EFFECT OF FOLLICLE SIZE AND CUMULUS OOCYTE COMPLEX GRADE ON *IN VITRO* EMBRYO DEVELOPMENTAL COMPETENCE FOR BORAN COWS

A thesis submitted to the University of Nairobi in partial fulfillment of the requirements for a Master of Science degree in Clinical Studies

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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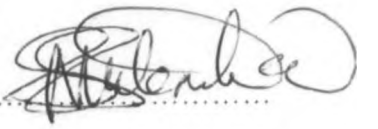
Date 30/6/10 Signature 

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DEDICATION

This thesis is dedicated to my family, my mother Grace Muli, siblings Sebastian, Francis, Carol and Veronica, and to Ivy and Carter for their unwavering support.

Within us, we all have a David, yearning for a chance to face Goliath.

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LIST OF ABBREVIATIONs

ART	- Assisted Reproductive Technologies
ASAL	- Arid and semi arid Lands
BCB	- Brilliant Cresyl Blue
CAIS	- Central Artificial Insemination Station
CO_2	- Carbon Dioxide
COC	- Cumulus oocyte complex
FCS	- Fetal calf serum
FSH	- Follicle Stimulating Hormone
GVBD	- Germinal vesicle break down
ICM	- Inner cell mass
IVC	- *in vitro* culture
IVF	- *in vitro* fertilization
IVM	- *in vitro* maturation
LH	- luteinizing Hormone
MOET	- Multiple Ovulations and Embryo Transfer
OPU	- Ovum Pick Up

TALP - Tyrode albumin Lactose Lactate

TCM - Tissue Culture Medium

TE - Trophoectoderm

ABSTRACT

Zebu cattle are the predominant cattle breed in the arid and semi arid lands (ASAL) of Kenya. The Boran, a zebu is one such breed whose full utilization is yet to be optimized as it has been viewed as a low grade animal. This is in spite of its adaptive superiority to the ASAL's. A feasible, practical and economic plan for utility of this breed is to produce its 1st filial generations (F1) cross-bred animals adapted for various production systems. This study sought to utilize a combination of emerging reproductive technologies, especially the *in vitro* embryo production (IVEP), to explore the potential of constant production of F1 embryos for delivery of the desired F1s. Such innovations would enhance the utility of the indigenous cattle breeds such as the Kenyan Boran beyond beef production.

This study embarked on research to understand how the follicle size and oocyte grade quality would influence blastocyst output in an IVEP system. Thus, the association between the follicle size and the grade of the cumulus oocytes complex (COC) was evaluated. In addition the extent to which these factors affected blastocyst development was studied. Brilliant Cresyl Blue (BCB) stain was used to evaluate competence of the COC's while rates of *in vitro* maturation, cleavage and blastocyst formation were used to determine parameters of the IVEP process.

Some 308 ovaries collected from 154 Boran cows at slaughter from local abattoirs were utilized

for the study. Follicles were evaluated and divided into 3 groups: - group 1: 1-3 mm, group 2; 3-6 mm and group 3; > 6 mm in diameter. The total numbers of grade A and B COC's aspirated from each of follicle group sizes graded and were recorded and used for IVEP process. The IVEP process involved *in vitro* maturation (22-24hrs), *in vitro* fertilization (18-22hrs) and *in vitro* embryo culture (7days). The COC's were fertilized with pre-tested semen from a proven bull and the resulting zygotes cultured to the blastocyst stage. Cleavage and blastocyst rates were assessed 72 hrs post-fertilization and 7 day post-culture, respectively. Some 2658 oocytes were aspirated, 1628 COC were ran through the IVEP process while BCB stain test was conducted on 1030 oocytes.

This thesis reports maturation, cleavage and blastocyst rates of 90.95%, 60.48% and 27.55% respectively, for COCs from Boran cows during the IVEP process. There was no relationship between the follicle size and the grade of COC aspirated ($P > 0.05$). It was shown that both grade A and grade B COC's can be used for IVEP process for the Boran. There was also no relationship ($P > 0.05$) between the follicle size, COC grade and the rate of maturation However there was a strong relationship ($P<0.05$) between the follicle size, COC grade and cleavage rate and blastocyst formation.

The follicle size and grade significantly influenced the BCB competence rate, ($P<0.05$) with COC's from group 3 being the most competent (88.33 %) followed by those from group 2 (77.33%) and then those from group 1 being the least (73.09%). The study revealed that in the

CHAPTER 1

1 Introduction

Zebu cattle are the predominant cattle breeds in Kenya. They are kept in the arid and semi arid (ASAL) lands. The ASAL areas constitute over 80% of the country (Republic of Kenya 2003). In Kenya the Boran is traditionally kept by the pastoralist community as a source of milk and by commercial beef ranches as the main indigenous beef breed (Kimenye, 1985; Okeyo *et al.*, 1998; Rewe *et al.*, 2006). When compared to the *Bos taurus* breeds, the Zebu cattle are better adapted to survive in the harsh and unfavorable climatic conditions of the ASAL's that are characterized by high ambient temperature, poor feed quality and high disease challenges (Herlocker, 1999). In spite of its adaptive superiority to the ASALs, the Boran's milk production ability is very low compared to the *Bos taurus* commercial dairy breeds such as Holstein Friesian, Ayshires, Guernsey's and Jerseys.

Crossbreeding Boran with exotic dairy cattle breeds is one way of enhancing its dairy productivity in agriculturally low potential areas of Kenya. Cross-bred (Boran x *Bos taurus)* offspring, especially the 1st filial generations (F1) produce more milk than the Boran and are better adapted to the tropics than the *Bos taurus* (Gregory *et al.*, 1984; Mwandoto *et al.*, 1988). However, the challenge is that, whereas the F1 is the ideal genotype that most suits the

smallholder farmers' production environment, maintaining this proportion of *Bos taurus* and *Bos indicus* genes is difficult as the temptations are usually to go for higher levels of *Bos taurus*, by continuously crossing back the F1 to the *Bos taurus* as there is no commercially available crossbred semen. This leads to loss of hybrid vigor through a process known as recombination loss (Cunningham and Syrstad, 1987).

A feasible, practical and economic plan for production of such F1's is therefore needed in order to exploit the full potential of the F1 cross-bred animals. Adoption of a combination of emerging reproductive technologies, especially the *in vitro* embryo production (IVEP), has the potential to constantly produce F1 embryos and at the same time deliver the desired F1s, thus enhancing the utility of the indigenous cattle breeds such as the Kenyan Boran beyond commercial beef production (Henshall *et al.,* 2006; Mutembei *et al.,* 2008 a,b).

Although bovine IVEP embryos are being produced in great numbers worldwide (Thibier, 2005), a major challenge to this technology is variability of the results by the different laboratories and production systems (Carmago *et al.,* 2006). These differences have been attributed to several factors including the breed, oocyte quality, follicular environment, fertilization, and embryo culture environment (Lonergan *et al.,* 2006*).*To optimize IVEP system for any cattle breed, it is important to first understand how the donor cows' follicular parameters, including size of the follicles influence the oocyte competence and grade of cumulus oocyte complex's (COC).

Studying such ovarian factors enables a clear understanding of various breed characteristics that would affect embryo output during the IVEP process.

While these ovarian follicular factors have been previously studied in other breeds, information was scarce for the Boran breed. The research reported here therefore focused on the effects of follicular size and grade of cumulus oocyte complex on oocyte and *in vitro* development competence (cleavage and blastocyst rates) in the Boran cow.

CHAPTER 2

2 Objectives and Justification

2.1 General objectives and Purpose of the Study

The overall objective of the study was to increase dairy cattle productivity in the low input production systems in Kenya through innovative application of assisted reproductive technology, specifically *in vitro* embryo production.

The purpose of this study was to design optimum conditions for an *in vitro* embryo production system for Boran cattle. This study utilized oocytes collected from Boran cows at slaughter and used them to set a basis for ovum pick up which would be the ideal technology for such a production system. This study succeeded in setting the protocol for use in an *in vitro* set up in Kenya.

2.2 Specific Objectives

- To assess the effect of follicle size on the grade of the aspirated Cumulus Oocyte Complex(COC) in Boran Cattle
- To investigate the effect of follicle size of origin and Cumulus oocyte grade on oocyte developmental competence as assessed by the Brilliant Cresyl Blue stain in Boran cattle

- To investigate the relationship between follicular size and COC morphological grade on in *vitro* embryo developmental competence as assessed by cleavage and blastocyst development in Boran cattle.

2.3 Justification

It was justified to carry out studies on factors that affect IVEP systems for the Boran cattle because this is the major commercial beef breed in Kenya. The Boran presents an ideal potential for rural dairy setups in terms of future genetic and commercial improvements.

Limited experiences with Boran cows during *in vitro* embryo production (IVEP) had demonstrated that variability of oocyte donors at slaughter warranted ovum pick up (OPU) from live animals as a future option of application in Kenya (Mutembei *et al.,* 2008 a,b). However, before embarking on OPU, preliminary studies using cost effectively available ovaries obtained from Boran cows at slaughter were necessary so as to evaluate oocyte developmental dynamics. Slaughter house ovaries presented an advantage of providing oocytes in the numbers required to cost effectively study the relationship between follicle size, oocyte competence, oocyte morphology and grade on fertilization (cleavage) and embryonic development and viability (blastocyst and conception rates) for the Boran. Once follicular dynamics of Boran cows are understood, a clear foundation of information will be available for timed ovum pick up (OPU) and timed embryo transfer (TET) (Gordon, 1996, Barros and Nogueira, 2005). Ovum pick up will control the source of the oocyte and can utilize oocyte donor's whose genealogy is well known.

CHAPTER 3

3 Literature Review

3.1 Assisted Reproductive Technologies (ART)

Biotechnologies in farm animal reproduction are greatly responsible for the significant progress made in breeding and genetics in cattle (Thibier, 2005). Various techniques have been developed and refined to obtain a large number of offsprings from genetically superior animals. Artificial insemination(AI) and embryo transfer alone have resulted in an almost complete transformation of the breeding work, especially in the dairy industry. In Kenya, AI has been available since 1946 and currently provides about 400,000 doses of semen annually to Dairy farmers (CAIS, 2009). Other biotechnologies that have developed over time include Multiple Ovulations and Embryo transfer (MOET), Sex determination of sperm or embryos, cryopreservation of embryos and semen, nuclear transfer or cloning and *in vitro* embryo production.

3.1.1 *In vitro* Embryo Production

In vitro embryo production (IVEP) is one of the relatively new assisted reproductive technologies in cattle breeding and husbandry (Carmago *et al.,* 2006). This IVEP biotechnology comprises of: oocyte recovery, oocyte maturation, *in vitro* fertilization and embryo culture.

Oocytes can be obtained from either live or dead animals. Follicular oocytes recovered from ovaries obtained from cows at slaughter are commonly used to study factors that affect the process of IVEP (Alm *et al.,* 2005).IVEP is also faced with the usual ART challenges, with the *in vitro* development of bovine oocytes to the blastocyst stage yield of about 30% (Rizos *et al.,* 2002). The quality of embryos produced *in vitro* is affected by various factors, both intrinsic and extrinsic (Merton *et al.,* 2003). Extrinsic factors include efficiency of recovering oocytes from follicles (Boland *et al.,* 1993, Cognie *et al.,* 2004) and the fineness of processes of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and In vitro culture (IVC) (Mermilliod *et al.,* 1999, Merton *et al.,* 2003). Intrinsic factors that determine outputs in an IVEP system include, among others, the effect of breed (Dufour *et al.,* 2000); influence of age of the animals used as donors (Driancourt and Avdi/ 1993) and the plane of nutrition of donor cows (O'Callaghan *et al.,*, 2000). The follicle size of origin and the quality of the oocyte are also key factors affecting IVEP (Lonegran *et al.,* 1994).

The effect of breed on developmental competence of oocytes and embryos is evident because significant differences between breeds occur (Rocha *et al.,* 1998; Block *et al.,* 2002; Paula-Lopes *et al.,* 2003 Carmago *et al.,* 2006). In cattle, oocyte source, whether from a dairy or a beef breed, influences the blastocyst rate (Fischer *et al.,* 2000; Boediono *et al.,* 2003). In addition, follicle size has been shown to influence oocyte competence and grade of the obtained cumulus oocytes complex (COC) (Lonergan *et al.,* 1994; Hazeleger *et al.,* 1995). Both, the oocytes competence

and grade of COC also affects fertilization (cleavage rate) and embryo development (blastocyst rate) (Lonergan *et al.,* 1994).While numerous studies have been carried out to investigate these factors in other breeds, studies for the Boran breed were scarce and it was therefore prudent to investigate how oocytes and embryos of this breed would behave in an IVEP system.

3.2 Development of the Follicle and the Oocyte

3.2.1 **Folliculogenesis**

Folliculogenesis is the process of development of follicles. An ovarian follicle is a highly complex unit comprising of a fluid-filled cavity or the antrum, which bathes a somatic cell-surrounded oocyte (for review see Gordon, 1994). The fluid within the antrum is known as follicular fluid. It is the follicle which provides a microenvironment for oocyte growth and production of hormones.During folliculogenesis, follicles develop and ovulation of one or more mature oocytes occurs. This process starts at puberty and it involves sequential sub-cellular and molecular transformations by various components of the follicle (Gordon, 1994).

During postnatal life, ovarian follicles continue to grow, mature and either ovulate or regress. Each follicle contains one oocyte and the follicles are recruited continuously until the original store is exhausted. The bovine follicle grows 300 to 400 fold in diameter from the primary (50 μm) to the pre-ovulatory (10-15 mm) stage (Lussier *et al.,* 1987). The entire process of follicular growth from the primordial stage (50-60 μm) to the ovulatory stage (10-15 mm) takes

approximately 180 days in cattle and in this time the follicles grow at different times following the follicular waves resulting in different sized follicles at any one time (Lussier *et al.*, 1987).Some physiological reproductive parameters, like folliculogenesis differ among cattle breeds and this can imply differences in COC grade or oocyte quality. For instance, Brazilian zebu cattle usually have three or four follicular waves with small diameter follicles (Viana *et al.*, 2000) while European breeds usually have two or three follicular waves and greater diameter follicles (Lequarre *et al.*, 2005).

