Ovarian follicular dynamics, super-ovulatory response, and *in vivo* embryo production potential of Boran (*Bos indicus*) and Boran *Holstein cross cattle in Ethiopia

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By

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DEDICATION

This work is dedicated to my sons, Lati Tamrat and Risa Tamrat for their unfailing Encouragement. I hope this work will inspire them to great heights.

DECLARATION

I declare that this dissertation titled as “Ovarian follicular dynamics, superovulatory response, and *in vivo* embryo production potential of Boran (*Bos indicus*) and Boran *Holstein cross cattle in Ethiopia” presented for the degree of Doctor of Philosophy in Veterinary Obstetrics and Gynecology is an original report of my research work, and has not been submitted for any other degree or professional qualification.The collaborative contributions and references were clearly indicated and acknowledged.

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LIST OF ABBREVIATIONS

AD - After Delivery
AI - Artificial Insemination
AMP - Adenine Monophosphate
AnGRs - Animal Genetic Resources
ATP - Adenosine Tri Phosphate
BSA - Bovine Serum Albumin
BCS - Body Condition Score
bFSH - bovine Follicle Stimulating Hormone
CIDR - Control Intravaginal Drug Release
CL - Corpus Luteum
COC - Cumulus Oocyte Complexes
DF - Dominant Follicle
DMI - Dry Matter Intake
DMSO - Di Methyl Sulph Oxide
DNA - Diribo-neuclic acid
DZARC - Debre Zeit Agricultural Research Center
eCG - Equine Chorionic Gonadotropin
EB - Energy Balance
ET - Embryo Transfer
ET-1 - Endothelin-1
ETL - Economical Trait Loci
FAO - Food and Agriculture Organization
FSH - Follicle Stimulating Hormone
FSH-P - Follicle Stimulating Hormone - Porcine
FSH-R - Follicle Stimulating Hormone Receptors
GnRH - Gonadotropin Releasing Hormone
GH-IGF -Growth Hormone-Insulin like Growth Factor
hMG - Human Chorionic Gonadotropin
ICM - Inner Cell Mass

IETS	International Embryo Transfer Society
IIF	Intracellular Ice Formation
IGF-I	Insulin-like Growth Factor I
IOI	Inter Ovulatory Interval
IVC	In Vitro Culture
IVEP	In Vitro Embryo Production
IVF	In Vitro Fertilization
IVM	In Vitro Maturation
LH	Luteinizing Hormone
LH-R	Luteinizing Hormone Receptors
MOET	Multiple Ovulation and Embryo Transfer
mRNA	messenger Ribonucleic acid
NEB	Negative Energy Balance
NEFA	Non-Esterified Fatty Acid
OARI	Oromia Agricultural Research Institute
OPU	Ovum Pick up
P4	Progesterone
PGF2α	Prostaglandin F2α
PGI2	Prostaglandin I2
PLP	Prolonged Luteal Phase
PMSG	Pregnant Mare Serum Gonadotropin
PRID	Progesterone Release Intravaginal Devise
QTL	Quantitative Trait Loci
rbFSH	Recombinant bovine Follicle Stimulating Hormone
rhFSH	Recombinant human Follicle Stimulating Hormone
rpFSH	Recombinant porcine Follicle Stimulating Hormone
ROS	Reactive Oxygen Species
SOF	Synthetic Oviductal Fluid
TCM	Tissue Culture Media
TQE	Transferable Quality Embryos
UFO	Unfertilized Ovum

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ABSTRACT

Boran (27) and Boran*Holstein crossbred (26) cows were used to study the characteristics of ovarian follicular dynamics, superovulatory response and embryo production potential. Follicular population and growth, CL number and size were monitored by ultrasonography for 61 days. Ovulation or formation of CL was used as a reference to compute the number of follicular waves and the inter-ovulatory-interval. Serum progesterone was determined every other day by immunoassay. Cows were superovullated with a combination of CIDR implant for 7days and three doses of FSH (300, 250 and 200IU), administered in a divided decreasing morning and afternoon doses over 4 days. Prostaglandin was injected on Day 6 of CIDR implantation. Cows were inseminated twice based on manifestation of standing heat. Time to estrus, duration of estrus, and CL count were used to determine superovulatory response. Embryos were flushed on Day 16/17 by non-surgical closed gravitational method. Recovered embryos/UFOs were evaluated for development stage, and quality grade. Viable embryos at stage 4-7 with quality grade 1-2 were separately loaded on to a 0.25ml straw and stored in liquid nitrogen. Some embryos (~12%) were fresh transferred to synchronized recipients. Frozen embryos were thawed and transferred to warmed slide for post thawing microscopic evaluation. Viability test was performed by culturing embryos in TCM 199 containing 10% BSA at 37^0C for 24 hours. The overall mean Inter-ovulatory-interval was 19.8±0.21 days and breed difference was not significant. All Boran and 56.2% of the Boran*Holstein crossbred cows had two follicular waves/cycle with a significant (P=0.001) breed difference. The mean Inter-ovulatory-interval for the cows with two and three waves/cycle was 19.48±0.94 and 20.86±2.14 days, respectively. The mean serum P4 level in Boran and Boran*Holstein cross cows were 2.01ηg/ml and 0.88 ηg/ml, respectively, and breed difference was significant (P=0.001). The total CL count was significantly higher (p=0.01) in Boran (10.1CL/cow/cycle) than Boran x Holstein crossbred cows (7.2CL/cow/cycle). A mean number of 4.1 and 2.67embryos“/cow were flushed from Boran and Boran*Holstein crosses, respectively. The average number of transferable embryos/ cow was 3.8±0.57 and 2.67±0.99 in Boran and Boran*Holstein crosses, respectively. A significantly higher (p= 0.001) number of UFOs were collected from Boran cows (8.17±1.82). In total, 94% of the embryos were at stage 4 while 88.6% of the embryos were quality grade 2. The viability test confirmed development in 24% and 40% of the incubated Boran and Boran*Holstein embryos, respectively. Boran breeds showed a relatively better potential for application of embryo technology in terms of ovarian follicular growth, superovulatory response, and embryo production. The uniformity of waves of follicular growth in each cycle is an additional virtue of the Borans for application of biotechnology tools such as estrous synchronization and superovulation for breed improvement or conservation.

