weight data was missing, values were interpolated based on the mean of the values of the previous and following weighing, or using the same BW as the second or second last weighing date for the first or last weighing session respectively. Certain continuous or categorical data were categorised into 2 categories for inclusion as binary variable in certain models, based on observation of scatter plots to interpret where the most appropriate cut-off levels should be applied. Data were analysed using NCSS 2007 (NCSS, Kaysville, UT, USA) and STATA 14 (StataCorp, Texas, USA). Proportions and means were compared using the Fisher exact test and Students t-test respectively. Medians were compared using the Wilcoxon rank-sum test and were reported for variables that were not normally distributed based on the kurtosis normality test ($P < 0.05$). Multivariable mixed effects Poisson or negative binomial regression models were used for count data (number of antral follicles, numbers of COCs harvested and numbers of COCs and embryos reaching certain stages during the In vitro embryo production procedure). Overdispersion in the Poisson model was assessed by fitting the equivalent negative binomial regression model with over dispersion parameter α and then using a likelihood ratio test to test the null hypothesis that $\alpha = 0$ (Dohoo et al. 2009). Mixed effects multiple linear and logistic regression models were used for continuous and binary outcomes, respectively. After univariable screening, all potential

predictor variables that were associated with the outcome with $P \leq 0.20$ were initially included in the multivariable models, thereafter variables were removed one by one based on the highest Wald P-value in the model. When only variables with $P_{Wald} \leq 0.10$ remained, excluded variables were included into the model again one by one, as well as in combinations of two variables at a time to test for confounding. Confounding was considered to occur if a variable changed the coefficient, odds ratio or count ratio of other variables by ≥15%, in which case the confounder was retained in the model. Animal identification was included as a random effect in all models and was retained if it tended to be significant ($P < 0.10$). The number of antral follicles per cow, and the number of COCs cultured in a pool were included as exposure variables in models of numbers of COCs harvested, and number of embryos reaching certain stages of development on certain days during the IVEP process, respectively. In the models of the numbers of embryos that reached certain stages of development on certain days in the pooled IVEP data, weighted mean serum chemistry values were included, and were calculated from individual cow data based on the proportion of oocytes contributed by each individual cow to the total pool of oocytes cultured.

Results

The Nguni cows in this trial tended to be lighter than the Hereford cows over all weighing sessions on 13 sampling days (mean ± SD 413.7 ± 89.9 kg and 430.5 ± 67.8 kg respectively, $P = 0.07$) whereas the median BCS of Nguni cows was higher than that of the Hereford cows at these same sessions (4.0, IQR 2.5, 4.5 vs 3.0, IQR 2.5, 3.0, $P < 0.01$).

Urea inclusion rate in the TMR significantly affected BUN in both breeds ($P < 0.01$) (Table 3). Overall, mean BUN levels did not differ between breeds ($P = 0.44$) but Nguni cows receiving ≥150 g of feed grade urea per day had lower mean BUN levels than their Hereford counterparts (Table 1); however, this effect was not evident after adjusting for other covariates. Sampling day, serum albumin level and dietary urea inclusion level were the independent predictors of BUN level that remained in the multiple regression model ($P \leq 0.01$) (Table 4). Feed grade urea included at 100 g/cow/day and ≥150 g/cow/day independently increased BUN levels by 4.26 and 10.28 mg/dL respectively, whereas 1mg/dL higher serum albumin was independently associated with an increase in the BUN levels of 0.22 mg/dL.

Table 3: Serum chemistry and oocyte aspiration data by dietary urea inclusion rate and breed.

	Dietary urea inclusion level					
	0 g/cow/day		100 g/cow/day		≥150 g/cow/day	
	Nguni* (n = 78)	Hereford* (n = 63)	Nguni* (n = 17)	Hereford* (n = 15)	Nguni* (n = 58)	Hereford* (n = 52)
Blood urea nitrogen†	$8.0^{a} \pm 2.4$	$7.2^{a} \pm 3.2$	$11.7^{b} \pm 2.8$	$10.9^{b} \pm 3.5$	$17.5^{c} \pm 3.8$	$19.3^{d} \pm 3.9$
Total serum protein†	$74.0^{a} \pm 4.6$	$67.6^{b} \pm 2.8$	$73.1^{a} \pm 5.3$	$67.2^{b} \pm 2.4$	$75.7^{a} \pm 56$	$67.4^{b} \pm 3.2$
Serum albumin†	$34.6^{a} \pm 3.7$	$35.9^{b} \pm 1.9$	$32.2^{c} \pm 4.3$	$36.0^{b,d} \pm 2.0$	$35.2^{a,b} \pm 4.5$	$36.6^{d} \pm 1.7$
Antral follicle count	$8.9^{a} \pm 2.6$	$12.0^{b} \pm 7.1$	$11.2^{b} \pm 4.7$	$12.7^{b} \pm 5.5$	$8.6^{a} \pm 3.1$	$11.0^{b} \pm 5.2$
Number of oocytes aspirated	$2.9^{a} \pm 2.1$	$4.6^{b} \pm 3.7$	$4.5^{b,c} \pm 2.1$	$4.4^{b,c} \pm 3.4$	$2.9^{a,c} \pm 2.5$	$3.9^{b} \pm 2.9$
Grade 1-3 oocytes aspirated	$1.0^{a} \pm 1.3$	$1.4^{a,b} \pm 2.6$	$2.4^{b,c} \pm 2.0$	$2.4^{c} \pm 2.5$	$0.8^{a} \pm 1.3$	$0.9^{a} \pm 1.3$

***Mean ± SD, per oocyte aspiration session**
†mg/dL
[a,b,c] Means in rows with differing superscripts differ significantly ($P < 0.05$)

Table 4: Mixed effects multiple regression model of factors associated with blood urea nitrogen level (mg/dl)

Predictor	level	Coefficient	95% CI		*P*
Dietary urea inclusion level (g/cow/day)	0	1.00	-	-	-
	100	4.26	2.88	5.64	<0.01
	≥150	10.28	9.57	10.99	<0.01
Serum albumin (mg/dL)		0.22	0.10	0.33	<0.01
Repeat	1	1.00	-	-	-
	2	1.70	1.04	2.37	<0.01
Sampling day (within repeat)	1	1.00	-	-	-
	2	2.60	1.13	4.07	<0.01
	3	-0.89	-2.46	0.68	0.27
	4	2.24	0.70	3.79	<0.01
	5	3.45	1.89	5.01	<0.01
	6	2.07	0.48	3.66	0.01
	7	3.11	1.54	4.68	<0.01
Random effects, estimate (SE); 95% CI					
Animal identification		0.32 (0.27); 0.06, 1.64			

Nguni cows had higher total serum protein levels than Hereford cows (mean ± SD 74.5 ± 5.2 mg/dL and 67.4 ± 2.9 mg/dL respectively, $P < 0.01$), however Nguni cows had lower serum albumin levels than Hereford cows (mean ± SD 34.6 ± 4.2 mg/dL and 36.2 ± 1.9 mg/dL respectively, $P < 0.01$). Total serum protein levels were similar between dietary urea inclusion levels ($P > 0.19$). In the mixed effects linear regression model of albumin level, BCS≤2.0 (coefficient -1.12, $P = 0.03$), body weight (coefficient 0.01, $P < 0.01$) and BUN (coefficient 0.07, $P < 0.01$) were independently associated with albumin level.