3.2.2 **Oocyte Maturation**

In the antenatal life oocyte maturation is arrested at the diplotene stage of meiosis I to remain dormant until puberty. The maturation commences again at puberty during the folliculogenesis phase in a complex phenomenon that facilitates nuclear maturation of the oocyte so as to transform from the diplotene state to the Meiosis II (MII) stage (Yang *et al.*, 1998).

During folliculogenesis the oocyte resumes meiosis in response to the ovulatory luteinizing hormone (LH) surge or the trigger to remove it from the follicle. In cows, it has been shown that the beginning of nuclear maturation of the oocyte, that is, Germinal Vesicle Breakdown (GVBD) occurs within hours after removal of the oocytes from the follicle or the ovulatory LH signal (Yang *et al.*, 1998). The oocyte remains arrested at the M II stage until fertilization takes place, when the oocyte completes meiosis and forms the pronucleus (sign of complete nuclear

maturation).However the completion of nuclear maturation alone does not guarantee subsequent embryo development (Yang *et al.,* 1998). Oocytes must also undergo cytoplasmic maturation, which involves transformations at the cytoplasmic level that prepare the cell to support fertilization and early embryonic development (Blondin and Sirard, 1995).

Oocytes matured *in vitro* or *in vivo* have similar rates of nuclear maturation, fertilization and cleavage, but clearly differ in their developmental potential (Farin and Farin, 1995). In cows the differences in development between *in vivo* and *in vitro* cultured bovine oocytes are expressed at the morula and blastocyst stages (Farin and Farin, 1995). This shows that important factors either in the form of proteins or stable mRNAs are stored during oocyte growth and final follicular maturation after the growth has been completed may be responsible for the differential developmental competence (Blondin and Sirard, 1995).

3.2.3 **Oocyte Developmental Competence**

Developmental competence is the ability of the oocyte to produce normal, viable and fertile offspring after fertilization (Duranthon and Renard, 2001). The developmental competence of the oocyte is acquired within the ovary during the stages that precede ovulation or in case of IVEP, during the stages preceding isolation of the oocyte from its follicle (Mayes, 2002).The ability of the oocyte to complete meiosis is known as meiotic competence, which is acquired gradually during follicular growth. It is closely correlated with oocyte size, which in turn is correlated with

follicle size and the size of the antral follicle at which the oocyte acquires meiotic competence is species-specific (Mayes, 2002). Cleavage and blastocyst rates increased in parallel with meiotic competence and significantly higher developmental rates have been obtained when the diameter of fertilized oocytes is greater than 120 µm (Hazeleger *et al.,* 1995).

Once the oocyte becomes meiotically competent, inhibitory factors are necessary to maintain the oocyte in meiotic arrest. The nature of meiotic arrest in bovine follicles is poorly understood. The meiotic resumption is characterized by GVBD, chromosomal condensation, progression to metaphase of the first meiosis and release of the first polar body and then arrest at the meiosis II (Motlik and Kubelka, 1990). Most mammalian oocytes are ovulated at MII and remain arrested at this stage until activated by a fertilizing spermatozoon or by artificial stimuli (Ozil, 1990).Oocyte developmental competence is a difficult parameter to assess since embryonic development may fail due to reasons independent of oocyte quality. The IVEP is commonly used as a means to diagnose the competence of oocyte batches representing different physio-pathological situations (Gandolfi *et al.,* 1997). Developmental competence is usually expressed as the percentage of oocytes that can develop to the blastocyst stage (Gandolfi *et al.,* 1997).However, development to the blastocyst stage does not guarantee that the embryo will develop to term. Additional criteria may be used to investigate the quality of resulting embryos such as the evaluation of the level of expression of specific gene sets, embryo metabolism, or *in vitro* survival of embryos after freezing or vitrification. Other aspects used to evaluate developmental competence include morphological evaluations, such as number of blastomeres or the ratio between inner cell mass

(ICM) and trophoectoderm (TE) cells number and metabolic rates (Crosier et *al.*,, 2001). Nevertheless, embryo transfer, pregnancy results and offspring viability remain the ultimate way to finally conclude about oocyte quality.

3.2.4 **Development of Preimplantation Embryo**

During the early development from day 1 to day 8 the bovine embryo remains within the zona pellucida, approximately 170μm in diameter (Morris *et al.,* 2001). The embryos enter into several divisions after fertilization. The zygote is a large cell, having a low nuclear to cytoplasmic ratio. To attain a ratio similar to somatic cells, cell divisions occur without an increase in cell mass. This process is referred as cleavage and the resulting daughter cells are called blastomeres. The first cleavage occurs at 2 days after fertilization. Between day 3 and 4 after fertilization the embryo contains 8-16 cells. At day 5 and 6 the embryo begins to form junctions and resulting into a compact of cells termed the morula.

Compaction is known to be the first essential step in differentiation and is fundamental and essential for viable blastocyst formation. The most significant event occurring at compaction is the emergence of 2 distinct cell populations: the blastomeres remaining in contact with the outside are destined to form the trophoectoderm (TE) cells lineage while the blastomeres inside the embryo are destined to form the Inner Cell Mass (ICM).

In mammalian embryos, during the 8, 16 and 32-cell stages, specific cells are induced to change

their morphological and functional phenotype to a polarized form. This commences with the division of the 8-cell stage embryos generating an average of 9 outside and 7 inside cells in the embryo (Johnson and Ziomek 1981), with the outer cells being polarised and larger than the inner cells that remain apolar.

Trophoectoderm cells therefore are polar, enveloping and fluid transporting (Gardner and Johnson, 1972, Ducibella and Anderson, 1975) whereas ICM cells are highly adhesive, compact readily on each other and, when aggregated to a morula, move to its center (Johnson and Ziomek, 1981). The embryo forms blastocoelic cavities at day 8. At this stage, the cells differentiate into ICM, destined to become the fetus, surrounded by TE cells, destined to become the placental tissue. Around day 9 and 10, expanded blastocysts hatch from the zona pellucida and the hatched blastocysts undergo further expansion before they start to elongate at day 13 (Morris *et al.,* 2001).

Cleavage rate is normally assessed 72 hours post fertilization and cells are analyzed for the number of divisions present. Blastocyst rate, on the other hand, is analyzed on day 7, 8 and 10 post-culture. Blastocysts are subdivided as early blastocyst, the smallest in size with the blastocoelic cavity are just beginning to form. The blastocyst is the next stage which is characterized by a clearly defined blastocoelic cavity and inner cell mass. The expanded blastocyst is larger than the blastocyst and signifies the stage prior to hatching of the zona pellucida. The hatched blastocyst is the final stage and is characterized by the presence of an embryo that has hatched for its zona pellucida.

3.2.5 **Factors that Influence Oocyte Development**

3.2.5.1 Follicle size

It is already known, from studies with *Bos taurus* breeds, that follicle size is associated with oocyte developmental competence, which is usually higher in oocytes from follicles greater than 8 mm in diameter (Hendriksen *et al.,* 2000, Lequarre *et al.,* 2005). Nevertheless, the relationship between oocyte competence and follicle size in Zebu breeds, like the Boran, whose follicles are known to be naturally smaller, is not yet known. One may expect to find oocytes with higher competence in follicles smaller than 8 mm in diameter. Zebu breeds are also more sensitive to external hormones used for superovulation than European ones while their oocytes seem to be more resistant to heat stress (Camargo *et al.,* 2007). However, for Boran breed no information has been published about follicular dynamics, follicle sizes, hormonal treatments as well as oocyte competence.

Oocytes grow inside the primordial follicles through a process that lasts about six months in cattle. During this period, the oocyte acquires the competence to undergo meiotic maturation through a process involving interactions between the oocytes, the theca and granulosa cells (Miyano, 2003). Growing evidence suggest that oocyte competence is dependent upon storage of gene products (messenger RNA or protein) that support early stages of embryo development before full activation of embryonic genome. It appears that oocytes also require additional pre-

maturation to express their complete competence (Hendriksen *et al.,* 2000). *In vivo*, this pre maturation occurs during pre ovulatory growth before the luteinizing hormone (LH) surge, while for *in vitro* embryos this process is critically influenced by ovarian morphology, number and size of the follicles present in the ovary at the time of aspiration and composition of the follicular fluid (Madison *et al.,* 1992, Hazeleger *et al.,* 1995, Lonergan *et al.,* 2003).

The oocyte quality is, therefore, related to its follicular environment. The size and the quality of the follicle of origin (Hazeleger *et al.,* 1995) influence the developmental capacity of bovine oocytes. In cattle differences exist between breeds on how the size of the follicle influences oocyte competence and oocyte grade, which also determines embryonic development and viability (Hazeleger *et al.,* 1995).For the breeds studied so far, oocyte competence increases as the follicle enlarges (Lonergan *et al.,* 1994) and greater rates of embryonic development are obtained by using oocytes aspirated from follicles greater than 2-3 mm in diameter (Yang *et al.,* 1998). Oocyte competence is higher in follicles greater than 8 mm in diameter (Hendriksen *et al.,* 2000). For the Boran this information is not available and studies were needed to establish how the follicular size influences COC grade and oocytes quality. Such information is useful in making vital decisions during ovum pick sessions on developmental competence of the oocytes recovered from different sized follicles.

3.2.5.2 Oocyte Morphology and Grade

The developmental competence of the oocyte is also highly dependent upon COC morphology and grade (Blondin and Sirard, 1995; Gandolfi *et al.,* 1997). This demonstrates the importance of the number and quality of cumulus cells surrounding the oocyte in this process.Morphological appearance is a criterion routinely used to select the cumulus oocyte complexes (COC) for developmental potential (Yotsushima *et al.,* 2007). Selection of oocytes for *in vitro* maturation is generally based on the morphology which is refers to the quality of the cytoplasm and the characteristic of the cumulus cell invests around the oocyte (De Loos *et al.,* 1989).

COC's are classified into four grade categories depending on their morphological appearance (de Loos *et al.,* 1989; Tamasia et *al.,* 2003); grade A corresponds to intact immature COCs with three or more layers of dense cumulus cells and homogeneous cytoplasm. Grade B COCs have fewer layers of compact cumulus investment with homogeneous cytoplasm, grade C is a partially denuded oocyte that has lost some of its cumulus cells but sustains a homogeneous cytoplasm or oocyte with some minor irregularities in the cytoplasm and grade D consists of degenerated oocytes as well as oocytes with irregular cytoplasm presenting dark areas. Oocytes with fragmented cytoplasm and cracked zona pellucida as well as COCs with abnormal or expanded cumulus investments are also classified as grade D.

The absence of reliable markers for the identification of viable embryos for transfer at the early cleavage stage contributes to low implantation rates in IVEP setups (Fenwick *et al.,* 2002). To

improve the selection of the embryo with the highest implantation potential, Van Montfoort et *al.,* (2004) suggested that selection of embryos for transfer should not be based on cell number and morphology on the day of transfer alone but also on early cleavage status.To improve on early cleavage status, it is essential to select COCs of only grade A or B for IVM and also to screen for markers indicating oocytes developmental competence (Carmago *et al.,* 2006).However, COC grading has neither been done for the Boran breed nor any studies conducted to establish the association of the oocyte grade and *in vitro* embryo development and viability for this breed. Therefore this study aimed to correlate COC morphology with grade and effect of morphology and grade on cleavage and blastocyst yield for the Boran cows in order to optimize on its IVEP outputs.

3.2.5.3 Developmental Competence

Early cleavage, which is highly correlated to developmental competence of oocytes, is indicative of increased developmental potential in embryos and may be useful as a criterion in the selection of oocytes for ET (Alm *et al.,* 2005).

Low level of efficiency achieved using *in vitro* embryo production is almost related to the quality of the oocyte at the beginning of maturation (Alm *et al.,* 2005). Immature oocytes, especially those from cows with reduced reproductive performance or which are slaughtered at old age, are heterogeneous in quality and developmental competence (Gordon, 2003).Although

morphological grading criteria provide reasonable means of identifying maturity and fertilization potential (Madison, *et al.,* 1992), results reported by De Loos *et al.,* (1992) suggest that there is considerable morphological variability among oocytes capable of normal development. Thus, morphological grading criterion provides only a limited means of identifying oocytes that will develop *in vitro*.

Brilliant Cresyl Blue (BCB) staining is easy and a vital method to evaluate glucose 6-phosphate dehydrogenase (G6PDH) activity in oocytes (Alm *et al.,* 2005). BCB staining is non invasive and does not interfere with the ability of the oocytes to develop.

Immature oocytes are known to synthesize a variety of proteins (Wassarman 1988), such as glucose-6-phosphate dehydrogenase (G6PDH) enzyme. This enzyme is active in growing oocytes (Mangia and Epstein, 1975) and its activity is decreased in oocytes that have finished their growth phase (Wassarman, 1988). G6PDH can convert Brilliant Cresyl Blue stain (BCB) from its blue colour into a colourless state (Ericsson *et al.,* 1993). This test is used to determine G6PDH activity in the tested oocytes. G6PDH is synthesized within oocytes during oogenesis as a component of the pentose phosphate cycle that provides ribose phosphate for nucleotide synthesis, and NADPH is utilized as a hydrogen or electron donor in reductive biosynthetic reactions such as the formation of fatty acids (Alm *et al.,* 2005).

A BCB colour change represents high G6PDH activity which is a sign of incompetence (immaturity) in oocytes tested (Ferrandi *et al.,* 2002). BCB stain can be used to select oocytes with a higher developmental competence in cows, with the BCB- oocytes (colorless cytoplasm, high G6PDH) being of lower developmental competence than BCB + oocyte (blue cytoplasm, lower G6PDH activity) (Ericsson *et al.,* 1993). This study utilized BCB test to evaluate competence of Boran oocytes.

CHAPTER 4

4 Materials and Methods

4.1 Summary of Activities

Data for this study was collected between January and August 2009. Ovaries were collected from S.M Thiani slaughter house in Dagoreti, Kikuyu. Samples were collected two days in a week on Mondays and Wednesdays between 6.30 AM and 9.00 AM to coincide with the peak slaughtering period. The time between collection and transportation to the laboratory was maintained below 4 hours.The activities carried out in this study included: pre-slaughter assessment of the donor cow, ovary collection and transportation from the abattoir to the lab, ovary and follicle assessment, oocyte aspirations, COC assessment and grading, Brilliant Cresyl blue (BCB) test for competence of oocytes, oocyte maturation, *in vitro* fertilization and embryo culture.