Key words: ovarian follicular dynamics, follicular waves, superovulatory response, embryo, Boran, Boran * Holstein cross

CHAPTER I

Boran --- handle the harsh conditions well and get into calf sooner.They are well adapted and efficient. "Eloff Muller, 2007"

1. INTRODUCTION

Pages 1-3

Over the centuries, natural selection and human intervention have produced strong, hardy animals that can thrive under higher disease prevalence, fairly low-quality feed and forage condition in the tropics. Having a wide range of agro-ecologies, Ethiopia is a home to these diverse indigenous cattle breeds of which Boran is one. Boran cattle are the most suitable types of breed for arid and semi-arid regions in the country due to their adaptive characteristics like tolerance to heat, ticks infestation, feed and water shortage, and hardened hooves and lighter bones that enabled them to endure long migrations (Solomon, 2001; Mekonnen *et al.,* 2010; OARI, 2010). Different literatures showed that they are superior to other highland cattle in Ethiopia in birth weight, weaning weight, faster growth rate, mature weight for cows, higher fertility, daily milk yield, milk yield/lactation, lactation length, milk fat, age at first calving, calving interval, and calving rate, (DAGRIS, 2008; Azage *et al.,* 2009) and mature weight for bulls, good source of beef for local and international markets in Ethiopia and other African countries such as Kenya, Tanzania, Uganda and Zambia (Trail *et al.,* 1984; Coppock, 1994; Azage *et al.,* 2009). They have large body size with good body conformation (large and long-legged, tall height, broad back and wide pin-bones, well developed dewlap and udder, and well-developed hindquarters) and body weight ranging from 318 - 680 kg in male and 225 - 454 kg in female (Mekonnen *et al,* 2010; OARI, 2010). However, effort made so far to increase the productivity on Boran cattle in Ethiopia was fully based on crossing with exotic breeds either through AI or natural breeding. As a result, crossbreeding of Boran with Holstein has resulted in improved growth, milk production and reproductive performance, and these traits exhibited an increasing trend with increasing exotic inheritance level (Demeke *et al.,* 2000; Haile *et al.*, 2009).

Boran cattle breed is also the only indigenous breed exposed to assisted reproductive technology other than AI in the process of breed improvement with the promising findings. A study made by Tegegne *et al.*, (1994) on superovulatory response of Boran cows treated with Pregovet (human menopausal gonadotropin), and Pluset (procine pituitary extract gonadotropin) showed excellent potential of the breed for superovulation and embryo production. Another study made on the seasonal variation in superovulatory response of Boran and Boran*Holstein crosses has shown that Boran cows responded to superovulatory treatments better than Boran* Holstein crosses, and yielded more numbers of embryos/ova and transferable embryos than Boran* Holstein cows (Tegegne *et al.,* 1997). However, in study made by Million *et al.,* (2011), the Boran breed tended to have a longer interval to estrus, shorter duration of estrus and an estrus behavior score lower than Boran*Holstein crossbred cattle treated under similar conditions. There are two main challenges facing the Boran breeds in Ethiopia: 1) Boran cattle breed is endangered due to recurrent drought (Solomon, 2001; FAO, 2004) and faced genetic erosion from introgression of highland cattle recruited for replacement stock after drought (Solomon, 2001; OARI, 2010); 2) Reproductive technologies so far in use are mostly developed for *Bos taurus* breeds.

Some reports on reproductive physiology of Zebu cattle indicated that their sensitivity to gonadotropins, estrus behavior and ovulation moment, timing of estrous expression, number of follicular waves during estrous cycle, size of follicular diameter at deviation and luteal tissue size are not similar to that of *Bos taurus* breed cows (Barros *et al.,* 1995; Medrano *et al.*, 1996; Figueiredo *et al*., 1997; Gambini *et al*., 1998; Pinheiro *et al*., 1998; Viana *et al.*, 2000; Baruselli *et al.,* 2006). Knowledge of ovarian follicular dynamics, however, could lead to development of methods for improving fertility, synchronizing estrus with more precision, and enhancing superovulatory responses for the application of other reproductive technologies for conservation and sustainable utilization of the genotype. These variables are largely studied in temperate breeds, but very limited on Zebu cattle breeds (Roche & Boland, 1991; Badinga *et al.*, 1994; Figueiredo *et al.,* 1997; Gambini *et al.,* 1998). This holds true and yet to be studied in Ethiopian Boran cattle breed. Generally, the knowledge of these differences is very important to the establishment of the correct evaluation parameters and management procedures for Zebu cows, and also to the development of new technologies to increase herd reproductive performance (Adams, 1994). Therefore, it is hypothesized that Ethiopian Boran cattle breed would exhibit a comparatively different reproductive physiology from *Bos taurus* in terms of ovarian follicular dynamics, sensitivity to superovulatory treatments and embryo production. Therefore, the objectives of this study were:

- To characterize the ovarian follicular dynamics of Boran & Boran*Holstein cows
- To assess the superovulatory response of purebred Boran in comparison to Boran*Holstein crosses
- To study embryo production potential of Boran in comparison to Boran*Holstein crosses
- To assess the quality of *in vivo* produced embryos from Boran & Boran*Holstein cows

CHAPTER 2


"Boran "God's Gift to Cattlemen"
Wayne Southwood, 2007

2. LITERATURE REVIEW

Pages 4-43

2.1 The Boran cattle breed

The Boran developed in eastern Africa and the main Boran hotspot was the Borana plateau in southern Ethiopia. That was a point where all the different breeds migrated through to their various destinations in Africa. The DNA analysis on the genetic make-up of the Boran identified that it is constituted of 24% European *Bos taurus*, 64% *Bos indicus* and 12% African *Bos taurus* (Wayne, 2007). As far as can be determined this is the only breed in Africa (and therefore the world) to have this specific combination of genes (Wayne, 2007). Indeed, it must be properly understood that the Boran is a breed that had its last infusion of "new" genes in 700 AD, and is therefore not a synthetic or compound breed that has been cross-bred in the last few decades. It has been bred as a pure breed for 1 300 years (Wayne, 2007). These unique features of Boran made them suitable for the harsh environment in the lowlands, and hence, ever been part of the pastoralists „identity: traditional and cultural rites and income of the pastoralists. The importance of this to the commercial breeder is that the Boran has much stronger hybrid vigor than modern compound breeds (Homann *et al.,* 2003a; Kerstin and John, 2004). Others more peculiarities of Boran breed were reported by different researchers (Table 1).

From the phenotypic perspectives two sub types of the Boran cattle are distinguished in its place of origin: the large-framed Qorti and the smaller Ayuna (Haile Mariam *et al*., 1998). The true type of the Ethiopian Boran cattle, the Qorti, has been known for its physiological adaptation to heat stress, drought tolerance and walkability, good mothering ability, docility and longevity with tall height, comparatively long legs, broad back, long neck, pending dewlap, short horns, small hump, short tail, and pendulous preputial sheath in male and umbilical fold in female (Haile Mariam, 1994). The second type, Ayuna, is known for high fertility, good growth and its milk producing capacity under range conditions (Homann *et al.,* 2003a; Kerstin and John, 2004). The weight of mature Ethiopian Boran cattle ranges from 300 to 400 kg for cows and 550 to 675 kg for bulls under ranch conditions and 225 and 400 kg under pastoral conditions. Both type of Ethiopian Boran are known for their good body conformation with coat color dominated by white or grey, short horns, well developed pyramidal shape hump over-hanging to the rear or to one side in males.