Nguni cows had higher BHBA levels than Hereford cows (mean ± SD 0.30 ± 0.10, and 0.27 ± 0.06 respectively) whereas mean BHBA levels were similar between dietary urea inclusion levels ($P > 0.20$). In the mixed effects linear regression model of BHBA, BCS 2.5 to 3.0, 3.5 to 4.0 and >4 relative to BCS≤2.0 (coefficients 0.07, 0.11, 0.12, $P < 0.01$) and albumin level (coefficient -0.01, $P = 0.02$) were the remaining independent predictors of BHBA level

Mean AFC was lower in Nguni cows than Herefords (mean ± SD 9.1 ± 3.1 and 11.7 ± 6.2 respectively, $P < 0.01$) and mean number of oocytes harvested was also lower in Nguni cows than in Herefords (mean ± SD 3.1 ± 2.3 and 4.3 ± 3.4 respectively, $P < 0.01$). In the count models of AFC and number of Grade 1-3 oocytes, the over dispersion parameter α differed from zero and therefore the negative binomial regression models were used (Tables 5 and 6). In the model of factors associated with AFC, sampling day within repeat, the

veterinarian performing the aspiration and the breed were independent predictors of AFC (Table 5).

Table 5: Mixed effects negative binomial regression model of factors associated with antral follicle count

Predictor	level	Count ratio	95% CI		*P*
Sampling day (within repeat)	1	1.00	-	-	-
	2	0.77	0.67	0.90	<0.01
	3	0.67	0.58	0.78	<0.01
	4	0.69	0.59	0.80	<0.01
	5	0.69	0.59	0.80	<0.01
	6	0.70	0.61	0.82	<0.01
	7	0.72	0.62	0.83	<0.01
Breed	Hereford	1.00	-	-	-
	Nguni	0.80	0.66	0.98	0.03
Vet	1	1.00	-	-	-
	2	0.72	0.67	0.79	<0.01
	3	0.84	0.72	0.96	0.01
Random effects, variance (SE); 95% CI					
Animal identification		0.05 (0.02); 0.02, 0.09			

Breed was not associated with the number of grade 1 to 3 oocytes aspirated after adjusting for AFC as the exposure variable ($P = 0.41$). Urea inclusion rate fed at 100 g/cow/day tended to increase the number of grade 1-3 oocytes harvested (count ratio 1.34, 95% CI 0.95 – 1.89, $P = 0.09$) after adjusting for sampling day, the veterinarian performing the OPU and AFC. Sampling day, the veterinarian performing the aspiration and the order in which animals were sampled (day sampling order) were significant predictors of the number of grade 1-3 oocytes that were harvested (Table 6).

Table 6: Mixed effects negative binomial regression model of factors associated with the number of Grade 1-3 oocytes harvested.

Predictor	level	Count ratio	95% CI		*P*
Sampling day (within repeat)	2	1.00	-	-	-
	3	0.61	0.35	0.53	1.06
	4	1.37	0.86	2.18	0.19
	5	1.52	0.96	2.44	0.08
	6	0.21	0.43	1.28	0.28
BUN[1] >20 mg/dL		0.53	0.32	0.86	0.01
Day sampling order (linear, from 1 to 22)		0.97	0.95		0.02
Vet	1	1.00	-	-	-
	2	0.66	0.29	0.84	0.01
	3	0.50	0.20	0.42	<0.01
Antral follicle count		Exposure variable			
Random effects, variance (SE); 95% CI					
Animal identification		0.09 (0.06), 0.03, 0.30			

[1]Blood urea nitrogen

Hereford cows on the HUN ration for less than 7 days had a lower proportion of oocytes reaching morula stage by day 7 compared to Hereford cows on the NUN ration during the same period (5/40 vs 16/51, $P = 0.05$). This difference was not apparent in the Nguni cows under the same circumstances. However, when adjusted for other covariates, breed, treatment and BUN levels were not significantly associated with the number of oocytes that cleaved by day 2 or that reached morula stage by day 7 ($P > 0.1$) (Table 7).

Table 7: *In vitro* fertilisation and culture data of cohorts of oocytes pooled per dietary urea inclusion level and per breed.

	Dietary urea inclusion level			
	0 g/cow/day		≥150 g/cow/day	
	Nguni* (n=9)	Hereford* (n=9)	Nguni* (n=9)	Hereford* (n=9)
Number of oocytes cultured	11.2[a] ± 5.8	15.6[a] ± 5.5	11.6[a] ± 5.1	12.4[a] ± 5.8
Number of oocytes that had cleaved by day 2 post insemination	5.4[a] ± 2.9	8.4[a] ± 4.4	5.8[a] ± 4.0	7.9[a] ± 5.8
Number of embryos that had reached the morula stage by day 7 post insemination	1.1[a] ± 1.4	2.4[a] ± 1.5	1.3[a] ± 1.2	1.8[a] ± 2.4

*Mean ± SD, per *in vitro* fertilisation and culture run
[a]Means in rows with differing superscripts differ significantly ($P < 0.05$)

Pools of oocytes from cows with weighted mean albumin levels between 31.5 and 35.9mg/dL were found to be less competent than those which had a weighted mean serum albumin level between 36.0 and 38.5 mg/dL (23/237 and 37/132 respectively. $P = 0.018$). Pools of oocytes from cows with weighted mean BHBA levels between 0.35 and 0.55 mmol/L were found to be less competent than those collected from cows that achieved values 0.15 and 0.34 mmol/L (2/70 and 58/387 respectively, $P < 0.05$) (Table 8).

Table 8: Effect of weighted mean serum albumin and weighted mean BHBA on oocyte viability and competence.

	Weighted mean albumin		Weighted mean BHBA	
	31.5 – 35.9 mg/dL	**36.0 – 38.5 mg/dL**	**0.35 – 0.55 mmol/L**	**0.15 – 0.34 mmol/L**
n	237	220	70	387
Cleaved by day 2	116 (0.49)[a]	132 (0.60)[b]	21 (0.37)[c]	221 (0.57)[d]
Reached morula by day 7	23 (0.10)[a]	37 (0.17)[b]	2 (0.03)[c]	58 (0.15)[d]

[2]Proportions in columns with different superscripts differ significantly ($P < 0.05$)

In the count models of the number of oocytes that cleaved and reached the morula stage, the over dispersion parameter α did not differ significantly from zero; therefore, Poisson models were used. Increasing weighted mean serum albumin was independently associated with an increasing number of oocytes (count ratios > 1) that cleaved by day 2 and the number that reached the morula stage by day 7 ($P = 0.02$ and $P = 0.01$ respectively), whereas increasing weighted mean BHBA tended to be associated with a decreasing number of oocytes (count ratios < 1) that reached the morula stage by day 7 ($P = 0.08$) (Table 9).