4.2 Donor Cow Assessment

Ovaries were collected from Boran cows at slaughter. Pre-slaughter parameters were evaluated during ante-mortem examination and recorded in a designed data collection form (Appendix 3)

Basic data on details of breed, body condition score, age and pregnancy status of the cow were

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obtained and recorded. The breed was ascertained phenotypically (Ojango et *al.*,, 2006). The Boran cattle are usually white or grey fawn. But light brown and black or dark brown shading on the head, neck and shoulder and hindquarter also occur. The hooves and muzzle are always black and any light colouration in these parts is indicative of presence of foreign blood, usually of European taurine type. They have a well-developed dewlap and preputial sheath; they have small ears, which are occasionally pendulous and are greater in males than in females. They have deep chest, slightly slopping rump and well-developed hindquarters. The body condition score (BCS) was based on the 5 point score with 1 being thin and 5 being fat (van Niekerk and Louw. 1980). The age of the cow was determined and recorded as heifer, adult or old as conditions in the abattoir did not allow for accurate determination of age.

4.3 Ovary Collection and Transportation

The ovaries were harvested in a hygienic manner using a clean knife 5- 10 minutes after stunning of the cow. They were then placed in a thermos flask containing Dulbecco's phosphate buffered saline (Dulbeccos's PBS) that was supplemented with 1ml/L of Gentamycin (Dulbecco's PBS+). Dulbecco's PBS was pre-heated by leaving it in a sterile bottle overnight in an incubator set at 39^{o}C. Ovaries were brought to the laboratory within 3-4 hrs of collection.

4.4 Ovary and Follicle Assessment and Measurement

At the laboratory, ovaries were washed twice using Dulbecco's PBS+ and dried using a paper

towel. Each ovary was examined for visible follicles and the ovaries without visible follicles were discarded.The number of follicles on the ovary was counted and their sizes determined. Size determination was achieved by dissection of the follicles from the ovary and measurement of the diameters done with the aid of a calibrated piece of string as shown in Figure 1. Briefly, a ruler was used to roughly estimate the size from the surface. Then a scalpel blade was used to dissect and isolate the individual follicles, which were then placed in a Petri dish containing warmed Tyrodes-Albumin-Lactose-Pyruvate (TALP) Hepes media (Parrish *et al.,* 1986). Each of the isolated follicles was picked up and its diameter determined using a string. The measured follicle was then placed into another Petri dish containing TALP- Hepes. The length of the string covering the diameter of the follicle was then determined by measuring the string against a calibrated ruler.

The follicles were then grouped into 3 categories: - Group 1: follicle size of diameters between 1 – 3 mm, Group 2: follicles of between >3-6 mm in diameter and Group 3: follicles of diameter length greater than 6 mm. From each group, oocytes were aspirated, graded, number oocytes falling in each grade counted and recorded

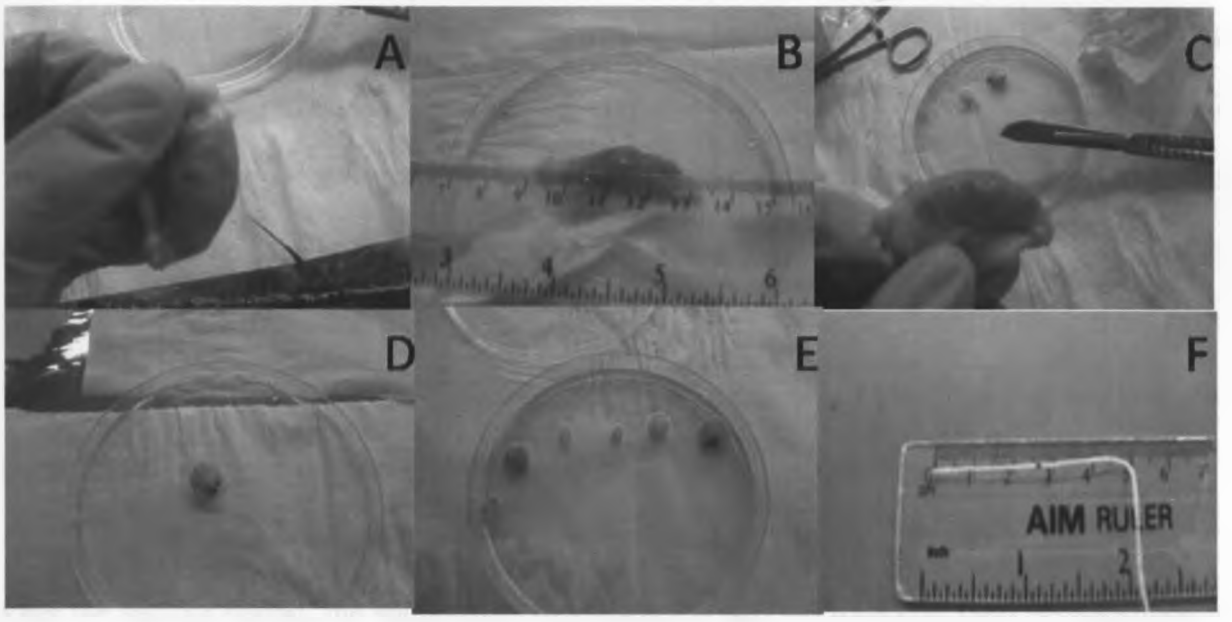

Plate 1: *Determination of follicle size; assessment of visible follicles (A),; Rough measurement of visible follicle's (B), isolation of the follicle using a scalpel blade (C) , Isolated follicle in a Petri dish being measured with a thread (D), isolated follicles in a Petri dish containing TALP Hepes (E), Measurement of the string against a ruler (F).*

4.5 Aspiration, Assessment and Grading of COC'S

The follicular fluid containing the COC's was collected by puncturing the follicles using a sterilized 18G ×1½ needle (1.20x40mm). The needle was attached to a 20ml plastic disposable syringe with a plastic plunger containing TALP –Hepes media.The follicular fluid from different follicle group sizes was collected separately into sterilized centrifuge tubes warmed to 38.5°C and then left to stand in a water bath at 38.5°C for the COC's to settle down.

The settling down period varied for different follicle group sizes. Follicular fluid containing group 3 COC was left to stand in the water bath for 10-15 minutes while those containing group 2 COC'S stood in the water bath for 30 minutes. Follicular fluid containing group 1 COC'S was left to stand for up to 1 hour. This variation was purely logistical because selection of COC's from the aspirated follicular fluid begun with group 3 as follicular fluids arising from large follicles gels with time making selection of COC's a difficult task Again, this task was done for group 3, then group 2 and lastly group1 follicle COC's.

After the settling period, the supernatant was discarded and the follicular fluid re-suspended in TALP Hepes media. Subsequently the fluid containing the COC's was emptied into Sterilin plates (Beckton Dickson USA) for selection of COC's using a D1000 Gilson pipette fitted with a 1000µl tip. Using D20 Gilson pipette fitted with 10 µl tips, the COC's were then individually picked up from the follicular fluid under a stereo scopic microscope into another Sterilin plate containing the TALP-Hepes media. The COC's were then selected, graded and grouped. Grading was performed according to the methods of De Loos *et al.,* (1989) with modification from Tamasia et *al.,* (2003). In summary grade A COC's corresponds to intact immature COCs with three or more layers of dense cumulus cells and homogeneous cytoplasm while Grade B COCs had fewer layers of compact cumulus investment with homogeneous cytoplasm. The discarded COC's were those with a heterogeneous cytoplasm or those with a homogenous cytoplasm but with little or no cumulus cell cover.

4.6 Brilliant Cresyl Blue Staining

Staining with Brilliant Cresyl Blue (BCB) was done for the compact COC's of grade A and B from follicle group sizes 1, 2 and 3. The selected COC's were washed twice in TALP-Hepes media and then put in an ependoff tube containing 500ul of modified Dulbecco's phosphate buffered saline containing 26 mM of BCB stain. The COC's were exposed to BCB stain for 60 minutes at 38.5 ^{o}C in a water bath (Alm et *al.*, 2005).

After the 1hr BCB exposure period, the COC's were transferred to modified Dulbecco's phosphate buffered saline and washed twice before being examined under a stereo microscope. During this examination, COC's were divided into two groups according their cytoplasm coloration and counted. BCB positive oocytes were the ones with any degree of blue cytoplasmic coloration while BCB negative oocytes were those without blue coloration in the cytoplasm. The COC's were then transferred to TALP hepes media and washed twice before being placed into maturation media for *in vitro* maturation procedure.

4.7 *In vitro* Maturation (IVM)

Only Grade A and grade B COC's were used for this procedure. Before IVM the COC's were washed two times with TALP hepes media and once in Tissue culture medium (TCM) that was supplemented with 10% Fetal calf serum (FCS), and 1ul/ml each of Follicle stimulating hormone

(FSH), Luteinizing Hormone (LH) and Estradiol. The COC's were then placed in a sterile maturation Nunc plate containing 400ul of a pre-prepared TCM that was warmed at 38.8°C in an incubator overnight or at least two hours prior to incubation. During IVM, high levels of hygiene and an incubation temperature of 38.8°C at 5% CO_2 were maintained. Oocytes were left in the incubator for 22-24hrs to mature.

4.8 Semen Preparation

One superior bull's from the Central Artificial Insemination Station in Kenya (CAIS JE 684 FAME) semen was selected and used for this study based on successful pilot testing in the IVEP process. One straw of frozen semen was used during the IVF process. The straw was picked, waved in the air for about 10 seconds and thawed in a water bath at 38.5°C for 30 seconds. The straw was then disinfected with 70% alcohol and wiped dry. The ends of the straw were then cut using a pair of sterilised scissors and the semen contents released into an ependoff tube.

A drop of the semen was placed on a pre-warmed glass slide and evaluated microscopically for vigour and motility. Only semen that attained above 50% motility was used. 0.25 ml of the semen was gently released at an angle of 45° at the bottom of the 10ml sterile centrifuge tube containing 1.0 ml of swim up -TALP (SPTL) medium as shown in figure 2. As described by Parrish *et al.*, (1986), the swim up was done in a Carbon Dioxide (CO_2) buffered incubator for 60 minutes at 38.5°C to allow vital sperm to swim up and undergo capacitation. Approximately 0.8 ml of the

supernatant (the SPTL medium with vital sperm), was collected in an eppendorf tube and centrifuged at 10,000 rotations per minute for 5 minutes at room temperature.

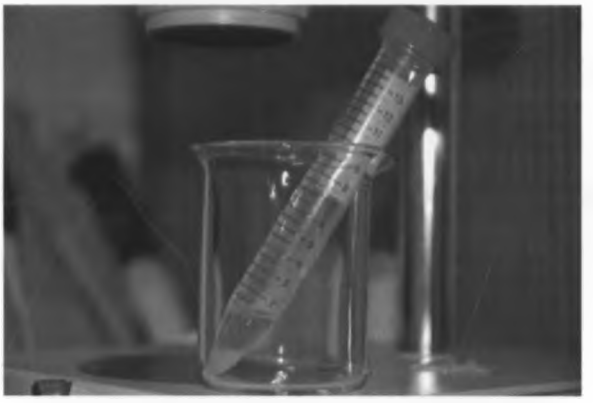

Plate 2: Swim Up technique after layering 0.25ml of semen at the bottom

The supernatant was then discarded and the sperm cell pellet located at the bottom of the tube re-suspended with 100ul of fertilization (Fert-TALP) medium.

Concentration of the sperm was determined by use of a Neuber chamber; 5 µl of the final semen preparation was mixed with 95 µl of water to achieve a 1:20 dilution. This ensured that the sperm was killed for ease of counting under a microscope. A drop was put on both sides of Neuber chamber and the numbers of sperm cells on both fields of the Neuber chamber counted under a microscope. The total number of sperm cells counted was used to determine the spermatozoa concentration in 1 ml of semen.

It is estimated that using a dilution of 1:20 in Neuber chamber concentration estimation, a final concentration of 1 X 10^6 sperm cells per ml can be obtained by using semen volume equivalent to

the volume of IVF droplet divided by the number of counted cells after taking into account the % post-thaw motility. This is in accordance with procedures done by Carmago *et al.*, (2006). Thus;-

$$\text{Volume of semen} = \frac{volume\ of\ IVF\ droplet}{\left[\left(\frac{no\ of\ cells\ counted}{10}\right) X\ \%\ post\ Swim\ up\ motility\right]}$$

The calculated final volume of semen was then drawn and added to a droplet of IVF to be part of the final 100ul IVF droplet.

4.9 *In vitro* Fertilization (IVF)

After 22-24hours of maturation, COC's were examined and judged on the basis of their cumulus expansion. COC's with full expansion of cumulus cells were considered mature and the number of matured COC's were counted and recorded.

The matured oocytes were then washed twice with TALP-hepes media and one time with Fert - TALP medium. 15-30 matured COC's in 20ul volume were then transferred into a prior prepared IVF droplet of Fert-Talp media. The semen concentration was adjusted to contain 1 X 10^6 sperm per ml and the determined volume of semen picked up and added to this droplet. The droplet volume was then topped up to 100ul using Fert- Talp media. The droplets containing mature oocytes and capacitated sperms were then cultured in an incubator at 38.8°C at 5% CO_2 for a period of 18- to 22 hrs.

4.10 *In vitro* Culture, Cleavage and Blastocyst Rate Assessment

After 18-22 hours of incubation, the fertilized zygotes were transferred into a Petri-dish containing TALP-hepes medium. The zygotes were then washed two times in TALP-hepes media and one time in CR2aa culture medium (Moore and Bondoli 1993, Wilkinson, 1996). The zygotes were then co-cultured with their cumulus in 100ul droplets of the semi-defined CR2aa culture medium covered with a film of oil for 72 hrs in an incubator at an atmosphere of 5% CO_2 at 38.8°C and 95% humidity.

After 72hrs of culture, half of the medium (50ul) was drawn out and replaced with a fresh CR2aa culture media. The cleavage was assessed at this point by counting and recording the number of cleaved cells at either 2, 4-7, or >8 cell-stages as shown in figure 3.