Table 1. Special characteristics traits of Boran cattle breed reported by different researchers

No	Authors	Evaluated parameter	Year
1	MacFarlane,	Adaption to harsh environment &larger body size	1964
2	D. Robertshaw & V. Finch, Nairobi	Tolerance to heat, predators & theft	1973
3	Trail, J.C.M., Gregory, K.E., Durkin, J and Sandford J.	Beef production and quality	1984
4	Coppock, D.L.	Growth performance, milk yield, mature body weight	1994
5	Freetly H.C. and L.V. Cundiff	Beef quality, tolerance to heat & ecto-parasite infestation	1997
6	Tegegne A.	Excellent superovulation response & embryo quality	1994; 1997
7	Haile-Mariam, M.	Phenotypic character	1994
8	Sprinkle et al,	Low Maintenance requirement	1998
9	Haile Mariam M.	Phenotypic characterization, mothering ability, defensive nature	1998
10	Demeke et al.,	Better Crossbreeding performance	2000
11	Dolan R.,	Trypanotolerance	1998
12	Solomon Desta	Adaption to harsh environment &larger body size	2001
13	Homann, S., Rischkowsky B., J. Steinbach And Ayalew W.	Hybrid vigor, growth performance, milk yield, fertility	2003a
14	Kerstin Zander and John Mburu	Hybrid vigor, growth performance, milk yield, fertility	2004
15	Wayne Southwood	Boran and Tomorrow; God"s gift to cattleman"	2007
16	Haile A, Joshi BK, Ayalew W, Tegegne A, Singh A	Milk production performance	2009
17	Mekonnen Haile-Mariam, Birgitta Malmfors & Jan Philipsson	Potential for performance improvement	2010
18	Oromia agricultural research institute (OARI)	Adaption to harsh environment &larger body size	2010

The dewlap is well developed and a pendulous preputial sheath in male while the females have a well-developed udder (Hailemariam, 1994; Haile Mariam *et al.,* 1998). The functional efficiency of the sheath of the Boran seldom results in a prolapse and/or infection due to three anatomically unique feature: the angle of the sheath points forward rather than downward, the preputium (tip of the sheath) has a strong sphincter that contracts to close the tip of the sheath to prevent protrusion of the lamina interna, and the sheath contains an active preputial muscle around the sheath that retracts the sheath up against the body of the bull. The skin fold in front of the sheath then acts as protection to the opening of the preputium (Wayne, 2007).

While living under relatively harsh environmental conditions Boran cows produce 680 to 1000 kg over a lactation period of 7 to 13 months, which is adequate milk for their calves and some extra for human consumption (Coppock, 1994). Furthermore, results from a number of breed evaluations for beef production in the region and elsewhere indicated that Boran and their crosses are comparable or even better than other breeds (Trail *et al.,* 1984). In Ethiopia, Boran cattle are good sources of beef for local and international markets (over 90 % of the livestock exported for slaughter from Ethiopia come from lowland areas, where Boran cattle form the majority (Coppock, 1994)). They are also considered an important dam breed for crossbreeding with temperate cattle for dairy production. Boran cattle have as well proven to be excellent beef animals, especially under commercial ranching conditions in Kenya and other East Africa countries, including Tanzania, Uganda and Zambia. Studies at the US Meat Animal Research Centre have also shown that heifers with 50% of their genes from the Boran breed reached puberty earlier and had higher fertility, calving and weaning rate than Brahman sired heifers (Freetly and Cundiff, 1997). Besides, cattle with 50% of their genes from Boran were proven to be superior in marbling score, an important measure of meat quality. Thus, by using Boran cattle as a source of genes, livestock breeders in tropical and subtropical areas could increase tolerance to heat and ticks in their breeding populations without sacrificing important traits like meat quality and reproductive performance (Freetly and Cundiff, 1997). Specially, the general width of the Boran, from the chest through to the wide pin-bones, originates from the *Bos taurus* genes in the Boran not only help to produce more beef, but the wide pin-bones have an added advantage for ease of calving (Haile Mariam *et al.,* 1998; Wayne, 2007).

To live and reproduce in a changing environment, Boran cattle have developed adaptive traits of crucial importance for their survival. Some of these characters are ability to withstand periodic shortage of water and feed due to higher level of lipoprotein-lipase activity in the subcutaneous fat depot, ability to walk long distances in search of water and feed, ability to digest low quality feeds, some degree of tolerance to heat stress, resistance to tick infestation and some tick-borne diseases (Coppock, 1994; Hailemariam, 1994; Hailemariam *et al.,* 1998; Wayne, 2007). The subcutaneous fat depot enables them to survive the periodic feed shortages that frequently occur during drought in their homelands and dramatic recoveries after drought years when pasture conditions improve. The high concentration of sweat glands that are larger in size and increased skin surface due to the extra skin folds are the reasons for their tolerance to heat. Further, the skin has a waxy secretion which is unpleasant for an insect to bite through. The hair coat is extremely short hence provides less footholds for insects attaching and holding onto clumps of hair to get into position to bite into the skin of the Boran. A smooth coat and motile skin provides the Boran with a useful degree of protection against tick and buffalo fly infestation, the dark skin pigment protects them against sunburn while the shiny coat reflects a high proportion of the solar radiation. Boran recovers from Foot and Mouth Disease (FMD) faster than *Bos taurus*, and suffers less damaging after effects (Wayne, 2007). Boran cows have also a long reproductive life, a good maternal ability and a good herd instinct (Hailemariam *et al.,* 1998). Particularly, the herding ability of the Boran breed has shown to be of great value in South Africa as it is difficult for thieves or predators to separate individual cows from the rest of the herd (Wayne, 2007).

The Borana cattle in northern Kenya and southern Ethiopia have unique traits. In 1992 FAO identified the Boran to be one of the five tropical cattle breeds that should be given priority for further development and conservation (Philipson, 1992). This is because in Ethiopia the area where Boran cattle originate is dominated by a semi-arid climate characterized by pastoral or agro-pastoral systems, and the major limiting resource is surface water (Hailemariam, 1994). By adopting a relatively sophisticated rangeland management and a coherent, well-structured social organization, the Borana pastoralists achieved a high level of production efficiency, while maintaining an optimum balance between people, livestock, grass and water. Today the Boran, a breed developed by Borana pastoralists in Ethiopia and improved by ranchers in Kenya (Homann *et al.,* 2006), is reported from 11 countries (nine in East, Central and Southern Africa, plus Australia and Mexico) (FAO, 2007). Nevertheless, mainly due to external pressure and uncoordinated, or sometimes inappropriate interventions, the stability of the Borana pastoral society and the source of their livelihood, the Boran cattle, are facing serious challenges. Current predictions indicate that one year in five can be considered as a dry year during which the annual rainfall is less than 75% of the average. Two consecutive dry years usually mean deaths of up to 50% of the animals, due to feed and water scarcity (Coppock, 1994; Haile Mariam *et al.*, 1998). As a result the breed is subjected to genetic erosion contributing immense threats to the livelihoods of the local pastoral communities. Thus conservation efforts of these important animal genetic resources (AnGRs) by governments and other stakeholders would ensure not only the well-being of the pastoralists but also prevent losses in genetic materials for future use.