Table 9: Poisson regression model of factors associated with the number of oocytes that cleaved and reached the morula stage:

	Cleaved by day 2				Reached the morula stage by day 7			
Predictor	**Count ratio**	**95% CI**		***P***	**Count ratio**	**95% CI**		***P***
Weighted mean serum BHBA[1] (mmol/l)	0.14	0.02	1.10	0.06	0.02	0.00	0.08	0.08
Weighted mean serum albumin (mg/dL)	1.07	1.02	1.12	0.01	1.21	1.06	0.01	0.01
Number of oocytes cultured	Exposure variable				Exposure variable			

[1]Beta-hydroxybutyric acid

Discussion

Within the South African beef farming system, cattle farmed in the sourveld region are routinely supplemented with licks containing variable levels of non-protein nitrogen during the winter months when the protein levels in the natural grazing drops below levels needed for growth, maintenance, lactation and pregnancy (Milton. 1999). Increasing CP in the diet leads to an elevated BUN. This has repeatedly been shown to have a detrimental effect on fertility (Elrod B. 1993; Ferguson *et al*. 1993; McCormick *et al*. 1999; Rhoads *et al*. 2004). In this study two breeds of cattle, the Nguni and the Hereford, were fed either a normal blood urea inducing ration or a high blood urea inducing ration. Blood urea nitrogen levels, the quantity, quality and viability of oocytes aspirated via trans-vaginal OPU as well as the developmental competence of the oocytes were investigated to determine if there were any breed difference in metabolism of non-protein nitrogen provided in a TMR and its effect on fertility. Further objectives of this study were to determine if any of the other factors measured were independently associated with the level of BUN, or with the quantity, quality, viability or developmental competence of oocytes.

The Nguni cow is a *Bos taurus africanus* while the Hereford is a *Bos taurus taurus*. The difference in AFC between *Bos taurus* and *Bos indicus* has been well documented, with the latter having a significantly higher AFC (Pontes *et al*. 2009). To the author's knowledge there has been no published data on the comparison of AFC between the *Bos taurus africanus* and *Bos taurus taurus*. In this study, the AFC and the mean number of oocytes harvested were significantly lower in the Nguni cows than in Hereford cows. This is an important consideration when initiating either an embryo flushing or IVF programme in these breeds. Antral follicle count varied significantly between individual animals. This has been well described by researchers, with AFC being highly variable between animals yet highly repeatable within individuals (Mossa *et al*. 2012))

The veterinarian responsible for sampling was a significant determinant of AFC and Grade 1–3 oocytes aspirated. These findings emphasise the importance of experience when performing OPU, as follicles are not counted effectively if the ovary is not fully manipulated trans-rectally in order to visualise all aspects of it ultrasonographically (Qi, 2013). The potential to damage the cumulus of the COC during aspiration as a result of inexperience is likely to have resulted in the association between the veterinarian performing the aspiration

and the number of oocytes with cumulus. Sampling order within each day was also shown to be associated with the grading of oocytes. As the day progressed the quality of the oocytes deteriorated and this is most likely to be as a result of aspirator fatigue causing excessive trauma to COC's.

The sampling day was found to be a predictor of AFC and number of grade 1–3 oocytes harvested. In this study animals were aspirated twice weekly, as it has previously been shown that this protocol yielded more oocytes than a once weekly aspiration, with stimulation of a new follicular wave (Garcia and Salaheddine. 1998; Goodhand *et al*. 1999). In this study it was found that with repeated sampling, the AFC decreased and oocyte quality deteriorated which is contradictory to previous researchers. This may be as a result of cows included in this study having poor follicular reserves. No effect of diet was found on AFC in this study despite it being reported by previous researchers (Garnsworthy et al. 2009).

The urea inclusion level in the TMR significantly affected the BUN level in both breeds. Previous investigations in Nguni cattle showed that they had a greater ability to maintain condition over winter. This was suspected to be due to an ability to maintain a high BUN as a result of an efficient nitrogen recycling pathway (Osler *et al*. 1993). Scholtz and Lombard. (1984) as cited by (Schoeman. 1989) found a significant difference in BUN levels between Nguni and Hereford cattle managed under the same (restricted) environmental conditions. They suggested this may be due to different habits such as the time spent grazing compared to browsing between the two breeds. In this study we found no difference in mean BUN levels between Hereford cattle and Nguni cattle when they were fed urea levels ranging between 0 and 100g/cow/day. However, when the TMR contained urea ≥ 150g/cow/day Hereford cows had a higher BUN than Nguni cows (Table 3). It was also noted that Nguni cattle reached serum BUN levels ≥ 20mg/dL less frequently than Hereford cows. However, in the multivariable analysis of factors associated with BUN, albumin and BCS remained as independent predictors, and may have confounded the association between breed and BUN levels at high dietary inclusion levels due to breed differences in albumin and BCS. Therefore, although our results could not conclusively accept or reject the hypothesis that breed differences in BUN metabolism exist, these findings strengthen the argument that it is browsing habits and not metabolic differences that are responsible for the findings of previous researchers, as cows in the current study were limited to the formulated diet only, with no access to grazing or browse. Dry matter intake for individual cows was not recorded

in this study. Intake of the ration could have played a role in the amount of urea ingested by individuals and as a result could have influenced the BUN levels between individuals.

In this study no difference was noted between breeds or diets on the number of oocytes that cleaved by day 2 or on the quantity which reached the morula stage by day 7. Similar findings were described by (Rhoads *et al*. 2006) who recorded no difference in the number, quality or stage of development of embryos from cows exposed to a high protein ration. It was only once these embryos had been implanted into heifers that the effect of the high PUN was seen with a higher pregnancy rate in the control animals vs. the high PUN animals. In contrast (Blanchard T *et al*. 1990) showed a significant increase in the percentage of embryos that were recovered post flushing as well as in the quantity of embryos which were of transferrable quality in cows receiving a relatively to protein ration. From the use of IVF studies to monitor the progress of embryo development from collection through fertilisation and embryonic development it would appear that the impact of BUN on oocyte competence only becomes apparent at later stages of development.

Total serum protein levels were higher in Nguni cows than Herefords, although both breeds remained within the normal reference range of 58–73 mg/dL (Russell and Roussel. 2007). Total serum protein consists of two major components: albumin and globulins. The higher TSP in the Nguni cattle is due to an elevated globulin fraction. Total serum protein is a very sensitive indicator of hydration status with a hyperproteinemia occurring as a result of hyperalbuminemia as well as hyperglobulinemia during dehydration. In a study by Roussel *et al* (1982) it was found that as dairy cows got older their globulin concentrations increased. With chronic antigenic stimulation, there is an increase in globulin fraction with a slight decrease in the albumin fraction (Russell and Roussel. 2007). Within our study the Nguni cows were all older than the Herefords due to availability of animals. The Nguni cows, however, had a lower serum albumin concentration than the Hereford cows. A possible explanation for this could be an underlying parasitaemia as these animals were sourced from an extensive bushveld farm.