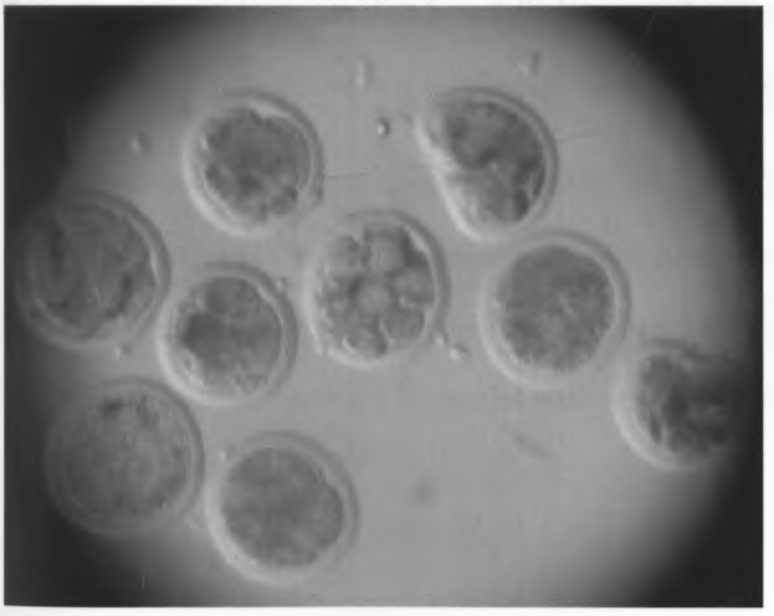

Plate 3: *An in vitro culture droplet showing cleaved zygotes.*

Cleavage rate was calculated as a percentage of the proportion of divided cells from the oocytes placed into the culture medium. Blastocyst formation was assessed at day 7, 8, and 10, post-fertilization, and recorded as either in early blastocyst (EB), blastocyst (B), expanded blastocyst (EXB) or hatched blastocyst (HB) stages. The blastocyst rate was calculated as the proportion of blastocyst formed from the total number of cleaved cells in the culture media.

4.11 Experimental Design

In order to fulfil the objectives of the study three experiments were designed and carried out.

4.11.1 **Experiment 1: To assess the effect of follicle size on the grade of aspirated cumulus oocyte complex in the Boran cow**

This objective sought to establish how follicles were distributed among the various group follicle sizes and how follicle size of origin influenced grade of the aspirated COC.

In this experiment all ovaries were assessed for visible follicles. The follicles were measured, counted and grouped into three categories, according to their respective sizes. Then the COC's falling into the various group categories were aspirated separately from each of the group follicle size. For experiment 1 and 3, 170 ovaries were sampled from 85 Boran cows and a total of 1628 COC's were studied.

4.11.2 **Experiment 2: To investigate the effect of follicle size of origin and COC grade on oocyte developmental competence as determined by BCB staining**

This experiment assessed the effect of follicle size and COC grade on the level of oocyte developmental competence using the Brilliant Cresyl blue stain. In this experiment, 138 ovaries sampled from 69 Boran cows were used. A total of 1030 COC's were used. After 1 hour exposure to BCB staining, all the oocytes from each group were assessed and then sorted out as either BCB positive (competent oocytes) or BCB negative (incompetent oocytes). The numbers of COC's for each outcome were counted and recorded.

4.11.3 **Experiment 3: To investigate the relationship between follicular size and COC morphological grade on in vitro developmental competence as assessed by the rates of maturation, cleavage and blastocyst development in Boran cattle**

This experiment was designed to investigate the relationship, if any, between follicular size and COC morphological grade on actual *in vitro* embryo developmental competence when assessed using rates of *in vitro* maturation, cleavage and blastocyst development.

For this experiment 1355 grades A and B were used. All the oocytes that fell in groups 1, 2 and 3 and were of similar grades were mixed and run through the *in-vitro* embryo production procedures. After the IVM, IVF and IVC procedures the respective number of oocytes matured, zygotes cleaved and blastocyst formed were counted and recorded.

4.12 Data: Description, Collection, Entry and Analysis

The overall minimum sample size (n) for the animals was determined as 384 follicles as determined by the formula:-

$$n = \frac{t^2 \alpha p q}{L^2} = 384$$

Where n= sample size,
t= confidence interval = 1.96,
α = error, p = proportion 0.5,
q =1-p 0.5,
L = precision = 0.05.

Data collected from the abattoir each day was entered into an abattoir collection form and later transferred to a Microsoft Excel sheet. From the abattoir, information of the donor cow body condition score, pregnancy status, age and general condition was noted and recorded. In the laboratory the number of ovaries used and the number of follicles from each group was counted and recorded in an Excel data sheet. For the IVEP process the number of COC in each procedure were recorded in a daily summary sheet (appendix 4) and later transferred to a Microsoft Excel sheet.

Data analysis was done using chi square in SAS ® 9.1(2008) statistical package.

Statistical analysis for differences/similarities between the observed and expected frequencies for three experiments from the 3 group sizes were tested using chi square test a 5% level of

significance.

The equation below was used to calculate the Chi` square value

$$X^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i},$$

Where

X^2 = the test statistic.
O_i = the observed frequencies for follicles within the various follicle group sizes;
E_i = the expected (theoretical) frequencies for follicles within the various follicle group sizes; *
n = the number of outcomes of each event.

For experiment 1 since the expected frequency distribution of follicles had already been determined as 60% for group 1, 30% for group 2 and 10% for group 3(Carolan *et al.,* 1996), and based on the laboratory data any ovary in cow is expected to have an average of 11 follicles. However for the other experiments the expected frequency for the COC's , the BCB competence rate , maturation, cleavage and blastocyst rates were calculated from the formula below:-

$$E_{i,j} = \frac{T_i T_j}{T}$$

Where $E_{i,j}$ is the expected frequency for cell i,j, T_i is the total I th row, T_j is the total for the j^{th} column, and T is the total number of observations.

CHAPTER 5

5 Results and Discussion

5.1 Basic Data

Data was collected on the donor cows and results obtained in the laboratory at various levels.

Table 1 and 2 show a summary of the raw data collected.

Table 1: Summary of data from Experiment 1 and 3; the In Vitro Embryo Production process*

Follicle Size group	Grade	Aspirated	Matured	Cleaved	Blastocyst
1-3 mm	A	393	368	180	21
1-3 mm	B	394	341	160	38
1-3 mm	Discards**	151	-	-	-
>3 -6 mm	A	226	213	121	36
>3 -6 mm	B	196	167	120	42
>3 -6 mm	Discards**	97	-	-	-
>6 mm	A	85	81	71	24
>6 mm	B	61	56	44	7
>6 mm	Discards**	25	-	-	-

*For the IVEP experiments, 11 runs were carried out with each run signifying a different week.
** The discards were removed from the study after the aspiration stage.

Table 2: Summary of data from Experiment 2: Brilliant Cresyl Blue stained Cumulus oocyte complexes

	BCB +		BCB -	
Follicle Size group	Grade A	Grade B	Grade A	Grade B
1-3 mm	181	265	65	100
>3 -6 mm	116	120	20	53
>6 mm	52	45	2	11

*For the BCB experiments 10 runs were done each run representing different week.

5.1.1 **Donor Cows**

The body condition scores (BCS) of the sampled animals ranged from 1 to 5. Seventy four percent (120/163) of the cows had BCS of 1.5 – 2.5 as shown in Figure 4. Forty two percent of the cows from which oocytes were collected were pregnant at different stages of gestation.

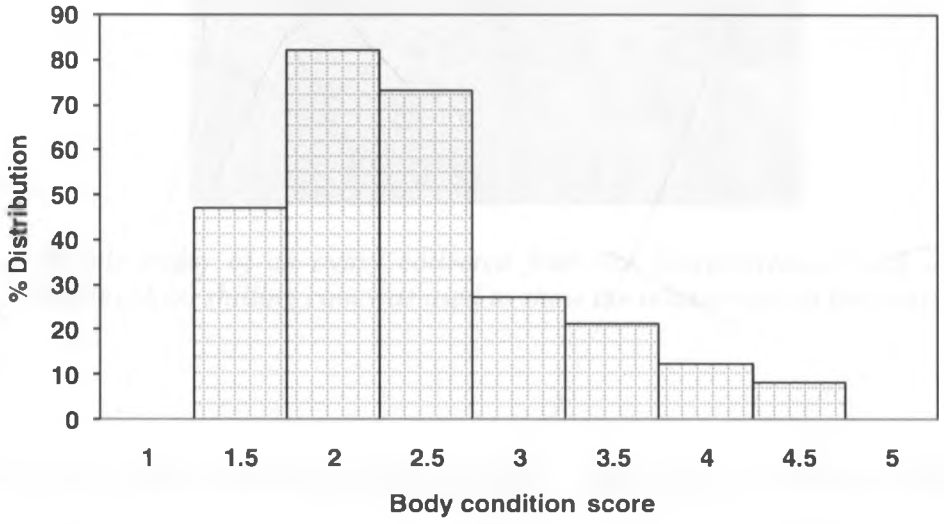

Figure 1: Percentage distribution of body condition scores of the sampled animals

5.1.2 **Ovaries**

A total of 163 cows were sampled from the abattoirs and 326 ovaries were collected from them. Only 308 ovaries contained follicles and thus used for the study. Thus, 94.5% (308/326) of the ovaries sampled were active (had follicles in them). Some ovaries were discarded on the basis of absence of follicles and presence of cysts.

As shown in Figure 5, any sampled ovary contained groups of different sized follicles.

Plate4: *A sample ovary of an ovary collected from the slaughterhouse with different sized follicles. A 20 shilling coin was used to show the relative size of the ovary*

The average size of any sampled ovary was 1.6cm by 1.9cm by 0.9; the smallest measured 0.8cm by 1.2 cm by 0.4 while the largest was 2.5 by 3.8 by 1.6 cm in size.

5.1.3 **Discussion of the Basic Data**

An average BCS of between 1.5 and 2.5 for the slaughtered Boran cows was reported in this study. Some authors recommend ovaries be collected from donor cows of superior body condition score than 2.0 (Dunne *et al.* 1999). This is not disputable but results of this study demonstrate that Boran cows with a body condition of 1.5 – 4.5 were able to produce competent COC's and viable embryos.

It has been demonstrated in ovum pick up in *Bos taurus* that heifer cows fed on low or high energy diets showed differences in numbers of follicles and COC's aspirated, and cleavage and blastocyst rates obtained (Nolan *et al.*, 1998). Also, heifers fed on hay for a short period of time yielded more fertilized oocytes than heifers fed on silage although no difference was observed between the two groups for *in vitro* blastocyst production (Yaakub *et al.*, 1999). In order to produce optimal results in IVEP for the Boran breed, studies are necessary to shed more light on influence of nutrients and BCS of the cow on its embryo production *in vitro*.

All ovaries collected were put together in a common thermos flask and also COC's aspirated from similar group follicle size and grades were mixed together. Since the focus of this study was on effects of follicle size and grades of the COC's on *in vitro* embryo competence, there was no need to match oocytes aspirated to a particular donor cow. Thus the results of study can not be used to comment on effect of BCS of the cow on the grade of COC and or number of oocytes aspirated. However it is recommended that further investigation to elucidate on this important issue for the Boran breed.

The development of normal embryos with the capacity to develop into viable off-springs to term requires contribution from both the oocyte and spermatozoa. Both parental components influence the characteristics of the newly formed embryo; however, the cytoplasm of the early embryo is composed almost entirely of the cytoplasm from the oocyte (Tamasia *et al.,* 2003). As an important female contribution to embryonic development, the ooplasm contains elements

necessary for early embryonic development and an intrinsic cytoplasmic program that regulates early embryonic development. The female contribution from the donor cow is therefore of utmost importance and careful selection is required when recruiting donor animals. Donor cow selection should consider their nutritional status.

5.2 Experiment 1: Assessment of how the size of follicle influenced the grade of aspirated cumulus oocyte complex in the Boran cow

This experiment tested the distribution of follicles from different sizes and to asses the differences/similarities in numbers of follicles appearing in the different group follicle sizes and also to test if there was any association between the follicle size and the grade of the COCs aspirated. Table 3 shows the distribution of follicles as counted within follicle group sizes; group 1 (measured 1-3 mm), group 2 (3-6 mm) and group 3 (>6 mm) in diameter.

Table 3: Percentage distribution of follicles obtained from the various follicle group sizes

Follicle Size	*Number of follicles*	*Percent%*
1-3 mm	1132	58.7
>3 -6 mm	575	29.8
>6 mm	222	11.5

Proportionately, follicles of group 1 were more than group 2 and 3 while group 3 had the least number of follicles. However from the above table, the calculated χ^2 value was $P > 0.05$ indicating no association between the follicle size and the COC's. Thus the null hypothesis is accepted. More follicles were counted from group of 1 -3 mm diameter size, followed by group of 3-6 mm diameter size and least from group of over 6 mm diameter sizes. This observation was the same for the number of COC's counted.

Table 4 shows the frequency distribution of COC's grades within the various follicle group sizes. More COC's for each of grade A and B were obtained from group 1 follicles (1-3 mm in diameter), followed by group 2 follicles while group 3 follicles had the least number.

Table 4: Percentage frequency distribution for the aspirated Cumulus oocyte complex (COC'S) grades obtained from 3 different follicle size groups

	COC'S (%)		
Follicle Size group	**Grade A**	**Grade B**	**Discards**
1-3 mm	24.14	24.2	9.28
>3 -6 mm	13.88	12.04	5.96
>6 mm	5.22	3.75	1.54

A $\chi 2$ value of 6.312 and a probability of 0.1770 was reported thus the null hypothesis was accepted. This signifies that there is no relationship between the follicle size and the grade of

COC aspirated. This observation corresponds with previous studies carried out by Lonegran (1994), where in the *Bos taurus* cows, although more grade A COC from follicles larger than 6 mm in diameter were aspirated as compared to those measuring 2-6 mm in diameter, no significant differences were observed for grade A and B COC's. Carolan (1996) also showed a follicle distribution of 59%, 25%, and 16% from follicles that were less than 6 mm, between 6 and 8mm, and greater than 8 mm in diameter, respectively. This is in agreement with the results obtained in this study.