2.2 Factors affecting reproduction in cattle

Cattle are non-seasonal breeding animal and reproductive activity takes place throughout the year with cows exhibiting recurrent and regular estrous cycles once it reaches maturity. Many factors like breed, season, heat stress, body condition score, energy balance, reproductive stage, high milk yield and calf suckling affects reproductive performance and periodicity through follicular growth and atresia (Figueiredo *et al.,* 1997; Burke *et al.,* 1998; Wilson *et al.,* 1998; Crowe and Mullen, 2013). Physiological evidences indicates that the above mentioned factors governs bovine folliculogenesis and development through modulated and orchestrated activities of gonadotropins as well as metabolic hormones, including growth hormone, leptin, insulin and insulin-like growth factor-1 (IGF-I), and metabolites such as glucose, urea and non-esterified fatty acids (NEFA) (Ginther *et al.,* 1989; Figueiredo *et al.,* 1997; Viana *et al.,* 2000). Specifically, nutrition affects folliculogenesis indirectly by altering the activity of reproductive hormones, and directly by a decisive local impact on the development of follicles at the ovarian level (Garcia-Garcia, 2012; Do and Taylor-Robinson, 2015). With regards to the embryo production the major determinant extrinsic factor responsible for the differences is farm management practices including management of the donor, genetic composition, nutrition, age, lactation phase, quality of the semen and timing of the insemination that affects the results of the superovulation and ovarian response in the donor (Silva *et al.,* 2009). These factors are discussed in the following sections.

2.2.1 Nutrition and energy balance effect

Nutrition has an important impact on the reproductive performance of cattle. Chronic dietary energy deficits as well as energy surpluses has a detrimental impact on reproductive capacity modulating the hypothalamic GnRH neuronal network and/or the pituitary gonadotropin secretion (Garcia-Garcia, 2012). Inadequacy in energy intake or negative energy (NEB) balance impair reproductive activity of the female bovine expressed as loss of body condition, extended periods of anovulation, postpartum anestrus, and infertility. The metabolic condition of cows in NEB shifts to catabolic metabolism, which in turn causes increased plasma growth hormone (GH) and NEFA concentrations, decreased plasma IGF-I, insulin, and glucose as well as leptin serum concentrations (Do and Taylor-Robinson, 2015). Resumption of ovulatory cycles is associated with regaining of energy balance (EB) and the underlying mechanisms seem to be associated with metabolic signals and regulatory hormones primarily insulin and IGF-I, which link nutritional status with gonadotropin secretion, re-coupling of the GH-IGF system, and follicle maturation and ovulation. Feeding diets that promote increases in plasma glucose and insulin may improve the metabolic and endocrine status of cows in early lactation (Garcia-Garcia, 2012).

One of the most important events modulated by EB is folliculogenesis (Evans and Anderson, 2012). Dietary restriction and NEB reduce circulating concentrations of insulin and therefore could reduce androgen and estradiol production which compromise the ability of follicles to acquire LH receptors. Leptin is a potent stimulator of central GnRH and gonadotropin secretion, and has a direct inhibitory effect on ovarian steroidogenesis. In the ovary, leptin antagonizes the stimulatory effect of insulin on theca cell steroidogenesis, ultimately leading to a decrease in estradiol secretion, oocyte maturation, follicle rupture, corpus luteum formation, embryo implantation and pregnancy (Garcia-Garcia, 2012; Do and Taylor-Robinson, 2015).

In the puerperium period, nutritional requirements increase rapidly due to milk production. Consequently, cows may be affected by a NEB and/or disproportionate energy metabolism, diseases related to mineral imbalance, or immune function disorders and dehydration, and reduction in intake (Esposito *et al.,* 2014). Furthermore, dairy cows are highly susceptible to oxidative stress associated with metabolic adaptation processes in early lactation that increase the production of reactive-oxygen species (ROS) such as melondialdeíd, and cause a reduction in serum glucose (Turk *et al.,* 2008; José and Luiz, 2015). Therefore, cows affected by NEB can redirect nutrients from the reproduction limiting the number of follicular growth and maximum size, delaying the first ovulation, hindering estrus expression and decreasing plasma *P4* concentrations from the DF by inhibition of the LH pulse frequency and suppression of blood glucose, insulin, and IGF-1 (Leroy *et al.,* 2008). Conversely, increased food intake can suppress reproduction due to steroid metabolism. The increased food intake enhances hepatic perfusion, which increases the metabolism of estradiol and progesterone, and contributes to anovulation, multiple ovulation, or poor luteal function and delayed luteal regression. These events are most likely caused by the development of a DF to a no-estrogen-sensible resulting in inadequate endometrial PGF2α production (José and Luiz, 2015).

High yielding cows have been selected and bred to produce more milk, mostly through their ability to mobilize fat and muscle to support milk production in early lactation. The energy and protein available from body stores, however, can supply only a limited amount of the animal‟s needs, and the animal faces the NEB. This results in a loss of body condition and is associated with alterations in blood metabolite and hormone profiles which in turn, influence fertility (Kadokawa and Martin, 2006). The severity and duration of NEB are positively associated with the interval to first postpartum ovulation. In early lactation it exerts latent negative effect on the quality of oocytes ovulated 80-100 days‟ later reducing conception rates in the first weeks of the breeding season. It reduces the ability of the uterus to recover after calving and may result in persistent inflammatory mediated damage. The metabolic changes associated with tissue mobilization can probably cause damage to oocytes either directly or via alterations of the follicular environment. Adaptive alterations in the GH-IGF axis reduce the bioavailability of circulating IGF-I. This is likely to be a key mediator of EB status on reproductive function, delaying the time to first ovulation and reducing conception rates, possibly through actions on the oviductal and uterine environments (Gereš *et al.,* 2009). In ruminants circulating leptin concentrations are positively correlated with body fatness, feed intake, nutrients and hormones (especially glucose and insulin), and LH pulse. In short, this hormone is responsible for the linkage between nutritional status and reproductive function (Kadokawa and Martin, 2006). The decreased level of glucose and insulin, with an elevated NEFA level delays the beginning of pulsating level of gonadotrophins, LH and FSH, which are indispensable for folliculogenesis stimulation. A decreased level of insulin leads to reduction of IGF-1 production in the liver, with consecutive ovarian refractibility to gonadotrophins. Reduced secretion of progesterone after calving slows down uterine involution and ovarian activity. Ovarian inactivity was a consequence of severe loss of body mass and decreased levels of leptin and insulin, which consequently results in the blockage of GnRH and LH. A reduced level of LH causes mature follicles to keep producing estradiol, which in turn causes failure of ovulation and growth of intraovarian follicular cysts (Royal *et al.,* 2000; Gereš *et al.,* 2009). Thus minimizing the extent and duration of NEB in early lactation is an important factor for achieving optimum reproductive performance (Patton *et al.,* 2006). Addition of moderate amounts of supplemental fat to the diet improves energy intake, modulates PGF2α secretion by the uterus, affects ovarian dynamics, enhances luteal function and embryo quality, and has moderate positive effects on fertility. Excess of dietary protein has been suggested as detrimental to fertility, although the evidence is weak. On the other hand, feeding protein in excess to what is needed by the cows has been implicated in increased ammonia and urea concentrations in blood and milk, which have been used as markers for reduced fertility due to decline in uterine pH during the early luteal phase of the estrous cycle. A more acidic uterine environment is less conducive for early stages of embryo development and maintenance of pregnancy in cattle (Rhoads *et al.,* 2006).