Weighted mean serum albumin was a predictor of the cleavage rate and the number that reached morula stage by day 7. Serum albumin, a component of total serum protein, is synthesised in the liver and is important in maintaining the oncotic pressure within the vasculature as well as serving as a carrier for lipid soluble molecules such as hormones and free fatty acids which are essential for optimal fertility (Russell and Roussel. 2007). Serum

albumin is a product of protein anabolism while conversely urea and ammonia are by-products of protein catabolism (Leroy *et al*. 2008). From previous studies it would appear that serum albumin, being a positive predictor of growth and sufficient ME utilisation, will in turn be positively associated with oocyte viability and development, thereby strengthening the argument that it is not an excess of protein alone which has the detrimental effect on fertility but instead the interplay between protein and energy (Papadopoulos *et al*. 2001;Leroy *et al*. 2008). However, our data showed that the absolute level of serum albumin is an independent predictor of oocyte developmental competence, even after adjusting for negative energy balance, BUN and BCS, which all reflect the dietary balance between protein and energy. Further, in our data BUN and albumin were positively correlated (Table 4), which could have been a result of variation in hydration status, or variation in dry matter intake between animals. To the researcher's knowledge the role of serum albumin of the donor cow as potential predictor of developmental competence of oocytes has not been reported before; further research is needed to clarify this, and also to determine whether there may be a causal link or whether they are both simply determined by similar factors.

Previous investigators have shown that a BUN threshold of ≥ 20 mg/dL was associated with detrimental effects to the reproductive system (Ferguson *et al*. 1993; Boland et al. 2001;Sinclair *et al*. 2000). However this is not consistent through all studies with other investigators reporting detrimental effects at levels much lower than this (De Wit *et al*. 2001). In this study it was shown that as BUN exceeded 20 mg/dl the number of grade 1–3 oocytes was negatively affected. Grading of oocytes is predominantly based on the quality of cumulus surrounding them. Rooke *et al*. (2004) investigated the effect of protein by-products (ammonia and urea) on cumulus integrity and found that their function and ability to support growth was compromised when exposed to elevated levels.

If the energy concentration of a diet is inadequate for the level of production required from a cow, the detrimental effect of elevated BUN is enhanced. Mean BHBA levels were not affected by treatment in the current study. Nguni cows had higher BHBA levels than Herefords; however, when included into a mixed effects linear regression model the only significant predictor of BHBA was found to be BCS, which was higher in Nguni cows than in Herefords. BHBA is a good indicator of short term energy deficits. The reference range for BHBA is 0–0.9 mmol/L (Russell and Roussel. 2007); all the animals in this trial remained within this range, indicating that the diet had sufficient energy levels. This was confirmed by the body weight data of the cows that did not show any temporal pattern. However, as

weighted mean BHBA increased (within the range of what is considered normal) it was found that the viability and competence of the oocyte as expressed by cleavage and morula development was negatively affected (Table 8). Previous studies have confirmed the association of BHBA with oocyte quality and viability (Sales *et al*. 2015) (Leroy *et al*. 2008); however, these studies were performed on high producing dairy cows in early lactation. Our data show that even subtle increases (within the normal range) in BHBA concentration of non-lactating beef cows, which may be indicative of a ME deficit, may be associated with poor oocyte viability.

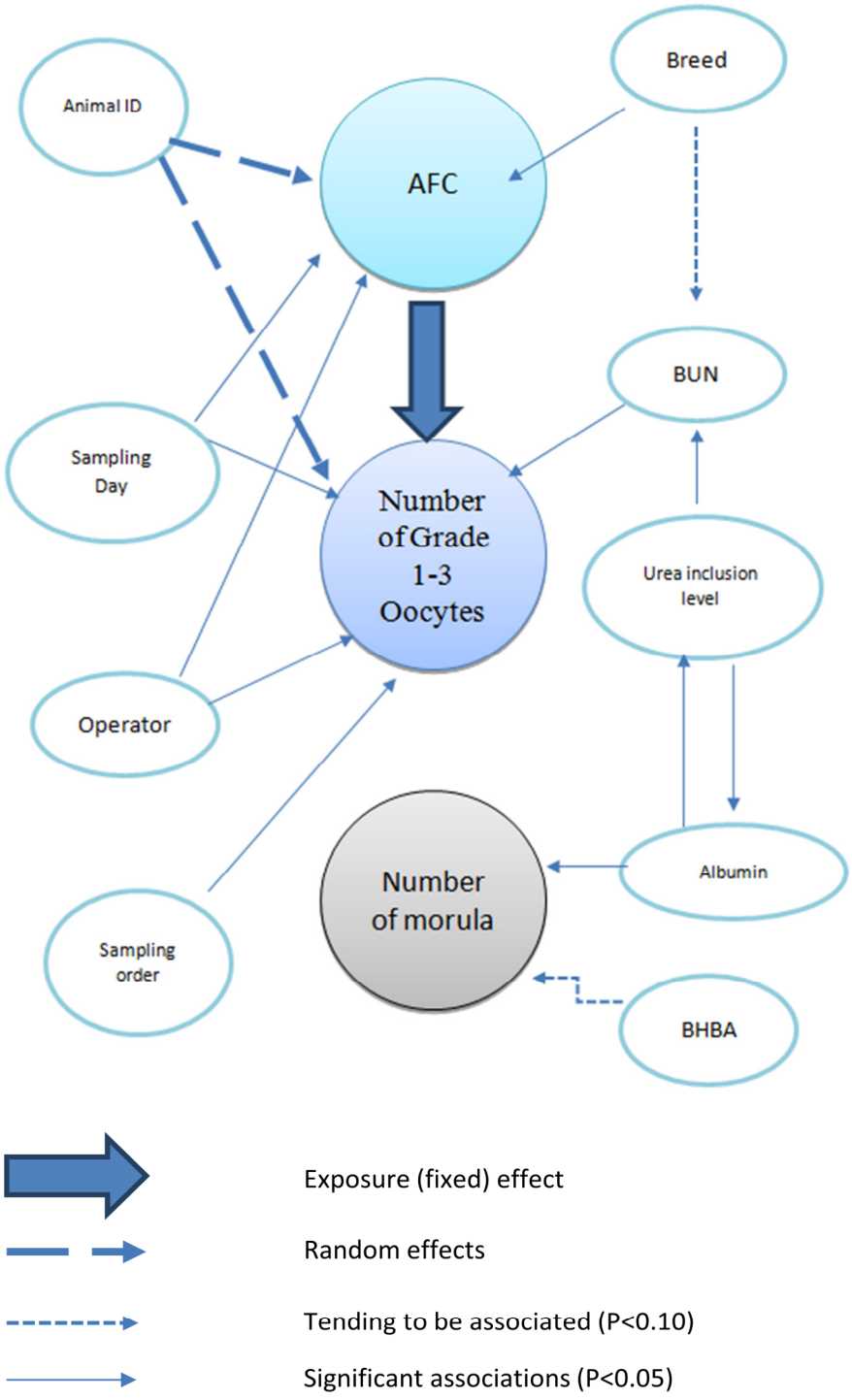


Figure 3 Factors associated with AFC and oocyte quality and developmental competence as detected within this study

Ultimately the most accurate method to determine the effect of elevated BUN on oocyte competence and fertility would be to assess the ability to establish a pregnancy. Due to the fact that the embryos produced from this trial were not placed into recipients the full effect of the BUN was not able to be assessed. It is at this stage that previous researchers

have found the most significant effects of urea on fertility (McEvoy *et al*. 1997; Rhoads *et al*. 2006).