No previous studies have been done for Boran cows on this aspect. The differences in numbers reported here were expected because small-sized follicles appear as cohorts of higher numbers during recruitment or selection phases of folliculogenesis. By the time the follicle is over 6 mm in diameter, it has entered the dominant phase of folliculogenesis while many other smaller follicles would have undergone atresia, making the numbers of follicles >6 mm in diameters to be significantly lower. More COC's were from group1 follicles than for group 2 and also for group 3 follicles. This was expected because each follicle contains one COC. Thus, the pattern for distribution of the number of follicles and COC's would be similar. However, some of the COC's would be naturally lost during aspiration processes. At any one given time, ovarian folliculogenesis in a cow can be at recruitment, selection or dominance phases of development (Binelli *et al.,* 2006, Viana and Carmago, 2007). It is known that different breeds of cattle exhibit variability in follicular dynamics, particularly on the number of follicular waves and follicles for

each wave (Mermillod *et al.,* 1999, Martinez *et al.,* 2004; Moore and Thatcher, 2006). For the Boran, it was not possible within this study to elucidate on number of follicular waves but it was clear that follicles aspirated were at different phases of development.

This study reveals that the grade of the aspirated COC's did not significantly depend on the size of follicle from which it originated ($P > 0.05$). Although both grade A and B COC's can be obtained from the three groups of follicles, the larger the follicle size the more grade A COC were aspirated. Lonegran (1994) reported that in *Bos taurus* cows significantly more COC's with 4 or 5 layers of cumulus cells (grade A) were obtained from the follicles of 2-6 mm diameter sizes. This study shows that in the Boran cow's follicles as small as 1mm in diameter can produce both grade A and B COC's. All the group sizes can harbor active follicles capable of yielding both grade A and B COCs. It has been shown previously for other breeds that grade A and B COC's are similarly good in all *in vitro* attributes (Rocha *et al.,*, 1998; Wiltbank *et al.,*, 2002; Senger, 2003; Martinez *et al.,*, 2004; Moore and Thatcher, 2006).

The efficiency in the recovery of good grade of COC from follicles has been shown to highly influence the output of *in vitro* production system (Boland and Roche, 1993). The number of good grade of COC's aspirated from an ovary of a cow can be influenced amongst other factors by influence of age of the animals used as donors (Driancourt and Avdi, 1993), plane of nutrition

of donor (Abecia *et al.,* 1997, O'Callaghan et *al.,* 2000), reproductive status of donor (season, postpartum) (Boland and Roche 1978, Driancourt and Avdi, 1993, Mitchell *et al.,* 2002, Gonzalez-Bulnes *et al.,* 2003) and the technique of the aspirating personnel (Carmago *et al.,* 2006). COC's of grade C is usually of low quality and mostly not used for *in vitro* work. As for other previous authors, this study demonstrates that the numbers of such grade were lower than for grade A and B COC's (Boland and Rochae 1993; Carmago *et al.,* 2006).

Like in other Zebu breeds (Viana *et al.,* 2000), the Boran cow had smaller ovaries with an average of 1.6 cm by 1.9 cm by 0.9 when compared to *Bos taurus* breeds (Hendriksen *et al.,* 2000, Lequarre *et al.,* 2005). From this study the small ovaries of the Boran cow seems to be very active and also harbouring good COC's. However, for this breed more studies are needed to provide further information on follicular dynamics in relation to follicle size as well as oocytes.

5.3 Experiment 2: Effect of Follicle size and COC grade on oocyte developmental competence as assessed by BCB staining

This experiment tested if the oocyte developmental competence was influenced by the follicle size and grade of COC, when assessed using BCB staining. This criterion assesses oocytes competence by observing the coloration of oocytes after 1 hr exposure to BCB staining. The extent of BCB stain coloration is a measure of the Glucose 6 Phospahte dehydrogenase levels in the oocytes and thus the level of cytoplamic/ metabolic maturation of the oocyte. The BCB

positive oocytes were judged as competent while BCB negative oocytes were judged as not competent.

As shown in Table 5a, more competent COC's were obtained from group 3 follicles (88.33 % of them were BCB positive). On the other hand groups 1 and 2 follicles had oocytes of competence rates of 73.09 and 77.33 respectively. Table 5b gives the frequency distribution rate of the competent COC's. Figure 6 shows the comparative competence rate from the 3 follicles size groups.

Table 5a: Mean Cumulus Oocyte Competence as evaluated by the Brilliant Cresyl Blue (BCB) stain

Follicle size	BCB competence (%)		
	GRADE A	GRADE B	Group Rate
1-3 mm	73.58	72.60	73.09
>3-6 mm	85.29	69.36	77.33
> 6 mm	96.30	80.36	88.33

Table 5b: Frequency distribution of Cumulus Oocyte Competence as evaluated by the Brilliant Cresyl Blue (BCB) stain

	% frequency	
Follicle Size group	Grade A	Grade B
1-3 mm	15.38	15.17
>3 -6 mm	17.97	14.62
>6 mm	20.07	16.79

A χ^2 value of 8.178 and a probability of 0.0168 were calculated thus the null hypothesis was rejected. Overall competence increased with increase in follicle size as shown by the meqan

competence rate in table 5a. The analysis shows that there was a relationship between the competence rate and the follicle size and grade. Both grade A and B COC's from follicle size 1 contributed the highest to the overall chi square (2.5941 and 2.9744 respectively). This suggests that the effect of the follicle size and grade begins to occur at this level.

BCB staining is an initial method of determining competence of oocytes involved in *in vitro* embryo production. This method is non-invasive and does not interfere with the ability of the oocytes to develop *in vitro* (Alm *et al.,* 2005).This study shows that competence of the Boran COCs significantly improved as the diameter of follicle size increased. In *Bos taurus* it was demonstrated that COC competence was higher in oocytes originating from follicles greater than 6-8 mm in diameter (Hendriksen *et al.,* 2000, Lequarre *et al.,* 2005). In the Boran this was different because this study has demonstrated that one can find oocytes with high competence from follicles smaller than 8 mm in diameter. This can be explained by the fact that some physiological reproductive parameters differ among cattle breeds, including differences in oocyte quality. For instance, Brazilian zebu cattle have been shown to have three or four follicular waves with smaller diameter follicles (Viana *et al.,* 2000) when compared to European breeds, which have two or three follicular waves of bigger diameter follicles (Boland and Roche, 1993). It is also an established fact with European breeds that follicle size is associated with oocyte developmental competence (Lequarre *et al.,* 2005) and therefore it was a normal expectation to find significant differences in BCB staining for COC's obtained from different groups of follicle for the Boran cows. Nonetheless, this is an important finding for this breed, whose ovaries and

follicles were observed to be small. However, this study agrees with others in that much as one can obtain morphologically good quality COC's even in small follicles 1-3 mm, larger follicles (> 6 mm) are the ones likely to have better competence as evidenced by the BCB staining.

5.4 Experiment 3: Effect of follicle size and COC grade on maturation, cleavage and blastocyst rate

This experiment was designed to test the actual developmental competence of the COC's during *in vitro* processes of embryo production. Competence for this purpose was assessed at different stages of oocytes and embryo development and this was judged according to the rates of success or failure of further development during maturation, cleavage and blastocyst stages of *in vitro* procedures. Successful rates of development were used as indicators of COC competence while failure rates of development indicated COC's were not competent.Table 6a shows the mean rates of maturation, cleavage and blastocyst development. This table shows that COC's from follicles larger than 3 mm progressed better than those less than 3 mm. On average the maturation rate was 90.95 %,(1226/1355) the cleavage rate was 60.48% (696/1226) and the blastocyst rate was 27.55% (168/698) for the COC's tested. Table 6b shows the frequency distribution for the rate of development for COC's obtained from the various follicle group sizes during maturation, cleavage and blastocyst stages.This table shows the COC are distributed at each level of evaluation. While there are minimal differences in the maturation rate, differences begin to appear with the cleavage rate and are more pronounced in the blastocyst stage. While Figure 7 shows the comparative rate of development for the 3 different group sizes. This graph also shows

the progressive development from maturation to blastocyst stage and how these differed with different group sizes.

Table 6a: Mean in-vitro developmental rates for the cumulus Ooocyte Complex within various follicle group sizes during maturation (MR), cleavage (CR) and blastocyst (BR) development

Follicle Size	Grade	Mean rate of development %		
		MR	CR	BR
1-3 mm	A	93.75	51.87	12.07
1-3 mm	B	87.16	45.29	23.96
>3 -6 mm	A	94.16	55.88	29.78
>3 -6 mm	B	85.02	67.14	40.95
>6 mm	A	94.53	84.87	41.25
>6 mm	B	91.37	76.21	19.91

Table 6b: Frequency distribution for in-vitro developmental rates for the cumulus Ooocyte Complex within various follicle group sizes during maturation (MR), cleavage (CR) and blastocyst (BR) development

COC grade within the Follicle Size		% frequency		
		MR	CR	BR
1-3 mm	A	17.17	13.60	7.19
1-3 mm	B	15.96	11.88	14.27
>3-6 mm	A	17.25	14.66	17.74
>3-6 mm	B	15.57	17.61	24.38
>6 mm	A	17.31	22.26	24.56
>6 mm	B	16.73	19.99	11.86
$\chi 2$		ns	P<0.0001	P<0.0001

ns – Not significant

Table 6c shows the results of the chi square analysis of the relationship between the follicle size and grade on maturation, cleavage and blastocyst rates.

Table 6c: Results of the Chi square analysis for In vitro developmental rates for the cumulus Oocyte Complex within various follicle group sizes during maturation (MR), cleavage (CR) and blastocyst (BR) development

Variable	Degrees of freedom	$\chi 2$	P
Maturation Rate	2	1.174	0.556
Cleavage rate	2	20.898	<0.0001
Blastocyst rate	2	144.24	<0.0001

For the maturation rate there was no relationship between the follicle size and the rate of maturation. However, both the cleavage and blastocyst rate showed a strong relationship between the follicle size and both cleavage and blastocyst rates. For both rates follicle size of above 3 mm contributed highly to the overall chi square. This may be attributed to increased competence.

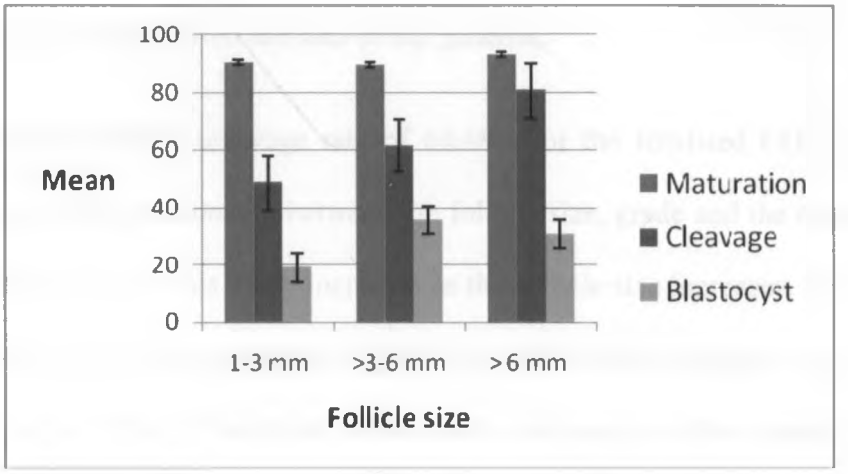

Figure 2: *Mean rate of development for COC's from three different follicle sizes in the Boran cow*

The outputs in an IVEP system are determined by various intrinsic factors, including, among others the effect of breed (Bindon *et al.,* 1986, Nuti *et al.,* 1987, Dufour *et al.,* 2000), influence of age of the animals used as donors (Torres *et al.,* 1987, Driancourt and Avdi, 1993), plane of nutrition of donors (Abecia *et al.,* 1997, O'Callaghan *et al.,* 2000) and reproductive status of donors (season, postpartum) (Boland *et al.,* 1978, Driancourt and Avdi, 1993, Mitchell *et al.,* 2002, Gonzalez-Bulnes *et al.,* 2003).

With oocytes maturation rate of over 80%, the Boran cow has a good potential for a successful IVEP system. Again, although the quality of ovaries collected from the slaughterhouse seemed low (majority of the ovaries being derived from animals in poor BCS and of uncertain genotypes), the study provides promising basic information to allow for accurate aspiration of oocytes from live cows during ovum pick up (OPU) to provide good oocyte numbers to be

matured per donor per session from animals of top genetics.

This study reports an average cleavage rate of 60.48% for the fertilized COCs for the Boran cows. There was a strong relationship between the follicle size, grade and the cleavage rate ($P < 0.0001$). The cleavage rate in this study increases as the follicle size increases. The cleavage rate obtained for Boran cows was within ranges reported for other breeds (Bindon *et al.,* 1986, Nuti *et al.,* 1987, Dufour *et al.,* 2000). Therefore, as previously indicated for other breeds (Dufour *et al.,* 2000, Merton *et al.,* 2003, Carmago *et al.,* 2006), these results suggest that both grades A and B COCs from Boran cows can be selected for use in an *in vitro* fertilization (IVF).

The output of the cleavage is highly dependent on fertilizing capabilities of the semen utilized (Camargo *et al.,* 2002; Ward *et al.,* 2002). To obtain such functional spermatozoon it is necessary to separate the motile from the dead ones as well as from diluents used to freeze semen. This can be performed by different methods, such as swim up and Percoll gradient (density gradient). Some studies have shown better results with Percoll (Somfai *et al.,* 2002; Cesari *et al.,* 2006) while others did not find any difference (Dode *et al.,* 2002). Recent studies in cattle showed higher cleavage and blastocyst rate with swim up method (Polisseni *et al.,* 2007). The results of cleavage reported for this study were not influenced by the method of semen processing because the study used pretested bull semen already shown to be good in IVEP and the swim up method was applied all throughout the study to obtain vital sperms. Semen from one jersey bull was used

to rule out to bull effect. This semen had been previously pre-tested for success in the IVEP system.

Cleavage rate can also be affected by sperm concentration of the semen used during IVF. High sperm concentration can increase polyspermy rate without increasing blastocyst rate (Camargo *et al.,* 2002; Ward *et al.,* 2002). A previous study found that different bulls may need different sperm concentrations (from 0.5 to 4.5 x 10^6/ml) for *in vitro* fertilization (Lu and Seidel, 2004) but a concentration of 1x10^6 sperms/ ml was used for this study.