Body condition score is usually used to monitor nutritional and health status of high producing cows during their reproductive cycle. Cows in low BCS at calving or that suffer from excess BCS loss early postpartum are less likely to ovulate, have a reduced submission rate to artificial insemination, increased calving to conception interval and have an increased likelihood for pregnancy loss. Each individual dairy cows has a genetically programmed target BCS that she attempts to reach approximately 10-12 weeks after calving. If her body condition is above this target feed intake is reduced and she loses condition; if her BCS is below this target, feed intake is increased and she gains condition (Garnsworthy and Wiseman, 2006). The ability of cows to reach their target BCS is also affected by diet composition.

- ✓ With high-energy diets, thin cows can be in positive energy balance and increase BCS, but fat cows will be in negative energy balance and decrease BCS. With low energy diets, feed intake is limited by physical capacity of the rumen and thin cows can‟t increase intake to match milk energy output; fat cows mobilize body condition at a faster rate to support milk production and are in a NEB longer than with high-energy diets (Jones and Garnsworthy, 1989). Conception rate decreases by 10% per 0.5 unit BCS loss due to NEB that strongly associated with attenuation of LH pulse frequency and low levels of blood glucose, insulin, IGF-I that collectively limit estrogen production by dominant follicle; with diminished quality of oocytes and capability for embryo development; and with reduced serum *P4* concentration (Butler, 2005).

- ✓ High-protein diets result in greater loss of body condition by fat cows, but either increase gain or decrease loss of body condition by thin cows which use excess protein for gluconeogenesis (Garnsworthy and Jones, 1987; Jones and Garnsworthy, 1989).

- ✓ Low fiber high-starch diets increase BCS gain in thin cows and decrease BCS loss in fat cows, probably by increasing insulin status (Garnsworthy and Jones, 1993).

- ✓ High fat diets decrease BCS loss in fat cows, but do not affect BCS change in thin cows (Garnsworthy and Huggett, 1992). The main signaling factor was suggested to be leptin, a peptide hormone secreted by adipose tissue, and leptin has been shown to decrease appetite by direct action on the hypothalamic satiety center (Vernon et al., 2001). It also modulate nutrient transfer and partitioning by interaction with other hormones including insulin, glucagon, glucocorticoids, GH, IGF-I, cytokines and thyroid hormones (Hill, 2004).

The influence of the body condition score on follicular development manifested only at the level of medium and large follicle populations, and the significant increase in number of large follicles with increase in body condition score indicate that changes in body nutrient reserve have an effect on follicular development and maturation in cattle (Maina *et al.,* 2008). The negative correlation between body condition score and the incidence of anestrous cows on one hand and that of cows with abnormal ovarian cyclicity on the other agrees with the report that cows that are losing body weight or are thin do not have estrus cycles or have irregular cycles. Apart from cessation of estrus cycle in seriously malnourished and emaciated cows, there is restoration of normal ovarian activity and conception when the state of under-nutrition or NEB is reversed (Maina *et al.,* 2008). On the contrary, fertility in cows that are over conditioned at calving (BCS≥3.5; 5-point scale) is also compromised as they have reduced dry mater intake (DMI) just prior to calving, take longer time to increase DMI postpartum, tend to have greater fat mobilization and therefore a more severe NEB early postpartum than cows with an optimum BCS at calving (Roche *et al.,* 2009).

2.2.2 Breed

Differences in the reproductive characteristics such as gestation length, puberty age, duration and intensity of estrus, and twinning rate between *Bos taurus* and *Bos indicus* cattle were documented. These breed disparities in several reproductive characteristics are based on gonadotropin and steroid secretion, follicular growth and luteal development, and/or the secretion of metabolites and metabolic hormones that have vital roles in reproduction. Study made by Alvarez *et al.,* (2000) on *Bos indicus* (Brahman) cows, temperate *Bos taurus* (Angus), and tropically adapted *Bos taurus* (Senepol) confirmed the idea. In this study the two breeds adapted to the tropics had greater follicular activity than the unadapted Angus cows, and greater number of small and medium follicles observed in the Brahman and Senepol cows. The greater concentration of metabolic hormones in Brahman *vs* Angus and Senepol cows suggests a greater activity of the somatotropic axis in Brahman cows than in Angus and Senepol cows. However, the activity of the gonadotropic axis was shown to be greater in Angus than in Brahman and Senepol cows revealed by the greater concentrations of follicle-stimulating hormone found in Angus cows (Alvarez *et al.,* 2000). Inevitably it is accepted that the dose of FSH needed to induce superovulation in *Bos taurus* is 30 to 50% higher than the dose recommended for *Bos indicus* cattle (Barati *et al.*, 2006; Silva *et al.,* 2009). Accordingly, lower amount of FSH needed to induce superovulation in Zebu cattle was suggested to be related to the smaller size of the ovarian structures (follicles and corpora lutea) which is the characteristics of the breed (Figueiredo *et al.* (1997).

2.2.3 Reproductive stage

The age of the animal is one of the determinant factors as lesser embryo production from aged cows may be related to follicular and endocrine changes that occur as age increases. Although the follicular wave pattern in older animals is similar to those in young cows, old cows have fewer small ovarian follicles recruited into a follicular wave, and have fewer large follicles after superovulation (Malhi *et al.,* 2006). Furthermore, old cows have elevated circulating concentrations of FSH, similar to aged women with a low storage of ovarian oocytes (Silva *et al.,* 2009).

High-producing dairy cows have been selected to produce more milk, in large part through their ability to mobilize fat and muscle to support milk production in early lactation, which results in a loss of BCS, alterations in blood metabolites and hormone profiles, which in turn affect fertility (Shrestha *et al.,* 2004). The NEB was associated with a greater incidence of irregular patterns of the postpartum luteal activity (Wathes *et al.,* 2007), and these irregularities consisted of a delayed first ovulation, prolonged luteal phase (PLP), short luteal phase and ovarian cysts. Substantial increase in energy requirements to facilitate the dramatic increases in daily milk yield, which peaks between 4 and 8 weeks postpartum is only partially met by increased feed consumption (due to limitations in intake and appetite) with remainder being met by mobilization of body reserves resulting in animals entering NEB (Grummer, 2007). The consequences of severe NEB are, therefore, increased risk of metabolic diseases, reduced immune function and a reduction in subsequent fertility.