Conclusions

The Nguni breed had a lower AFC and oocyte count than the Hereford breed. The Hereford cows reached the critical BUN threshold more frequently than the Nguni cows however once over the threshold the detrimental effect on oocyte viability was the same.

It was concluded that increasing dietary urea, low BCS and increasing serum albumin, but not breed, were associated with increasing BUN levels in cows.

It was further concluded that BUN above the critical threshold of 20mg/dL primarily affected the quality of oocytes as assessed microscopically and that concentrations of serum albumin and BHBA were independently associated with developmental competence of oocytes in this study, but neither dietary urea level nor breed were significantly associated with this variable.

Although the data was inconclusive about breed differences in protein metabolism, this study suggests that serum albumin has the potential to be used as predictor of the interaction between protein and energy metabolism and its effect on oocyte development.

References

Abbott EM, Parkins JJ, Holmes PH, 1985: Influence of dietary protein on the pathophysiology of ovine haemonchosis in Finn Dorset and Scottish Blackface lambs given a single moderate infection. Res.Vet.Sci. *38* 54-60.

Alavi-Shoushtari SM, Asri-Rezai S, Abshenas J, 2006: A study of the uterine protein variations during the estrus cycle in the cow: A comparison with the serum proteins. Anim.Reprod.Sci. *96* 10-20.

Andrews AH, Blowey RW, Boyd H, Eddy RG, 2004: Bovine Medicine: Diseases and Husbandry of Cattle.

Armstrong DG, McEvoy TG, Baxter G, Robinson JJ, Hogg CO, Woad KJ, Webb R, Sinclair KD, 2001: Effect of dietary energy and protein on bovine follicular dynamics and embryo production in vitro: associations with the ovarian insulin-like growth factor system. Biol.Reprod. *64* 1624-1632.

Bester, J., Matjuda, L. E., Rust, J. M., & Fourie, H. J. (2003). The Nguni: a case study. *Vilakati, D., Morupisi, C., Setshwaelo, L., Wollny, C., von Lossau, A. and Drews, A.(eds)*, 45-68.

Blanchard T, Ferguson J, Love L, Takeda T, Henderson B, Hasler J, Chalupa W, 1990: Effect of dietary crude-protein type on fertilization and embryo quality in dairy cattle Am J Vet Res *51* 905-908.

Boland MP, Lonergan P, O'Callaghan D, 2001: Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. Theriogenology *55* 1323-1340.

Butler WR, 2000: Nutritional interactions with reproductive performance in dairy cattle. Anim.Reprod.Sci. *60–61* 449-457.

Butler WR, 1998: Review: Effect of Protein Nutrition on Ovarian and Uterine Physiology in Dairy Cattle. J.Dairy Sci. *81* 2533-2539.

Collins-Lusweti E, 2000: Performance of Nguni, Afrikander and Bonsmara cattle under drought conditions in the North West Province of Southern Africa. South African Journal of Animal Science 30-33.

Connell A, Calder AG, Anderson SE, Lobley GE, 1997: Hepatic protein synthesis in the sheep: Effect of intake as monitored by use of stable-isotope-labelled glycine, leucine and phenylalanine. Br.J.Nutr. *77* 255-271.

Dawuda PM, Scaramuzzi RJ, Leese HJ, Hall CJ, Peters AR, Drew SB, Wathes DC, 2002: Effect of timing of urea feeding on the yield and quality of embryos in lactating dairy cows. Theriogenology *58* 1443-1455.

De Wit AAC, Cesar MLF, Kruip TAM, 2001: Effect of urea during in vitro maturation on nuclear maturation and embryo development of bovine cumulus-oocyte-complexes. J.Dairy Sci. *84* 1800-1804.

Dohoo I, Martin W, Stryhn H, 2009: Veterinary epidemiologic research. 2nd ed. VER Inc Charlottetown, Canada.

Elrod BW, 1993: Reduction of fertility and alteration of uterine pH in heifers fed excess ruminally degradable protein. Journal of Animal Science *71* 694-701.

Epstein H, 1971: The origin of domestic animals in Africa. Vol. 1. Africana Publishing Corporation New York.

Ferguson JD, Galligan DT, Blanchard T, Reeves M, 1993: Serum urea nitrogen and conception rate: The usefulness of test information. J.Dairy Sci. *76* 3742-3746.

Garcia A, Salaheddine M, 1998: Effects of repeated ultrasound-guided transvaginal follicular aspiration on bovine oocyte recovery and subsequent follicular development. Theriogenology *50* 575-585.

Garnsworthy PC, Fouladi-Nashta AA, Mann GE, Sinclair KD, Webb R, 2009: Effect of dietary-induced changes in plasma insulin concentrations during the early post partum period on pregnancy rate in dairy cows. Reproduction 137 759-768.

Goodhand KL, Watt RG, Staines ME, Hutchinson JSM, Broadbent PJ, 1999: In vivo oocyte recovery and in vitro embryo production from bovine donors aspirated at different frequencies or following FSH treatment. Theriogenology *51* 951-961.

Gordon I, 2003: Laboratory production of cattle embryos. CABI publishing Oxon, UK.

Guilbault LA, Beckers JF, Lapierre S, Zoli AP, Benites W, Roy GL, 1991: Peripartum concentrations of placental protein hormones (bPL and bPAG) in Holstein and Hereford recipients carrying purebred Holstein fetuses. Theriogenology *35* 208.

Gunaretnam I, Pretheeban T, Rajamahendran R, 2013: Effects of ammonia and urea in vitro on mRNA of candidate bovine endometrial genes. Anim.Reprod.Sci. *141* 42-51.

Hammon DS, Holyoak GR, Dhiman TR, 2005: Association between blood plasma urea nitrogen levels and reproductive fluid urea nitrogen and ammonia concentrations in early lactation dairy cows. Anim.Reprod.Sci. *86* 195-204.

Hasler JF, 2014: Forty years of embryo transfer in cattle: A review focusing on the journal Theriogenology, the growth of the industry in North America, and personal reminisces. Theriogenology *81* 152-169.

Helmer LG, Bartley EE, 1971: Progress in the utilization of urea as a protein replacer for ruminants. A review1. J.Dairy Sci. *54* 25-51.

Jooste F, 2005: The association between foot-and-mouth disease virus and bovine oocytes and embryos during in vitro embryo production. MMedVet dissertation, University of Pretoria.