An average blastocyst rate of 27.55% was reported for Boran cow in this study. There was a strong relationship between the follicle size, grade and the blastocyst rate ($P < 0.0001$). Following the trend from maturation rate to blastocyst rate, there was a progressive increase as the size of the follicle increases. Most IVEP setups for other breeds have reported blastocyst rates of between 20-40% (Bindon *et al.,* 1986, Nuti *et al.,* 1987, Dufour *et al.,* 2000, Carmago *et al.,* 2006). These results suggest that the Boran cow has similar blastocyst rate like other Zebu breeds. The COCs aspirated from group 3 follicles were significantly more competent than those in group 1 and 2. Thus it was not unexpected finding that they developed into blastocysts at higher rates than those from group 1 and 2. *In vitro* developmental competence is species-specific and it is closely correlated with oocyte size, which in turn is correlated with follicle size and the size of the antral follicle at which the oocyte acquires meiotic competence (Mayes 2002). Cleavage and blastocyst rates increase in parallel with meiotic competence and significantly higher

developmental rates have been obtained when the diameter of fertilized oocytes is greater than 120 μm (Hazeleger *et al.,* 1995).

Some of IVEP output differences for various research groups may be explained by the environment where the donors are and whether they are adapted to it. The results obtained by this study suggest that it is possible to produce F1 embryos using *Bos taurus X* Boran (*Bos indicus*) breed with acceptable success rates in IVEP systems. The Boran, being a better adapted breed to tropical environment is likely to give better results on F1 embryo production.

5.4.1 **Progressive evaluation of Blastocysts Development**

This attribute was used to test if the follicle size influenced the progress of blastocyst development. Blastocyst examination at day 7 of development were either in stages of early blastocyst (EB), Blastocyst (B), expanded blastocyst (EXB) or Hatched blastocyst (HB) (Figure. 8). Depending on the numbers for the various categories observed at day 7 of development, the rates of their appearances were calculated for evaluations.

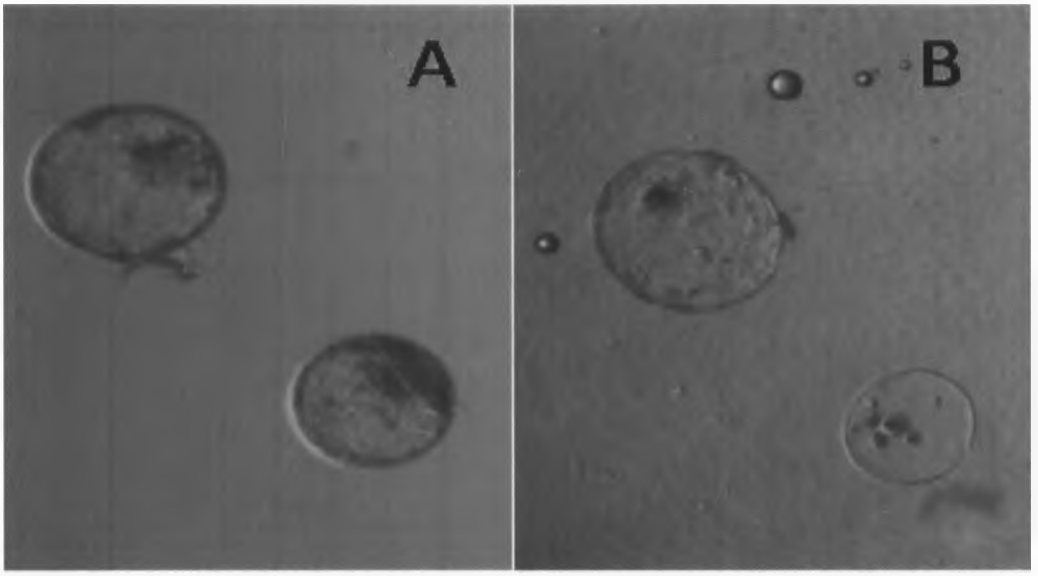

Plate 5: *Different stages of a blastocyst: A- Blastocyst (top) Early Blastocyst (bottom) – B: Hatched blastocyst (top) its zona Pellucida (bottom)*

Table 7 shows rate of development for various types of blastocysts. Blastocyst from 3-6 mm group developed to blastocyst and expanded blastocyst faster than the 1-3 mm group. Group 3A oocytes developed into blastocysts at a faster rate when compared to other group oocytes. Group 1 had no hatched blastocyst at day 7 for both grade A and B.

On day 7, which is the ideal day for transfer, oocytes that were from follicles larger than 6 mm had a faster rate of development than from the other two groups. Blastocyst from this group developed to blastocyst and expanded blastocyst faster than the other two groups. Group one had no hatched blastocyst at day 7.

Table 7: Rate of development for various stages of blastocyst at day 7

Follicle Size	COC grade within the	Embryo development (%)			
		EB	B	EXB	HB
1-3 mm	A	56.25	25.00	18.75	0.00
1-3 mm	B	67.86	14.29	17.86	0.00
>3-6 mm	A	26.47	47.06	26.47	0.00
>3-6 mm	B	25.00	50.00	17.86	7.14
>6 mm	A	0.00	42.86	50.00	7.14
>6 mm	B	66.66	16.16	0	16.16

EB early blastocyst
B blastocyst
EXB expanded blastocyst
HB hatched blastocyst

Blastocyst from 3-6 mm group developed to blastocyst and expanded blastocyst faster than the 1-3 mm group. None of the grade A or B COC's demonstrated constantly higher or lower rates of development throughout maturation, cleavage and blastocyst formation stages. This means this study cannot suggest that oocytes from any group size would do better than the others in any stage of development. This agrees with previous studies that suggest equal chances of developmental competence for grade A and B oocytes and further development after maturation is affected by many other factors (Hazeleger *et al.,* 1995). In summary, like for other breeds, the IVEP reproductive biotechnologies can be exploited to produce Boran F1 embryos. Thus, this technique has great potential to multiply genotypes.

CHAPTER 6

6 Conclusions and Recommendations

This study shows that *in vitro* production of embryos for the Boran cow in Kenya is technically feasible and that the Boran would be a good cow for ova harvesting. Unlike the *Bos taurus*, in the Boran, follicles as small has 1-3 mm can be utilized for IVEP processes. By the time the follicle size is about 6 mm in this species, the oocyte competence is fully attained. This study has set up baseline information for enhanced utilization of the Boran cow as oocyte donors. The following conclusions can be drawn from this study:-

- There was no relationship between the follicle size and grade of COC aspirated and between the follicle size, grade and the rate of maturation of the COC's from Boran cows.
- Follicles of sizes between 1-3 mm produce COC's that have the ability to develop to the blastocyst stage. However, embryos that developed from follicles greater than 6 mm had a faster rate of development than those that arose from follicles less than 6 mm in size.
- There was a strong relationship between follicle size and grade and the rates of BCB competence, cleavage and blastocyst rates.

From this study several aspects of the Boran cow remain unknown. In particular, follicular wave development and nutritional status of the cow on embryo development need further study.

CHAPTER 7

7 References

Abecia J. A., Lozano J.M., Forcada F., Zarazaga L. (1997): Effect of level of dietary energy and protein on embryo survival and progesterone production on day eight of pregnancy in Rasa Aragonesa ewes. Animal Reproductive Science; **48**:209-218.

Alm H., Torner H., Lo¨hrke B., Viergutz T., Ghoneim I.M., Kanitz W. (2005): Bovine blastocyst development rate *in vitro* is influenced by selection of oocytes by brilliant cresyl blue staining before IVM as indicator for glucose-6-phosphate dehydrogenase activity Theriogenology **63** 2194–2205

Barros C.M., Nogueira M.F.G. (2005): Superovulation in zebu cattle: protocol P-36. Embryo Transfer Newsletter *2005*;**23**:5-9.

Bergfelt D.R., Lightfoot K.C., Adams G.P. (1994): Ovarian synchronization following ultrasound-guided transvaginal follicle ablation in heifers. Theriogenology **42**:895-907.

Bindon B.M., Piper L.R., Cahill L.P., Driancourt M.A., O'Shea T. (1986): Genetic and hormonal factors affecting superovulation. Theriogenology; **25**: 53-70.

Binelli M., Ibiapina B.T., Bisinotto R.S. (2006): Physiological, pharmacological and endocrine basis of treatments aiming synchronization and ovulation of follicles in cattle. Acta Scientiae Veterinariae. **34** (Suppl 1): 1-7.

Block J., Chase C. C. Jr., Hansen, P.J. (2002): Inheritance of resistance of bovine preimplantation embryos to heat shock: relative importance of the maternal versus paternal contribution. Molecular Reproductive Development, **63**: 32-37.

Blondin P., Sirard M.A. (1995): Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Molecular Reproductive

Development; **41**: 54-62.

Boediono A., Suzuki T., Godke R.A. (2003): Comparison of hybrid and purebred *in vitro*-derived cattle embryos during *in vitro* culture. Animal Reproductive Science, **78**:1-11.

Boland M.P., Crosby T.F., Gordon I. (1978): Morphological normality of cattle embryos following superovulation using PMSG. Theriogenology **10**:175.

Boland M.P., Roche J.F. (1993):. Embryo production: alternative methods. Molecular Reproduction Development ;**36**: 266-270.

Bredbacka P. (1998): Recent developments in embryo sexing and its field application. Reproduction and Nutritional Development. **38**, 605–613.

Camargo L.S.A., Sá W.F.D., Ferreira A.D.M., Viana J.H.M., Araújo M.C.C.D .(2002): Effect of sperm concentration and oocyte-spermatozoa incubation period on *in vitro* fertilization of Gyr breed. Pesquisa Agropecuária Brasileira, Brasília, v. **37,** p. 709-715.

Camargo L.S.A., Viana J.H.M., Ramos A. A., Serapião R. V., Sá W.F., Ferreira A.M., Guimaraes M.F.M., Vale F., Vicente R.D.(2007): Factors influencing *in vitro* embryo production. Animal Reproduction, v. **3**, p. 19-28.

Camargo L.S.A., Viana J.H.M., Sá W.F., Ferreira A.M., Ramos A.A. , Vale Filho V.R. (2006): Factors influencing *in vitro* embryo production Animal Reproduction., v.**3**, n.1, p.19-28,

Carolan C., Lonergan, P., Monget p., Monniaux D and Mermillord P. (1996): Effect of follicle size and quality on ability of follicular fluid to support cytoplasmic maturation of bovine oocytes. Molecular Reproductive Development **43:**477-483.

Central Artificial Insemination Station (2009): Annual report on Artificial Insemination in Kenya

Cesari A., Kaiser G.G., Mucci N., Mutto A., Vincenti A., Fornés M.W., Alberio R.H. (2006): Integrated morphophysiological assessment of two methods for sperm selection in bovine embryo production *in vitro*. Theriogenology **66 (5):**1185-93.

Cognié Y., Poulin N., Locatelli Y., Mermillod P. (2004): State-of-the-art production,

conservation and transfer of in-vitroproduced embryos in small ruminants. Reproduction, Fertility and Development;**16**:437-445.

Crosier A.E., Farin P.W., Dykstra M.J., Alexander J.E., Farin C.E. (2001): Ultrastructural morphometry of bovine blastocyst produced in vivo or in vitro. Biology of Reproduction **64**, 375- 1385

Cunningham, E.P. and Syrstad O. (1987): Crossbreeding Bos indicus and Bos taurus for milk production in the tropics. FAO Animal Production and Health Paper **68.** Food and Agriculture Organization of the United Nations Rome.

De Loos F., van Vliet C., van Maurik P. and Kruip T.A. (1989): Morphology of immature bovine oocytes Gamete Research **24** 197–204

Dode M.A., Rodovalho N.C., Ueno V.G., Fernandes C.E. (2002): The effect of sperm preparation and co-incubation time on *in vitro* fertilization of Bos indicus oocytes. Animal Reproductive Science Jan 23; **69** (1-2):15-23

Driancourt MA, Audi M. (1993): Effect of the physiological stage of the ewe on the number of follicles ovulating following hCG injection. Animal Reproductive Science; **32**: 227-236.

Ducibella T. and Anderson E. (1975): Cell shape and membrane changes in the eight-cell mouse embryo: prerequisites for morphogenesis of the blastocyst. Developmental Biology 47, 45-58

Dufour J.J., Cognie Y., Mermillod P., Mariana J.C., Romain R.F. (2000): Effects of the Booroola Fec gene on ovarian follicular populations in superovulated Romanov ewes pretreated with a GnRH antagonist. Journal of Reproduction and Fertility **118**:85-94.

Dunne L.D., Diskin M.G., Boland M.P., O'Farrell K.J. and Sreenan J.M. (1999): The effect of pre- and post-insemination plane of nutrition on embryo survival in beef heifers. Animal Science **69**:411-17.

Duranthon V., Renard J.P. (2001): The developmental competence of mammalian oocytes: a convenient but biologically fuzzy concept. Theriogenology **55**, 1277–1289.

Ericsson S.A., Boice M.L., Funahashi H., Day, B.N. (1993): Assessment of porcine oocytes

using brilliant Cresyl Blue. Theriogenology **39**:214. abstr.

Fair, T., Corcoran, D. and Evans, A.C.O. (2006): Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos' Theriogenology , : **137**

Farin P. W. and Farin C. E. (1995): Bovine embryos produced in vivo or *in vitro*: Embryo survival and fetal development. Biology of Reproduction **52**:678–682.

Fenwick J., Platteau P., Murdoch A.P., Herbert M. (2002): Time from insemination to first cleavage predicts developmental competence of human pre implantation embryos in vitro. Human Reproduction **17,** 407-412

Ferrandi B., Cremonesti G., Consiglio A.L., Luciano A.M., Gandolfi F., Modina S., Carnevali A., Porcelli F.(2002): Microdensitometric assay of enzymatic activities in parthenogenetically activated and *in vitro* fertilized bovine oocytes. Acta Histochemistry; 104:193–

Fischer A.E., Bernal D.P., Gutierrez-Robayo C., Rutledge J.J. (2000): Estimates of heterosis for *in vitro* embryo production using reciprocal crosses in cattle. Theriogenology, **54**:1433

Gandolfi F., Luciano A.M., Modina S., Ponzini A., Pocar P. and Armstrong D.T. (1997): The *in vitro* developmental competence of bovine oocytes can be related to the morphology of the ovary. Theriogenology **48:** 1153-1160.