Cows nursing their own calves have reduced reproductive performance. This is because suckling depresses the duration of induced LH surge by suppressing follicular LH receptor populations, and preventing the reliability of exogenous hormonal treatments to counteract the effects of suckling. On the contrary, removal of the suckling stimulus increases pituitary responsiveness to GnRH, increases serum LH and FSH concentrations, and follicular fluid concentrations of prolactin, which together or separately cause an increase in LH and FSH receptors in the largest follicle (Walters *et al.,* 1982b; Margerison, 2002).

2.2.4 Season

On the evolution, animals were selected by environmental pressure to give birth in seasons when food supply was ample, and reproductively seasonal animal uses photoperiod as a cue, aiming a more favorable period for offspring birth. Furthermore, acclimation to changes in thermal environment and photoperiod are homeorhetic processes that are commonly referred to as seasonal changes in domestic animal biology involving changes in hormonal signals as well as alteration in target tissue responsiveness to hormonal stimuli over weeks rather than days. Hormones known to be homeorhetic regulators (thyroid hormones, prolactin, somatotropin, glucocorticoids, and mineralocorticoids) are also implicated in acclamatory responses to thermal stress and altered photoperiod (Collier *et al.,* 2006). The mechanism may include altering the GnRH secretion followed by LH and FSH release from the pituitary, so that ovulation is concentrated in a more successful season (Anivaldo *et al.,* 2012). However, the literature generally presents conflicting results regarding the seasonal reproduction in cattle. In *Bos indicus* both the absence of seasonal effect on serum progesterone concentrations, greater *P4* during decreasing photoperiod or even the opposite were described: CL showed larger area and volume during the summer compared to winter, associated to an increased capacity of *P4* production in response to LH (Anivaldo *et al.,* 2012).

Generally genetic differences in heat tolerance between animals appear to be exacerbated under high temperature conditions. For example, the success rate of superovulation was different during the particular seasons. In the spring, 88.89% of the cows positively responded to superovulation, only 80.00% in the summer and the highest proportion (89.47%) was recorded in the autumn (Stadnik *et al.,* 2014). There is evidence that *Bos indicus* embryos are less sensitive to heat stress than *Bos taurus* embryos. The superior thermoregulatory ability of *Bos indicus* cattle makes these animals better adapted for hot climates than many *Bos taurus* breeds. Thus, it seems that the thermoregulatory mechanisms of *Bos indicus*, as well as some adapted *Bos taurus* breeds (Barati *et al.,* 2006), are not severely affected by heat stress. As a result, tropical breeds are able to respond to the superovulation treatment even in a relatively warm environment (Silva *et al.,* 2009). Furthermore, preimplantation embryos from *Bos indicus* cattle are better able to withstand thermal stress than are embryos from *Bos taurus* cattle. Thus, major advances in environmental management of dairy cattle include improved housing and cooling systems, improved ration formulation based on altered requirements during thermal stress improves reproductive performance (Collier *et al.,* 2006).

Heat stress also alters the follicular development pattern in cattle. Exposing cows to heat stress during a complete estrous cycle altered the dynamics of the turnover of the first and second follicular waves by reducing the size of the dominant follicles. Roth *et al.,* (2000) and Gibbons *et al.* (1997) had characterized the effect of heat stress as immediate and delayed effects on follicular development and function. The immediate effect of heat stress was manifested in depression of dominance expressed by a pronounced decrease in plasma immuno reactive inhibin, and consequently increased FSH concentrations. This altered hormonal secretion led to early emergence of the second (preovulatory) follicular wave and an increased number of large follicles during the follicular phase. The delayed effect of heat stress was expressed by a marked increase in FSH secretion and a reduction in the number of medium sized follicles. Both the immediate and the delayed responses to heat stress were shown to be involved in reducing fertility of cattle during the summer and autumn (Roth *et al.,* 2000).

Previous studies have shown that reduction in follicular size in heat stressed cattle is related to decreased steroidogenesis within the theca cells, granulose cells or both. The possible mediators of heat stress response were reduced variability of granulose cells or more specific changes in steroidogenic enzyme aromatase activity in granulose cells. A direct effect of heat stress on small follicles explains that they are particularly sensitive to physiological changes that occur during heat stress. Torres *et al.,* (2008) demonstrated that in Gir cows (*Bos indicus*) heat stress exerted a delayed effect on reproductive functions, manifested by an increased incidence of large follicle, more follicular co-dominance and reduction in estrus cycle length, progesterone concentration and oocyte developmental capacity. Heat stress doesn"t affect follicular recruitment, since the population of >3 mm follicles were not affected. However, heat stress increased the number of large follicles and number of follicles >9 mm characterized as follicular co-dominance which may compromise oocyte quality in cow. It is agreed up on that heat stress inhibited follicular growth and follicular dominance, decrease proestrus rise in estradiol-17β and induce smaller size of the second wave dominant follicle and longer luteal phase, decrease biosynthetic capacity of theca cells but has less effect on granulose cells. These changes can account for the well documented low breeding efficiency during warm months in subtropical environments (Roth *et al.,* 2000). De Rensis and Scaramuzzi (2003) described the decreased intensity of estrus signs, reduced heat duration, increased incidence of anestrus, and more frequent silent heat under higher temperature, which may also disrupt follicular development and its dominance in Holstein dairy cows.

2.3 Ovarian follicular dynamics in cattle

2.3.1 Folliculogenesis and follicular development

Folliculogenesis is the progression of small primordial follicles into large preovulatory follicles that enter the estrous cycle in estimated time course of 80 to 100 days in bovine regulated by signals between the oocyte and surrounding epithelial cells, other somatic cells in the ovarian cortex and by signals emanating from systemic circulation (Britt, 2008). In follicular developmental hierarchy the primordial follicle includes an oocyte with a visible nucleus surrounded by a single flattened layer of pregranulosa cells where as the primary follicle includes a larger oocyte with a visible nucleus surrounded by cuboidal granulosa cells. Primordial follicles may undergo progressive development soon after they are formed or they may remain dormant for years before beginning further development (Cushman *et al.,* 2002; Yang and Fortune, 2008). The larger secondary follicle includes an enlarged oocyte surrounded by 3 to 4 layers of cuboidal granulosa cells and thicker cytoplasmic membrane due to the appearance of zona pellucida layer secreted by the oocyte. Once progressive follicle growth begins there are a number of local and systemic regulatory factors such as insulin, IGF-I, steroids (estradiol, testosterone), gonadotropins, and abroad array of growth factors required to sustain growth of the follicle to the fully mature Graafian stage preceding ovulation (Britt, 2008).