Kenny DA, Humpherson PG, Leese HJ, Morris DG, Tomos AD, Diskin MG, Sreenan JM, 2002: Effect of elevated systemic concentrations of ammonia and urea on the metabolite and ionic composition of oviductal fluid in cattle. Biol.Reprod. *66* 1797-1804.

Kenny DA, Boland MP, Diskin MG, Sreenan JM, 2001: Effect of pasture crude protein and fermentable energy supplementation on blood metabolite and progesterone concentrations and on embryo survival in heifers. Animal Science *73* 501-511.

Koeln LL, Schlagheck TG, Webb Jr. KE, 1993: Amino acid flux across the gastrointestinal tract and liver of calves. J.Dairy Sci. *76* 2275-2285.

Kohn RA, Dinneen MM, Russek-Cohen E, 2005: Using blood urea nitrogen to predict nitrogen excretion and efficiency of nitrogen utilization in cattle, sheep, goats, horses, pigs, and rats. Journal of Animal Science *83* 879-889.

Kurzer FS, PM., 1956: Urea in the history of organic chemistry. Isolation from Natural Sciences *33* 451-459.

Lapierre H, Lobley GE, 2001: Nitrogen recycling in the ruminant: A Review. J.Dairy Sci. *84, Supplement* E223-E236.

Laven RA, Dawuda PM, Scaramuzzi RJ, Wathes DC, Biggadike HJ, Peters AR, 2004: The effect of feeding diets high in quickly degradable nitrogen on follicular development and embryo growth in lactating Holstein dairy cows. Anim.Reprod.Sci. *84* 41-52.

Leroy JLMR, Soom Av, Opsomer G, Goovaerts IGF, Bols PEJ, 2008: Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? Part II: Mechanisms linking nutrition

and reduced oocyte and embryo quality in high-yielding dairy cows. Reproduction in Domestic Animals *43* 623-632.

Leroy JLMR, Vanholder T, Delanghe JR, Opsomer G, Van Soom A, Bols PEJ, de Kruif A, 2004: Metabolite and ionic composition of follicular fluid from different-sized follicles and their relationship to serum concentrations in dairy cows. Anim.Reprod.Sci. *80* 201-211.

Leroy J, Vanholder T, Van Knegsel A, Garcia-Ispierto I, Bols P, 2008: Nutrient prioritization in dairy cows early postpartum: Mismatch between metabolism and fertility? Reproduction in Domestic Animals *43* 96-103.

McCormick ME, French DD, Brown TF, Cuomo GJ, Chapa AM, Fernandez JM, Beatty JF, Blouin DC, 1999: Crude protein and rumen undegradable protein effects on reproduction and lactation performance of Holstein cows. J.Dairy Sci. *82* 2697-2708.

McEvoy TG, Robinson JJ, Aitken RP, Findlay PA, Robertson IS, 1997: Dietary excesses of urea influence the viability and metabolism of preimplantation sheep embryos and may affect fetal growth among survivors. Anim.Reprod.Sci. *47* 71-90.

Melendez P, Donovan A, Hernandez J, 2000: Milk urea nitrogen and infertility in Florida Holstein cows. J.Dairy Sci. *83* 459-463.

Milton SJ, 1999: Veld management in South Africa—N.M. Tainton (ed.) 1999. African Journal of Range & Forage Science *16* 126-127.

Morris CA, Baker RL, Cullen NG, Johnson DL, 1994: Rotation crosses and inter se matings with Angus and Hereford cattle for five generations. Livest.Prod.Sci. *39* 157-172.

Mossa F, Walsh SW, Butler ST, Berry DP, Carter F, Lonergan P, Smith GW, Ireland JJ, Evans ACO, 2012: Low numbers of ovarian follicles =3mm in diameter are associated with low fertility in dairy cows. J.Dairy Sci. *95* 2355-2361.

Ndlovu T, Chimonyo M, Okoh AI, Muchenje V, Dzama K, Dube S, Raats JG, 2009: A comparison of nutritionally-related blood metabolites among Nguni, Bonsmara and Angus steers raised on sweetveld. The Veterinary Journal *179* 273-281.

Ocon OM, Hansen PJ, 2003: Disruption of bovine oocytes and preimplantation embryos by urea and acidic pH. J.Dairy Sci. *86* 1194-1200.

Osler EH, Meyer JHF, Linington MJ, van der Merwe PJ, 1993: Adaptability of indigenous cattle. In: Proceedings of SASAS Developing Areas Branch Symposium, Oct 11-14, 1993.

Papadopoulos S, Lonergan P, Gath V, Quinn KM, Evans ACO, O'Callaghan D, Boland MP, 2001: Effect of diet quantity and urea supplementation on oocyte and embryo quality in sheep. Theriogenology *55* 1059-1069.

Pontes JHF, Nonato-Junior I, Sanches BV, Ereno-Junior JC, Uvo S, Barreiros TRR, Oliveira JA, Hasler JF, Seneda MM, 2009: Comparison of embryo yield and pregnancy rate between in vivo and in vitro methods in the same Nelore (Bos indicus) donor cows. Theriogenology *71* 690-697.

Qi M, Yao Y, Ma H, Wang J, Zhao X, Liu L, Tang X, Zhang L, Zhang S, Sun F, 2013: Transvaginal ultrasound-guided ovum pick-up (OPU) in cattle. J.Biomimetics Biomat.Tissue Eng. *18*.

Radloff FG, Van der Waal C, Bond AL, 2013: Extensive browsing by a conventional grazer? Stable carbon isotope analysis reveals extraordinary dietary flexibility among Sanga cattle of North Central Namibia. Isotopes Environ.Health Stud. *49* 318-324.

Ray DE, Itulya SB, Roubicek CB, Benson CR, 1989: Pregnancy rate, calf mortality and calving date in unsupplemented hereford range cows. Livest.Prod.Sci. *23* 305-315.

Rhoads ML, Rhoads RP, Gilbert RO, Toole R, Butler WR, 2006: Detrimental effects of high plasma urea nitrogen levels on viability of embryos from lactating dairy cows. Anim.Reprod.Sci. *91* 1-10.

Rhoads ML, Gilbert RO, Lucy MC, Butler WR, 2004: Effects of Urea Infusion on the Uterine Luminal Environment of Dairy Cows. J.Dairy Sci. *87* 2896-2901.

Rodriguez LA, Stallings CC, Herbein JH, McGilliard ML, 1997: Effect of Degradability of Dietary Protein and Fat on Ruminal, Blood, and Milk Components of Jersey and Holstein Cows. J.Dairy Sci. *80* 353-363.

Rooke JA, Ewen M, Mackie K, Staines ME, McEvoy TG, Sinclair KD, 2004: Effect of ammonium chloride on the growth and metabolism of bovine ovarian granulosa cells and the development of ovine oocytes matured in the presence of bovine granulosa cells previously exposed to ammonium chloride. Anim.Reprod.Sci. *84* 53-71.