Gardner R.L. and Johnson M.H. (1972): An investigation of inner cell mass and trophoblast tissues following their isolation from the mouse blastocyst. Journal of Embryology and Experimental Morphology **28,** 279-312

Gonzalez-Bulnes A., Carrizosa J.A., Díaz-Delfa C., García-García R., Urrutia B., Santiago-Moreno J., Cocero M.J. and López-Sebastián A. (2003): Effects of ovarian follicular status on superovulatory response of dairy goats to FSH treatments. Small Rumininant. Research; **48**: 9-14.

Gordon I. (1994): Laboratory production of cattle embryos. Wallingford, UK: CAB International,

Gordon I. (1996) Controlled reproduction in cattle & buffaloes. Cambridge International p 492.

Gordon I.,(2003): Recovering the bovine oocyte. In: Laboratory production of cattle embryos Biotechnology in Agriculture No. **27**). 2nd ed. Cambridge, UK: CAB International/Cambridge University Press; p. 79– 111.

Gregory K.E., Trail J.C.M., Sandford J., Durkin J. (1984): Crossbreeding cattle in beef production programmes in Kenya. I. Comparison of purebred Boran and Boran crossed with Charolais, Ayrshire & Santa Gertrudis breeds. Tropic*al.*, Anim*al.*, Health and Production, **16**, 181-186

Hazeleger N.L., Hill D.J., Stubbings R.B. and Walton J.S. (1995): Relationship of morphology and follicular fluid environment of bovine oocytes to their developmental potential *in vitro*. Theriogenology **43**: 509-522. 57

Hendriksen P.J., Vos P.L., Steenweg W.N., Bevers M.M and Dieleman S.J. (2000): Bovine follicular development and its effect on the *in vitro* competence of oocytes. Theriogenology; **53**: 11

Henshall J.M., Hill J.R. and van der Werf J.H.J. (2006): A new look at advanced reproduction technologies and their impact on livestock breeding. 8^{TH} World Congress on Genetics Applied to Livestock Production, August 13.18, 2006, Belo Horizonte, Brazil.

Herlocker D. (1999): Rangeland Resources in Eastern Africa: Their Ecology and Development. GTZ, Germany Technical Co-operation, Nairobi.

Johnson MH, Ziomek CA (1981): Induction of polarity in mouse 8-cell blastomeres: specificity, geometry, and stability. Journal of Cell Biology **91**, 303-308

Kimenye, D. (1985): Review of the Boran in Kenya. In: Animal genetic resources in Africa. High potential and endangered livestock. Second OAU Expert Committee Meeting on Animal Genetic Resources in Africa, 24–28 November, 1983, Bulawayo, Zimbabwe.

Lequarre A.S., Vigneron C., Ribaucour F., Holm P., Donnay I., Dalbiès-Tran R., Callesen H. and Mermillod P. (2005): Influence of antral follicle size on oocyte characteristics and embryo development in the bovine. Theriogenology. Feb; **63(3)**:841-59.

Lonergan, P., Monaghan, P., Rizos, D., Boland, M.P. Gordon, I. (1994): Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization and culture *in vitro*. Molecular Reproductive Development **37** :4&53.

Lonergan, P., Rizos, D., Gutierrez-Adan, A., Moreira, P.M., Pintado, B., De La Fuente, J., Boland, M.P. (2003): Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage *in vitro* or in vivo. Biology of Reproduction **69**, 1424-1431

Lu K.H. And Seidel G.E. Jr. (2004): Effects of heparin and sperm concentration on cleavage and blastocyst development rates of bovine oocytes inseminated with flow cytometrically-sorted sperm. Theriogenology Sep **1; 62**(5):819-30.

Lussier J.G., Matton P. and Dufour J.J. (1987): Growth rates of follicles in the ovary of the cow. Journal of Reproduction and Fertility; **81**: 301-07.

Madison V., Avery B. and Greve T. (1992): Selection of immature bovine oocytes for developmental potential *in vitro* Animal Reproduction Science; **27:1**–11.

Mangia, F. and Epstein, C.J. (1975): Biochemical studies of growing mouse oocytes: preparation of oocytes and analysis of glucose-6-phosphate dehydrogenase and lactate dehydrogenase activities. Development Biology*; 45:211*–20.

Martínez M.F., Bó G.A. and Mapletoft R.J. (2004): Synchronization of follicular wave emergence and ovulation for reproductive biotechnologies. In: 1o Simpósio Internacional de Reprodução Animal Aplicada, , Londrina. Anais do 1o Simpósio Internacional de Reprodução Animal Aplicada;**1**:26-55.

Mayes, M.A, (2002): The meiotic arrest of bovine oocytes. Philosophiae Doctor Thesis, University of Laval, Quebec, Canada, 1-80

Mermillod, P., Oussaid, B. and Cognié Y. (1999): Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. Journal of Reproduction and Fertility Supplement;**54**:449-460.

Merton J.S., de Roos A.P., Mollaart E., de Ruigh L., Kaal L., Vos P.L. and Dieleman S.J. (2003): Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology;**59**:651-674.

Mitchell L.M., Silveira M., Mylne M.J., Matthews K. and Dingwall W.S. (2002): Seasonal differences in lamb birthweight do not arise from inherent differences in the oocyte and/or early embryo. Reproduction Fertility and Development **14:** 207-213.

Miyano, T. (2003): Bringing up small oocytes to eggs in pigs and cows. Theriogenology, **59:61-**72,

Moore K. and Thatcher W.W. (2006): Major advances associated with reproduction in dairy cattle. Journal of Dairy Science **89**:1254-66.

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

Morris D.G., Grealy M., Leese H.J., Diskin M.G., Sreenan J.M. (2001): Cattle embryo growth, development and viability. Teagasc, Dublin, 32p

Motlik J, Kubelka M (1990): Cell-cycle aspects of growth and maturation of mammalian oocytes. Molecular Reproduction and Development **27**, 366-375

Mutembei H.M., Muasa B.S., Origa R., Jimbo S., Ojango J.K., Tsuma V.T., Mutiga E.R., Okeyo A.M. (2008b): *in vitro* Fertilization in cattle and its potential practical application in Kenya. Proced 6th FVM Biennial Scientific Conference, Sept 17th-19th 2008, 15.

Mutembei, H.M., Muasa, B.S., Origa, R, Jimbo, S., Ojango, J.M.K., Tsuma, V.T., Mutiga, E.R., Okeyo, A.M. (2008a). Delivery of appropriate cattle genotypes to eastern African smallholder farmers through *in vitro* embryo production Technologies. Proced Biodiversity Conference, Sept 10th-12th 2008, 16-17.

Mwandoto B.A.J., Carles, A.B. and Cartwright, T.C. (1988): Weaning and 18 month weights of Boran, East African Shorthorn Zebu and Sahiwal breeds or crosses in Kenya. Tropical Agriculture., **65**, 257–264.

Nolan R., O'Callaghan D., Duby R.T., Lonergan P. and Boland M.P. (1998): The influence of short-term nutrient changes on follicle growth and embryo production following superovulation in beef heifers. Theriogenology **50**:1263-74.

Nuti L.C., Minhas B.S., Baker W.C., Capehart J.S. and Marrack P. (1987): Superovulation and recovery of zygotes from Nubian and alpine dairy goats. Theriogenology **28**: 481-488.

O'Callaghan D., Yaakub H., Hyttel P., Spicer L.J. and Boland, M.P. (2000): Effect of nutrition and superovulation on oocyte morphology, follicular fluid composition and systemic hormone concentrations in ewes. Journal of Reproduction and Fertility;**118**: 303-313.

Ojango, J.M., Malmfors, B. and Okeyo, A.M. (Eds): (2006) The Boran breeds in Animal Genetics Training Resource, version 2, http:// agtr.ilri.cgiar.org/ Breed Information/ breedpg/ cattle/ Boran.htm

Okeyo A.M., Mosi R.O. and Langat L.K.I. (1998): Effects of parity and previous parous status on reproductive and productive performance of Kenya Boran cows. Tropical Agriculture (Trinidad) 75(3):384-389.

Ozil JP (1990): The pathenogenic development of the rabbit oocytes after repetitive pulsatile electrical stimulation. Development **109**, 117-127

Parrish J.J., Susko-Parrish J., Leibfried-Rutledge M.L., Critser E.S., Evestone W.H. and First NF. (1986): Bovine *in vitro* fertilization with frozen-thawed semen. Theriogenology'; **25**:591-600.

Paula-Lopes F.F., Chase Jr. C.C., Al-Katanani Y.M., Krininger C.E., Rivera R.M., Tekin, S., Majewski A.C., Ocon O.M., Olson T.A. and Hansen, P.J. (2003): Genetic divergence in cellular resistance to heat shock in cattle: differences between breeds developed in temperate versus hot climates in responses of preimplantation embryos, reproductive tract tissues and lymphocytes to increased culture temperatures. Reproduction **125**: 285- 294.

Polisseni J, Carvalho, BC, Pereira, MM., Serapião, RV, Batista, RITP, Silva, MR, Boite, MC, Camargo, LS, Viana, JHM, Sa, WF, Iguma, LT. (2007): Comparison among different spermatozoon separation methods: Swim up, Isolate® and Percoll® on viability of in vitro fertilized bovine embryos. In: 6 Congresso Internacional do Leite, 2007, Resende, Brasil. p. 01-05.

Republic of Kenya, (2003): National policy for sustainable development of the arid and semi arid areas of Kenya draft

Rewe T.O., Indetie D., Ojango J.M.K. and Kahi A.K. (2006): Economic values for production and functional traits and assessment of their influence on genetic improvement in the Boran cattle in Kenya. Journal of Animal Breeding and Genetics **123,** 23–36.

Rizos D., Lonergan P., Boland M.P., Arroyo-Garcia R., Pintado B., De La Fuente J. and Gutierrez-Adan A. (2002): Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality. Biology of Reproduction **66**, 589-595

Rocha, A., Randel, R.D., Broussard, J.R., Lim, J.M., Blair, R.M., Roussel, J.D., Godke, R.A, Hansel, W. (1998). High environmental temperature and humidity decrease oocyte quality in Bos taurus but not in *Bos indicus* cows. Theriogenology, **49**:657-665.

Senger P.L. (2003): Pathways to Pregnancy and Parturition. Pullman: Current Conceptions, Inc.,.

Somfai T., Bodó S., Nagy S., Papp A.B., Iváncsics J., Baranyai B., Gócza E. and Kovács A.. (2002): Effect of swim up and Percoll treatment on viability and acrosome integrity of frozen-thawed bull spermatozoa. Reproduction in Domestic animals. Oct; **37(5):**285-90.

Tamassia M., Heyman Y., Lavergne Y., Richard C., Gelin V., Renard J. P. and Chastant-Maillard S. (2003): Evidence of oocyte donor cow effect over oocyte production and embryo development *in vitro* Reproduction **126**, 629–637

Thibier, M. (2005): Data Retrieval Committee Annual Report, 2004. *IETS Newsl*, **23**:11-17.

Torres S., Cognie Y. and Colas G. (1987): Transfer of superovulated sheep embryos obtained with different FSH-P.Theriogenology; **27**, 407-419

Van Montfoort A.P.A., Dumoulin J.C.M., Kester A.D.M. and Evers J.L.H. (2004): Early cleavage is a valuable addition to existing embryo selection parameters: a study using single embryo transfers. Human Reproduction **19**, 2103-2108

Van Niekerk A. and Louw B. P. (1980): Condition scoring of beef cattle. Bulletin **N2/80,** Department of Agriculture and Fisheries, Natal Region, South Africa

Viana J.H.M, Ferreira A.D.M., SÁ W.F.D. and Camargo L.S.A. (2000): Follicular dynamics in zebu cattle. Pesquisa Agropecuária Brasileira, Brasília, v. **35,** n. 12, p. 2501-2509.

Viana JHM, Carmago LSA (2007): Bovine embryo production in Brazil: A new scenario. Acta Scientiae Veterinariae **35(Supl. 3),** 920-924.

Ward F., Enright B., Rizos D., Boland M. and Lonergan P. (2002): Optimization of *in vitro* bovine embryo production: effect of duration of maturation, length of gamete co-incubation, sperm concentration and sire. Theriogenology. May; **57** (8):2105-17.

Wassarman M. (1988): The mammalian ovum. In: Knobil E, Neil D, editors. The physiology of reproduction, vol. 1.New York, USA: Raven Press;. p. 69–102.

Wilkinson, R.F (1996): The use of Neural networks in developing novel embryo culture media fomulations. Theriogenology **45**: 41-49

Wiltbank MC, Gümen A, Sartori R. (2002): Physiological classification of anovulatory conditions in cattle. Theriogenology **57**:21-52.

Yaakub H., O'Callaghan D.O. and Boland M.P. (1999): Effect of roughage type , concentrate supplementation on follicle numbers and *in vitro* fertilization and development of oocytes recovered form beef heifers. Animal Reproductive Science **55**:1-12.

Yang X., Kubota C., Suzuki H., Taneja M., Bols P. E. and Presicce, G. A. (1998). Control of oocyte maturation in cows--biological factors. Theriogenology; **49**: 471-82.