Fundamental reproductive processes in farm animals are characterized by emergence, selection and dominance followed by either atresia or ovulation of the DF governed by the prominent role of FSH and LH. In bovine the beginning of gonadotropin dependent follicle development is typified by the emergence of 5–20 cohort of follicles correlated with a transient increase in FSH concentrations and FSH receptors (FSH-R) localized within the granulosa cells (Ginther *et al.*, 2002; Crowe and Mullen, 2013). This enables FSH to perform its required downstream signaling effects including promoting cellular growth and proliferation leading to an increase in aromatase enzyme activity that converts androgen to estrogen in the granulosa cells of ovarian follicles known as two cells/two gonadotropin model. The increase in diameter of the DF selected from the cohort of follicles leads to an increase in estradiol and inhibin concentrations in follicular fluid, and actively suppresses FSH, thus preventing further follicle wave emergence until the DF either undergoes atresia or ovulated (Crowe and Mullen, 2013).

Luteinizing hormone receptor (LH-R) is localized in the theca and granulosa cells of healthy follicles at different stages of follicle development (Camp *et al.*, 1991). As the follicle grows the theca cell LH-R increases, and granulosa cells acquire LH-R in the follicle undergoing selection to become the DF and estradiol producer since it is dependent on sufficient LH pulse frequency (Bao *et al.,* 1997; Braw-Tal and Roth, 2005). The binding of LH to its receptors in the theca cells drives the conversion of cholesterol to testosterone through a series of catalytic reactions. Testosterone, once produced in the theca cells, diffuses out into the granulosa cells where it is converted to estrogens by the aromatase enzyme (Crowe and Mullen, 2013). During the follicular phase of the estrous cycle, when progesterone concentrations are basal, the large concentration of estradiol produced by the pre-ovulatory DF induces a GnRH surge from the hypothalamus. The resulting LH surge is of sufficient amplitude and frequency to stimulate final maturation and ovulation of the DF (Sunderland *et al.,* 1994). The increased estradiol concentrations also induce expression of estrous behavior, required for successful mating (Crowe and Mullen, 2013).

During the early luteal phase lesser amplitude and greater frequency (20 – 30 pulses/ 24 hrs.) of LH pulses occur. In the mid-luteal period LH pulses are of greater amplitude and lesser frequency (6 – 8 pulses/ 24 h) both of which are of insufficient amplitude and frequency for final maturation and subsequent ovulation of the DF. Thus, the DFs produced during the luteal phase of the estrous cycle undergo atresia, E_2 and inhibin production decreases, and removes this negative feedback block to the hypothalamus/pituitary, FSH secretion can increase and a new follicle wave emerges (Crowe and Mullen, 2013).

2.3.2 Follicular waves pattern during estrous cycle

The wave pattern of follicular development refers to periodic synchronous growth of a group of antral follicles. The mechanism involved with follicular wave dynamics is based on differential responsiveness of the ovary to FSH and LH (Adams, 1994). Periodic surges in circulating concentrations of FSH are responsible for eliciting follicular wave emergence; hence, cows with two-wave cycles have two FSH surges and three-wave cycles have three surges (Adams, 1994). Circulating FSH is subsequently suppressed by negative feedback by estradiol and inhibin from the emerging follicles and the following nadir in FSH effectively prevents new wave emergence. The transient rise in FSH permits sufficient follicular growth so that some follicles acquire LH responsiveness which allows survival without FSH. At the time of follicle selection, 2 or 3 days after wave emergence, FSH is declining rapidly. The follicle destined to become dominant apparently acquires receptors for LH and has the competitive advantage over follicles destined to becoming subordinate (Mapletoft *et al.,* 2002). However, LH responsiveness and the ability to become a dominant follicle likely represent a quantitative rather than an absolute difference between follicles in a wave. Continued suppression of LH as a consequence of luteal-phase progesterone secretion causes atresia of the dominant follicle, and FSH is again allowed to surge. This surge has no effect on the dying dominant follicle, but is responsible for eliciting the emergence of the next wave. Relief from progestational suppression (i.e., luteolysis) allows LH pulse frequency to increase, permitting further growth of the dominant follicle and dramatically higher circulating concentrations of estradiol, which results in a surge of LH followed by ovulation (Adams 1994; Mapletoft *et al.,* 2002).

Ovarian follicular dynamics in cows and heifers is characterized by waves of follicular growth and regression showing one, two, three or four follicular waves during estrous cycle (Ginther *et al.,* 1989; Forde *et al.*, 2010; Crowe and Mullen, 2013). The waves start approximately on days 2 and 11 in cycles with two waves or on days 2, 9 and 16 of the cycle in cycles with three waves of growth while the CL begins to regress earlier in two-wave cycles (day 16) than in three-wave cycles (day 19) resulting in a correspondingly shorter estrous cycle (20 days *VS*. 23 days, respectively) (Mapletoft *et al.,* 2002; Rosales-Torres *et al.,* 2011). Fortune *et al.,* (2001) reported that in *Bos taurus* cattle, dominant follicle selection occurs when the follicle reaches a diameter of 8 mm while subordinates only reach 7 mm and deviation or selection occurs approximately on day 2 after onset of follicular wave (Fortune *et al.,* 2001). The dominant follicle of each wave of growth continues to grow at an accelerated rate (1.6 mm/day) and if its development coincides with CL lysis and the decrease of *P4*, it may ovulate with a diameter between 12 to 20 mm in *Bos taurus* (Adams, 1999; Evans, 2003) or 7 mm in *Bos indicus* (Gimenes *et al.,* 2008; Rosales-Torres *et al.,* 2011). Apparently, when the largest follicle reaches a decisive developmental stage, rapid development of the deviation mechanism blocks development of the second-largest follicle before it reaches a similar diameter. Thus, rather than selection of a dominant follicle, selection involves an action against the other follicles in the wave (Ginther *et al.,* 2003). Increasing endogenous FSH and/or administering FSH early in a wave for superovulation thus induce several follicles to become dominant. A subordinate follicle remains viable for at least 1 day after deviation starts, and can become the new dominant follicle if the existing dominant follicle fails or is removed. The FSH decline is necessary for the establishment of deviation, and with decreasing serum FSH concentrations, follicles begin to undergo changes including reduced production of estrogens, reduced levels of higher molecular weight (MW) inhibins and increased amounts of lower MW IGF factor binding proteins, and culminating in granulosa cell apoptosis (Sunderland *et al.,* 1996; Mihm *et al.,* 1997). In this environment, only the dominant follicle is able to continue to grow and produce steroids, and hence, blood estradiol concentrations begin to increase at the beginning of deviation in cattle (Ginther *et al.,* 2003a). Inhibin (probably inhibin-A) is produced by multiple follicles before deviation and by the developing dominant follicle after the start of deviation and suppresses FSH during the entire FSH decline. The largest and the second-largest follicles at similar diameters had similar follicular-fluid estradiol concentrations during the common-growth phase; increasing concentrations were detected beginning at 7.6–7.7 mm in heifers (Ginther *et al.,* 2003). Estradiol began to increase differentially in the largest follicle 4 h before diameter deviation, and these increase the expression of genes for aromatase, and receptors for FSH and LH in granulosa cells (Bao and Garverick, 1998).