Roussel JD, Seybt SH, Toups G, 1982: Metabolic profile testing for Jersey cows in Louisiana: reference values. Am.J.Vet.Res. *43* 1075-1077.

Russell KE, Roussel AJ, 2007: Evaluation of the Ruminant Serum Chemistry Profile. Veterinary Clinics of North America: Food Animal Practice *23* 403-426.

Sales JNS, Iguma LT, Batista RITP, Quintão CCR, Gama MAS, Freitas C, Pereira MM, Camargo LSA, Viana JHM, Souza JC, Baruselli PS, 2015: Effects of a high-energy diet on oocyte

quality and in vitro embryo production in Bos indicus and Bos taurus cows. J.Dairy Sci. *98* 3086-3099.

Schoeman SJ, 1989: Recent research into the production potential of indigenous cattle with special refernce to the Sanga. South African Journal Animal Science *19* 55-61.

Scholtz MM, Lombard PE, 1984: Nguni research . Genetic research and indigenous breeds exhibition. 2nd world Congress Sheep & Beef cattle breed. *3*.

Sinclair KD, Kuran M, Gebbie FE, Webb R, McEvoy TG, 2000: Nitrogen metabolism and fertility in cattle: II. Development of oocytes recovered from heifers offered diets differing in their rate of nitrogen release in the rumen. J.Anim.Sci. *78* 2670-2680.

Sirard M-, Richard F, Mayes M, 1998: Controlling Meiotic resumption in bovine oocytes: A review. Theriogenology *49* 483-497.

Sirard M, Richard F, Blondin P, Robert C, 2006: Contribution of the oocyte to embryo quality. Theriogenology *65* 126-136.

Spickett, A.M., de Klerk, D., Enslin, C.B. and Scholtz, M.M., 1989: The resistance of Nguni, Bonsmara and Hereford cattle to ticks in a bushveld region of South Africa. Onderstepoort J. vet. Res., 56, 245-250. Onderstepoort Journal Vet. Res. *56* 245-250.

Strydom, S., Agenas, S., Heath, M., Phillips, C., Rautenbach, G., Thompson,P., 2008: Evaluation of biochemical and ultrasonographic measurements as indicators of undernutrition in cattle. Onderstepoort J.Vet.Res. *75*.

Strydom PE, Naudé RT, Smith MF, Kotzé A, Scholtz MM, Van Wyk JB, 2001: Relationships between production and product traits in subpopulations of Bonsmara and Nguni cattle. S.Afr.J.Anim.Sci. *31* 181-194.

Thrusfield M, 2005: Observational studies. In: Anonymous Veterinary Epidemiology. Blackwell Publishing, pp. 280-282.

Wattiaux MA, 2014: Protein Metabolism in Dairy Cows. http://babcock.wisc.edu/node/142. *2014*.

Whöler F, 1828: On the artificial production of urea. Annalen Der Physik *88*.

Wright JM, 1998: Appendix D. Photographic illustrations of embryo developmental stage and quality codes. IETS. , pp. 141-144.

Appendices

Appendix I: Media preparation

HEPES TCM-100

	Total 250 ml
Milli Q	227.5 ml
TCM199	23.6 ml
$NaHCO_3$	0.0875 g
Na pyruvate	0.00875 g
Hepes Na Salt	1.3015 g
4N HCL	345 µl
Filter	

This medium is used for the washing of oocytes (add 2%FCS) and can be used for aspiration (add 4ml heparin per 200ml and 2% FCS)

1. Bovine IVM

1.1Final media

Maturation

FCS	5.0ml
Bicarb TCM 199 (base media)	20.0ml
Penstrep 50X	500 µ L
LH Stock	125 µ L
FSH Stock	12.5 µL
Cysteamine	3.857 µL
Filter	
Add Oestradiol (Stock (1µg/ml in ethanol)	5µL

Bovine Base Media

Maturation Base Media

BicarbTCM199

(Make fresh media for each day)

Bicarb TCM 199	Total 50 ml	100 ml	200 ml
Milli Q	41.75 ml	83.5 ml	167 ml
10x Stock g(TCM199)	4.8 ml	9.6 ml	19.0 ml

Stock B2 (No phenol red)	5.0ml	10.0 ml	20.0 ml
Stock C1	0.25 ml	0.5 ml	1.0 ml
Glutamine (Sigma)	0.005g	0.01g	0.02g
Osmolarity	272		
Filter			

Fertilization Media

BSA (fatty acid free)	0.06 gram
Bicarb-SOFM	10.0ml
Penstrep 50X	200 µL
Filter	

Bovine SPM

SPERM PREPARATION MEDIA

BSA (fatty acid free	0.05g
Caffeine	0.005g
Glutathione	0.003g
Hepes-SOFM (base media)	10ml
Filter	
Heparin (sodium salt)	50µL/10ml

2.Bovine IVC

2.1Culture

BSA (fatty acid free)	0.06 gram
Bicarb-SOFM	9.3 ml
Myo-Inositol (weigh required and add)	0.005g
Essential Amino Acids (50 x stock)	300µL
Non Essential Amino Acids (100x Stock)	100µL
Pen strep 50X	200 µL
Glutamine	10µL
Filter	

Glutamine only lasts 2 weeks so add fresh each week.

Bicarb-SOFM

(Fridge life for two weeks)

Milli Q	38.75 ml		
Stock A	5.2 ml		
Stock B1	5.0ml		
Stock C2	0.5ml		
Stock D	0.5ml		
Osmolarity	280		
Filter			

Hepes-SOFM

(Fridge life for two weeks)

Milli Q	38.85 ml		
Stock A	4.9 ml		
Stock B1	1.0 ml		
Stock C2	0.5ml		
Stock D	0.5 ml		
Stock E	4.0ml		
Osmolarity	280		
Filter			

Stock A (Store for 2 months)

Reagent	Sigma	100ml	200 ml
NaCl		6.29g	
KCl		0.534g	
KH_2PO4		0.162g	
$MgSO_4$		0.182g	
Penicillin		0.06g	
Milli Q		99.4 ml	
Sodium Lactate		0.6ml	

Stock B (make up Fresh)

Reagent	10 ml	20 ml	30 ml	40 ml	50 ml
$NaHCO_3$	0.21g	0.42g	0.63g	0.84g	1.05
Phenol Red	Zero				
Milli Q	10 ml	20 ml	30 ml	40 ml	50 ml

Stock C (Make up Fresh)

Reagent	10 ml	20 ml
Sodium pyruvate	0.05g	0.102g
Milli Q	10 ml	20 ml

Stock D (Store up to 2 months)

Reagent	10 ml	50 ml
CaCl	0.262g	1.31g
Milli Q	10 ml	50 ml

Stock E (25mM) (Store up to 2 months)

Reagent	50 ml	100ml
Hepes	2.97g	5.958g

Phenol Red	Zero	
Milli Q	48.3 ml	96 ml
pH 7.5 (3N NaOH)	1.7 ml	4 ml

3N NaOH

Reagent	50 ml
NaOH	6.0g
Milli Q	50 l

2.2 Stock solution for LH

Lutropin from Bioniche

Each mg Lutropin is 0.92mg NIH-LH-S19 standard

Weigh the contents of the vial

Multiply the contents by 0.92 to get the contents in S19 units.