Yotsushima K., Shimizui M., Koni H. and Izaike, Y. (2007): A Simple Method for Selection of Cumulus-Oocyte Complexes from Bovine Ovaries by Sedimentation with Percoll Journal of Reproduction and Development, Vol. **53**, No. 4,

8 Appendices

Appendix 1: List of materials and equipments used

Equipments

- Gilson pipettes D 100, D 200 and D 20 D10 (Pipettman, France)
- Stereo scopic microscope (Wild, Switzerland)
- S.E Inverted Microscope (Nikon, Japan)
- Stage warmer (Lec, Australia)
- Water bath (Kotterman Germany)
- Refrigerator (LG, Japan)
- Laminar flow hood (Baker group, USA)
- Weighing balance (Kern, Germany)
- PH meter (Thermo Scientific, USA)
- Osmometer (Thermo Scientific, USA)
- Eppendorf Centrifuge (Eppendorf, Germany)
- CO2 incubator (Herra Cell) and
- Magnetic Stirrer (Stuart, Britain)

Materials

- Pipette tips D1000, D200, D20 D10 (Eppendorf, Germany)
- Sterlin plates (Becton & Dickson USA),
- Sodium chloride (Alanar, England)
- Potassium chloride (Sigma Aldrich, Germany)
- Sodium hydrogen carbonate (Alanar, England)
- Sodium hydrogen phosphate(Sigma Aldrich, Germany)
- Soduim pyruvate (Sigma Aldrich, Germany)
- Sodium lactate (Sigma Aldrich, Germany)
- Caffeine benzoate(Sigma Aldrich, Germany)
- Phenol Red(Sigma Aldrich, Germany)
- Calcium chloride(Sigma Aldrich, Germany)
- Magnesium chloride(Sigma Aldrich, Germany)
- Hepes(Sigma Aldrich, Germany)
- Glycine (Alanar, England)
- Alanine (Sigma Aldrich, Germany)
- Calcium L lactate Heparin (Sigma Aldrich, Germany)
- Gentamycin (Prepared in the lab)
- Bovine serum albumin (Sigma Aldrich, Germany)
- Medium I99 (Sigma Aldrich, Germany)
- Penicillamine(Sigma Aldrich,Germany)

- Hypo taurine (Sigma Aldrich,Germany)
- Epinephrine (Sigma Aldrich,Germany)
- Dulbecos Phosphate buffered saline (prepared in the lab)
- Non essential amino acids (Gibco USA)
- Essential amino acids (Gibco USA)
- Glutamine(Gibco USA)
- Sterile Water (prepared in the lab)
- Fetal calf serum (Sigma Aldrich,Germany)

Appendix 2: Media Used for embryo production

1. Maturation media: IVM: TCM Ready to use preparation

Maturation Medium (Parker based) (10ml)		25ml
Sodium pyruvate	**113.6 µl**	284µl
Gentamycin	**0.1 ml**	0.25ml
Medium 199 (Ref. M4530)	**9 ml**	22.5ml
FCS	**1ml**	2.5ml
FSH (1µl**/ml)**	**10 µl**	25 µl
LH (1µl**/ml)**	**10 µl**	25 µl
Estradiol (1µl**/ml)**	**10 µl**	25 µl

Osmolarity mOsm) 275 – 290, pH 7.2

2. Fertilization, Swim up and Talp hepes(wash) Media

Stocks

	FERT. TALP.	**FERT. TALP.**	**SPTL**	**SPTL**	**TALP HEPES**
NaCl	0.1636 g	0.3272 g	0.146g	0.292 g	0.7304g
KCl	0.00595g	0.0119g	0.00595g	0.0119 g	0.0238g
$NaH_2PO_4.H_2O$	50 µl	100 µl	50 µl	100 µl	200µl
Na lactate (60%) syrup	46.5 µl	93 µl	92.3µl	184.6 µl	186.8µl
$NaHCO_3$	0.0525g	0.105g	0.105 g	0.105 g	0.0168
Phenol red (1%)	25 µl	50 µl	25 µl	50 µl	100µl
Cafein	0.00675g	0.0135g	0.0025 g	0.005 g	-
$CaCl_2 .2H_2O.$	0.00735g	0.0147g	0.00735g	0.0147 g	0.0294
$Mg Cl_2 . 6H_2O$	32 µl	64 µl	67.5 µl	135 µl	127µl
Hepes	0.05955g	0.1191g	0.05955g	0.1191 g	0.239
Tridest. Water qsp	**25ml**	**50 ml**	**25ml**	**50 ml**	**100 ml**
PH	**7.3 - 7.4**	**7.3 - 7.4**	**7.3 - 7.4**	**7.3 - 7.4**	**7.3 - 7.4**
Osmolarity (mOsm)	**280 – 295**	**280 – 295**	**285 - 300**	**285 - 300**	**275 - 285**
Expiration	**1 week**	**1 week**	**1 week**	**1 week**	**2 weeks**

Wash recipients 3 times with Milli –Q water

Measure and correct pH; Measure osmolarity;

Filter with 0.2 µl size membrane;

Keep in vials into the fridge for one week.

Ready-to-use media

	FERT. TALP.	**SPTL**	**TALP HEPES**
BSA FAF	0.06 g	0.06 g	0.15 g
Na Pyruvate	100 µl	500 µl	500 µl
Gentamycin	100 µl	100 µl	500 µl
PHE	10 µl	-	-
Stock media	10 ml	10 ml	50 ml
Heparin	**1 aliquot (10µl)**	**1 aliquot**	
PH	**7.4 – 7.8**	**7.4 – 7.5**	**7.4 – 7.5**

Measured and corrected pH;

3. Culture Media: CR2 Osmolarity (mOsm) 255 (260) – 265 (270)

Item	CR2 stock (25 ml)	mM
NaCl	0.15778 g	108.0
KCL	0.00559 g	3.0
$NaHCO_3$ (Na bicarbonate)	0.055 g	26.0
Glycine (G6388-Sigma)	0.01876 g	10.0
Alanine (A3534 – Sigma)	0.00223 g	1.0
Glutamax	**250** µl	**1.0**
Cálcium–lactate (L4388 – Sigma)	0.01364 g	4.3
Milli-Q water q.s.p.	25 ml	Filtered and kept in the fridge

Ready-to-use solution Osmolarity (mOsm) 255 (260) – 265 (270)	**10 ml**	**20 ml**
Na pyruvate stock	182 µl (0.36 mm)	364 µl
Penicillin / streptomycin stock	10 µl	20 µl
BSA	0.03 g	0.06 g
(X 100) MEM (M7145 – Sigma)essential amino Acids	100 µl	200 µl
(x50) BME (B6766 – Sigma) non essential amino Acids	200 µl	400 µl
CR2 stock	10 ml	20 ml
FCS (10%)	1 ml	2 ml

Appendix 3; Abattoir collection form

abattoir		date	.... / .... /09
Collected by			
general comment			

CODES	**breed**		**Age**
sex	SEA=small East African Zebu		
	B-		
B=bull	Boran	Z=Zebu	H = heifer
		G-	
C=cow	S- Sahiwal	guensey	M=middle aged
S=steer	BC-beef cross	J-Jersey	O = old
	H-holstein	DC-dairy cross	- = don't know
	Ay- aryshire		

comments			
1	C	cull	
	D	damaged in transit?	
	E	emaciated	
	F	fine looking animal	
2	I	injured	
	L	lactacting	
	M	moribund	
	P	pregnant	
3	S	sick looking	
	T	ticks++	

	Sex	Breed	BCS	Age	comment code	origin
1						

Appendix 4: Laboratory summary sheet

EXPERIMENT:
Batch /Date:

- IVM

Beginning of ovary collection (h):
No. ovaries:
No. oocytes to IVM:
No. oocytes/ovary:
Person in charge:

Arrival temperature:
Beginning of aspiration:
Beginning of IVM:
End of IVM:
Duration of IVM:

- IVF

Bull (Breed/ No.):
Motility post-thawing:
Motility post-swim up:
Motility post-centrifugation:
Beginning of IVF:
End of IVF:
Duration:
Person in charge:

Beginning of swim-up/(Percoll):
End of swim-up (h):
Centrifugation force (RPM):
Sperm concentration post-centrif:
Sperm concentration in IVF drop:
No. oocytes in IVF drop:
Volume of IVF drop:
Motility post-IVF:

- IVC

Wash medium:
Beginning of IVC:
Cleavage rate (%):
Transferd

Culture medium (date/batch):
Volume IVC:
Blastocyst rate (%):
Frozen

		Day/hour:			**Day/hour:**				**Day/hour:**			**Day/hour:**	
Plate	**No**	**No. cells**			**7°**				**8°**			**10°**	
	Ooc.	**2**	**4-7**	**≥ 8**	**EBl**	**Bl**	**ExBl**	**HBl**	**Bl**	**ExBl**	**HBl**	**ExBl**	**HBl**

Appendix 5: Statistical Analysis

A. Effect of size on grade of COC

FSIZE(FSIZE)	GRADE(GRADE)			
Frequency Expected Cell Chi-Square Percent	Grade A	Grade B	Discard	Total
1-3 mm	393 405.62 0.3927 24.14	394 375.08 0.9539 24.20	151 157.29 0.2518 9.28	938 57.62
2-6 mm	226 224.43 0.0109 13.88	196 207.54 0.6413 12.04	97 87.031 1.1418 5.96	519 31.88
>6 mm	85 73.946 1.6525 5.22	61 68.379 0.7963 3.75	25 28.675 0.471 1.54	171 10.50
Total	704 43.24	651 39.99	273 16.77	1628 100.00

Statistics for Table of FSIZE by GRADE

Statistic	DF	Value	Prob
Chi-Square	4	6.3122	0.1770
Likelihood Ratio Chi-Square	4	6.2502	0.1812
Mantel-Haenszel Chi-Square	1	1.1182	0.2903
Phi Coefficient		0.0623	
Contingency Coefficient		0.0621	
Cramer's V		0.0440	

Sample Size = 1628

B. Determination using BCB, effect of follicle size on COC competence

OBJECTIVE 2 - EFFECT OF FOLLICLE SIZE AND COC GRADE ON BCB COMPETENCE

The FREQ Procedure — Table of FSIZE by GRADE

FSIZE(FSIZE) / GRADE(GRADE)	Grade A	Grade B	Total
1-3 mm	735.8	726	1461.8
	780.81	680.99	
	2.5941	2.9744	
	15.38	15.17	30.55
	50.34	49.66	
	28.79	32.57	
2-6 mm	860	699.75	1559.7
	833.12	726.63	
	0.8669	0.994	
	17.97	14.62	32.59
	55.14	44.86	
	33.65	31.39	
>6 mm	960.3	803.6	1763.9
	942.17	821.73	
	0.3489	0.4	
	20.07	16.79	36.86
	54.44	45.56	
	37.57	36.05	
Total	2556.1	2229.35	4785.45
	53.41	46.59	100.00

Statistics for Table of FSIZE by GRADE

Statistic	DF	Value	Prob
Chi-Square	2	8.1784	0.0168
Likelihood Ratio Chi-Square	2	8.1703	0.0168
Mantel-Haenszel Chi-Square	1	4.9947	0.0254
Phi Coefficient		0.0413	
Contingency Coefficient		0.0413	
Cramer's V		0.0413	

Sample Size = 4785.4499855

C. Developmental competence as determined by maturation rate

The FREQ Procedure

Table of FSIZE by Grade

FSIZE(FSIZE)	Grade(Grade)		
Frequency Expected Cell Chi-Square Percent Row Pct Col Pct	Grade A	Grade B	Total
1-3 mm	1031.4 1029.5 0.0033 17.17 51.82 33.19	958.8 960.63 0.0035 15.96 48.18 33.07	1990.2 33.14
2-6 mm	1035.8 1019.7 0.2567 17.25 52.55 33.34	935.24 951.42 0.2752 15.57 47.45 32.26	1971.1 32.82
>6 mm	1039.8 1057.8 0.3067 17.31 50.85 33.47	1005.1 987.06 0.3287 16.73 49.15 34.67	2044.9 34.05
Total	3107.02 51.73	2899.11 48.27	6006.13 100.00

The FREQ Procedure

Statistics for Table of FSIZE by Grade

Statistic	DF	Value	Prob
Chi-Square	2	1.1740	0.5560
Likelihood Ratio Chi-Square	2	1.1740	0.5560
Mantel-Haenszel Chi-Square	1	0.3908	0.5319
Phi Coefficient		0.0140	
Contingency Coefficient		0.0140	
Cramer's V		0.0140	

Sample Size = 6006.1306538

D. Developmental competence as determined by cleavage rate

OBJECTIVE 4 - EFFECT OF FOLLICLE SIZE AND GRADE ON CLEAVAGE RATE

The FREQ Procedure

Table of FSIZE by Grade

FSIZE(FSIZE)	Grade(Grade)
Frequency Expected Cell Chi-Square	

Percent Row Pct Col Pct	Grade A	Grade B	Total
1-3 mm	570.61 540.01 1.7337 13.60 53.38 26.93	498.26 528.86 1.7702 11.88 46.62 24.01	1068.9 25.48
2-6 mm	614.79 683.77 6.9603 14.66 45.42 29.01	738.64 669.65 7.1071 17.61 54.58 35.59	1353.4 32.27
>6 mm	933.57 895.18 1.6464 22.26 52.69 44.06	838.31 876.7 1.6811 19.99 47.31 40.40	1771.9 42.25
Total	2118.97 50.52	2075.21 49.48	4194.18 100.00

OBJECTIVE 4 - EFFECT OF FOLLICLE SIZE AND GRADE ON CLEAVAGE

The FREQ Procedure

Statistics for Table of FSIZE by Grade

Statistic	DF	Value	Prob
Chi-Square	2	20.8988	<.0001
Likelihood Ratio Chi-Square	2	20.9186	<.0001
Mantel-Haenszel Chi-Square	1	0.0892	0.7652
Phi Coefficient		0.0706	
Contingency Coefficient		0.0704	
Cramer's V		0.0706	

Sample Size = 4194.1829412

E. Developmental competence as determined by blastocyst rate

OBJECTIVE 5 - EFFECT OF FOLLICLE SIZE AND GRADE ON BLASTOCYSTS

The FREQ Procedure

Table of FSIZE by Grade

FSIZE(FSIZE)	Grade(Grade)
Frequency Expected Cell Chi-Square Percent	

Row Pct Col Pct	Grade A	Grade B	Total
1-3 mm	132.87 196.23 20.462 7.19 33.51 14.53	263.65 200.29 20.048 14.27 66.49 28.25	396.52 21.46
2-6 mm	327.64 385.08 8.5673 17.74 42.11 35.84	450.47 393.03 8.3939 24.38 57.89 48.27	778.11 42.12
>6 mm	453.75 332.94 43.832 24.56 67.45 49.63	219.02 339.82 42.945 11.86 32.55 23.47	672.76 36.42
Total	914.257 49.49	933.141 50.51	1847.4 100.00

The FREQ Procedure

Statistics for Table of FSIZE by Grade

Statistic	DF	Value	Prob
Chi-Square	2	144.2477	<.0001
Likelihood Ratio Chi-Square	2	146.8805	<.0001
Mantel-Haenszel Chi-Square	1	131.9244	<.0001
Phi Coefficient		0.2794	
Contingency Coefficient		0.2691	
Cramer's V		0.2794	

Sample Size = 1847.3979324