Note that the content of each vial differs.

Supposing there is 8mg then 8 x 0.92=7.36mg

Add 7.36 sterile saline to give a stock of 1mg/ml

Aliquot 150µl lots in sterile Eppendorfs and freeze

Saline is made with MQ water rater than using commercial saline.

2.3 Stock solution for FSH

FSH is Follitropin from Bioniche

Add 20 ml to 400 mg using saline made with MQ water

Take out 1 ml (20mg) and add 3ml saline

Total volume is now 4 ml with a concentration of 5mg/ml = 5µg/µL

Aliquot 40µL and freeze

Appendix II: Oocyte pick up and field lab form

UNIVERSITY OF PRETORIA: OPU & FIELD LAB RECORD SHEET

Lab. No.: 2013/ Day -1

Date: Veterinarian:

Owner:

Address: Tel no:

Cel no:

Fax no:

Code:

Location where OPU was performed:

DONOR INFORMATION

Donor ID/Ear Tag: BCS:

Breed: Age/Parity:

Pregnancy Stage: Days Postpartum:

Stimulation Protocol:

OPU INFORMATION (Day -1)

Epidural: Sedation:

OPU Needle Guage: Vacuum Level:

Length: Flow Rate:

OPU Start Time: OPU End Time:

Total Time:

Comments:

Left Ovary	Right Ovary
Length:	Length:
Depth:	Depth:
Width:	Width:
Largest Fol. Diam:	Largest Fol. Diam:
CL Diameter:	CL Diameter:
Number Follicles Counted	**Number Follicles Counted**
Small:	Small:
Medium:	Medium:
Large:	Large:
Number Follicles Aspirated	**Number Follicles Aspirated**
Small:	Small:
Medium:	Medium:
Large:	Large:

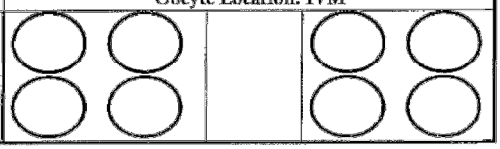
FIELD LABORATORY DATA

Vet / Vet Tech: Start Time: End Time:

Aspiration Media: Batch No. Total Time (Minutes):

Maturation Media: Batch No.: Time into Maturation Media/Inc.:

Media Temperature: Incubator Temperature:

Oocytes Aspirated	Oocyte Location: IVM
Grade 1	
Grade 2	
Grade 3	
Grade 4	
Degenerate	
Total No. Oocytes	

Appendix III: *In vitro* embryo production record

UNIVERSITY OF PRETORIA IVF LABORATORY RECORD SHEET

In Vitro Maturation (Day -1)

OPU Date: _____ Date Oocytes Received in Lab: _____
Time Oocytes Received in Lab: _____
Incubator Temp.: _____ Hours Post Aspiration: _____

Oocytes Aspirated: _____ Oocytes Recovered: _____

Oocytes Cultured: Grade 1: _____ Maturation Media: _____
Grade 2: _____ Batch No.: _____
Grade 3: _____ Final Mat. Media: _____
Grade 4: _____ Batch No.: _____

Sperm Preparation & Insemination (Day 0)

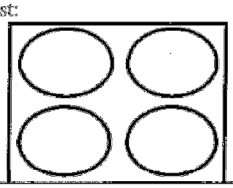
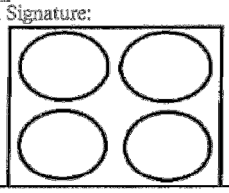
Date: _____ Fresh: _____ Frozen: _____

Bull/Sperm ID: _____
Time Collected: _____
Sample Collected by: _____ Time Received in IVF Lab: _____

Viscosity: _____ Color: _____
Volume: _____ Concentration: _____ / _____
Motility: _____ Final Concentration: _____

Preparation Method: _____ x 10 = _____ - 100
Gradient + Wash (x2) + Swim-up _____ = _____
Gradient + Wash (x2) _____ Final Volume: _____
Other: _____ Final Motility: _____

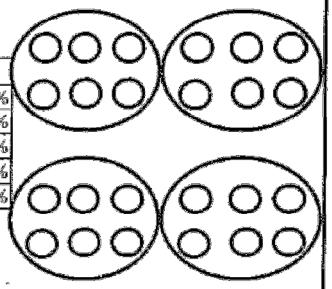
Insemination Amount: _____ Insemination Volume: _____ Insemination Time: _____
Vet. Technologist: Verification Signature:

Comments:  Hrs Post-Maturation: _____ 

Fertilization Check (Day 1)

Date: _____ Embryologist: _____
Time: _____

# Fertilized					
Dish	Drop 1	Drop 2	Drop 3	Total:	%
# 1				/	%
# 2				/	%
# 3				/	%
# 4				/	%
# 5				/	%
Total:					

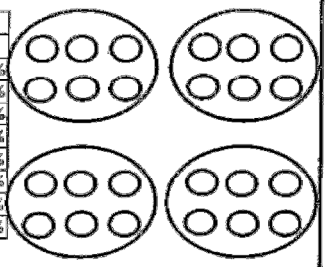


Time into IVC: _____ Hrs Post-Insem: _____
Culture Media: _____ Batch No.: _____
Comments: _____

IVC Embryo Evaluation (Day 8)

Date: _____ Embryologist: _____
Time: _____ Hrs Post Insemination: _____
Culture Media: _____ Batch No.: _____

No.	Stage	Quality Code 1	2	3	4	Total:	%
2	Cleaved					/	%
3	El Morula					/	%
4	Morula					/	%
5	El Blast					/	%
6	Blastocyst					/	%
7	Exp.Blast					/	%
8	Htch Blast					/	%
9	Exp Ht Bl					/	%



IVC Embryo Transfer / Cryopreservation (Day 7/8)

Date: _____ Embryologist: _____
Time: _____ Hrs Post Insemination: _____

Emb for ET: _____
Incubator Temp.: _____
Time Emb Left IVF Lab: _____
Owner: _____
Address: _____

Code: _____
Tel no: _____
Sel no: _____
Fax no: _____

Color of Straws:

Straw #:	Grade:	Straw #:	Grade:
1		11	
2		12	
3		13	
4		14	
5		15	
6		16	
7		17	
8		18	
9		19	
10		20	

Date: _____ Embryologist: _____
Time: _____ Hrs Post Insemination: _____

Emb Frozen: _____
Straws Frozen: _____
Time: _____
Embryologist: _____

Storage: Flask #: _____
Cannister: _____
Cane ID: _____

Freezing Media: _____
Batch No.: _____

Color of Straws:

Straw #:	Grade:	Straw #:	Grade:
1		11	
2		12	
3		13	
4		14	
5		15	
6		16	
7		17	
8		18	
9		19	
10		20	

Straw Inscriptions: _____ _____ _____