Many factors, mentioned earlier in the text, like breed, reproductive stage, season, heat stress, energy balance and body condition affects follicular growth and atresia (Figueiredo *et al.,* 1997; Burke *et al.,* 1998; Wilson *et al.,* 1998; Crowe and Mullen, 2013). Moreover, many authors characterized the luteal phase length as the main determinant of the number of follicular waves. Because it keeps high concentrations of progesterone, corpus luteum blocks ovulation of functional dominant follicles, inducing their atresia and the onset of a new follicular wave (Ginther *et al.,* 1989; Viana *et al.,* 2000).

2.4 Reproductive technologies in cattle

The first type of biotechnologies that affected the efficiency of male and female reproduction are: artificial insemination, estrous synchronization, multiple ovulations and embryo-transfer, ova pick-up and in-vitro-fertilization, twining, sexing of semen and embryos cloning (Renaville and Burny, 2005). The impact of these technologies is mainly advanced dissemination of superior germplasm increase the selection intensity, and increase the level of accuracy obtained when testing animals. In the past, artificial insemination has been a very successful biotechnology that enhanced greatly the genetic progress. A second generation of biotechnologies improved the determination of the genetic merit of animals.

2.4.1 Artificial insemination

AI is the manual placement of semen in the reproductive tract of the female by a method other than natural mating. It is the foremost biotechnological technique to be applied to improve farm animals‟ breeding, particularly in dairy cattle reproduction and genetics (Foote, 2002). Globally AI allowed the transfer of genes from a "superior" male to a large number of offspring, and breed improvement took place much more quickly than with natural mating. Furthermore, obsessive advantage of AI has been reduction of the risk of diseases transmission and physical injuries to cows from natural mating, make use of weak, old or crippled elite sire, early detection of infertile bulls, use of and elimination of the dangers of handling unruly bulls, and conservation breeding of rare or endangered species (Foote, 2002).

Potential use of marker-assisted selection can improve accuracy of early selection of candidate bulls to enter progeny test programs. However, despite the abundance of current research in genetic markers, genetic improvement for dairy cattle is still dependent primarily on a successful AI progeny test program, and evaluation of genetic merit of individual animals based on performance of offspring (Andrabi and Moran, 2007).

The use of AI in domestic animal reproduction was originally pioneered for sanitary reasons. Accordingly, several important developments including new AI techniques, novel sperm diluents, protocols for synchronizing estrus and ovulation, and the availability of gender-sorted semen have further increased the use and importance of AI as an assisted reproductive technique. However, achieving high fertility with AI requires excellent management of all phases of the AI program: semen collection, processing and delivery. Furthermore, fertility depends on the ability of others to correctly handle, thaw the semen and inseminate cows at the proper time (Kasimanickam, 2015). Damage to sperm during cryopreservation, storage, and thawing has been attributed to cold shock, ice crystal formation, oxidative stress, membrane alteration, cryoprotectant toxicity, and osmotic changes. Knowledge of semen tank management, proper thawing and semen handling techniques, and sanitary insemination in the correct location at the correct time are critical and should be periodically reviewed (Kasimanickam, 2015). For substantial success to be achieved it need due concentration and attention to the detail and clear understanding of reproductive anatomy, timing of insemination and the ability to identify the site of deposition for proper placement of the semen.

2.4.2 Estrous synchronization in cattle

Estrous synchronization is the manipulation of estrous cycle in order to bring a group of females at random stages of the estrous cycle into estrus at a precise time (Loony *et al.*, 2005). Naturally, emergence of follicles >4 mm in size is FSH dependent, and growth of normal estrogen-active follicles beyond 8-9 mm in size is dependent on the presence of adequate LH. Normal luteal phase LH pulse frequencies /amplitudes are required to cause DF turnover (atresia of DF); increased LH pulse frequencies maintained by the presence of continuous progesterone maintain estrogen-active DF for an extended period of time whereas follicular phase LH pulse frequencies stimulate final maturation and ovulation of DF (Crowe and Mullen, 2013).

Physiologically luteolysis by PGF2α is characterized by a cessation of *P4* production and a breakdown of cellular components through the cumulative effect of reduction of vascular supply, proliferation of connective tissue, increased cellular disorganization, and degeneration and phagocytosis of the luteal cells (Attila, 2005). Furthermore, the dramatic decrease in luteal blood flow, changes in membrane permeability, altered activity of steroidogenic enzymes, inhibition of lipoprotein stimulated steroidogenesis, alteration of nuclear chromatin conformation, decrease in the number of small lutein cells, decrease in gonadotropin receptors, vasoconstriction within the luteal vasculature resulting in hypoxia and endothelin-1 (ET-1) release from resident endothelin cells. ET-1 inhibits basal and LH-stimulated *P4* biosynthesis in small and large luteal cells directly via ET-1 action on ET receptors. Additionally, ET-1 alters arachidonic acid metabolism resulting in a net increase in the production of PGF2α and a reduction in the proposed luteotropin, PGI2, by bovine luteal tissue (Attila, 2005). Indeed, prostaglandin does not directly affect follicular wave dynamics, and the timing of estrous onset is dependent on follicular status at time of PGF administration. If administered at dominance of a follicular wave, the onset of estrus gets reduced (2–3 days). If PGF is given before follicular dominance is attained the interval from administration to onset of estrus get extended to 4–5 days to allow development of an ovulatory follicle followed by a rise in secretion of gonadotrophins and oestradiol-17β culminating in the pre-ovulatory surges, and eventual ovulation (Savio *et al.,* 1990).

Use of PGF2α and progesterone

In normally cycling cows and heifers the control of the estrous cycle is dependent on manipulation of the hormonal events occurring during the normal ovarian/estrous cycle. The fall in peripheral *P4* concentrations may be manipulated artificially in two ways: by artificial inducting premature luteolysis using luteolytic agents (prostaglandins), and by stimulation of CL function with administration of progesterone for a number of days followed by abrupt withdrawal. Injection of exogenous PGF2α or one of its analogues during the mid-luteal phase of the cycle results in a premature luteolysis and consequential fall in peripheral *P4* concentrations. The recent development of protocols designed to control both luteal and follicular function permit the initiation of synchronization of recipients at a self-appointed time. The most widespread approach to estrous synchronization in cattle is through the use of PGF2α because synchronized regression of the CL synchronizes a decline in progesterone and result in the final growth of the dominant follicle to produce estradiol and behavioral heat (Looney *et al.,* 2005; Day and Geary, 2007; Mapletoft *et al.,* 2009). However, in order for PGF2α to be effective, females must be cyclic and has to have matured CL on their ovaries. The responsiveness of cows to PGF at various times in the cycle is diagrammed in Figure 1.

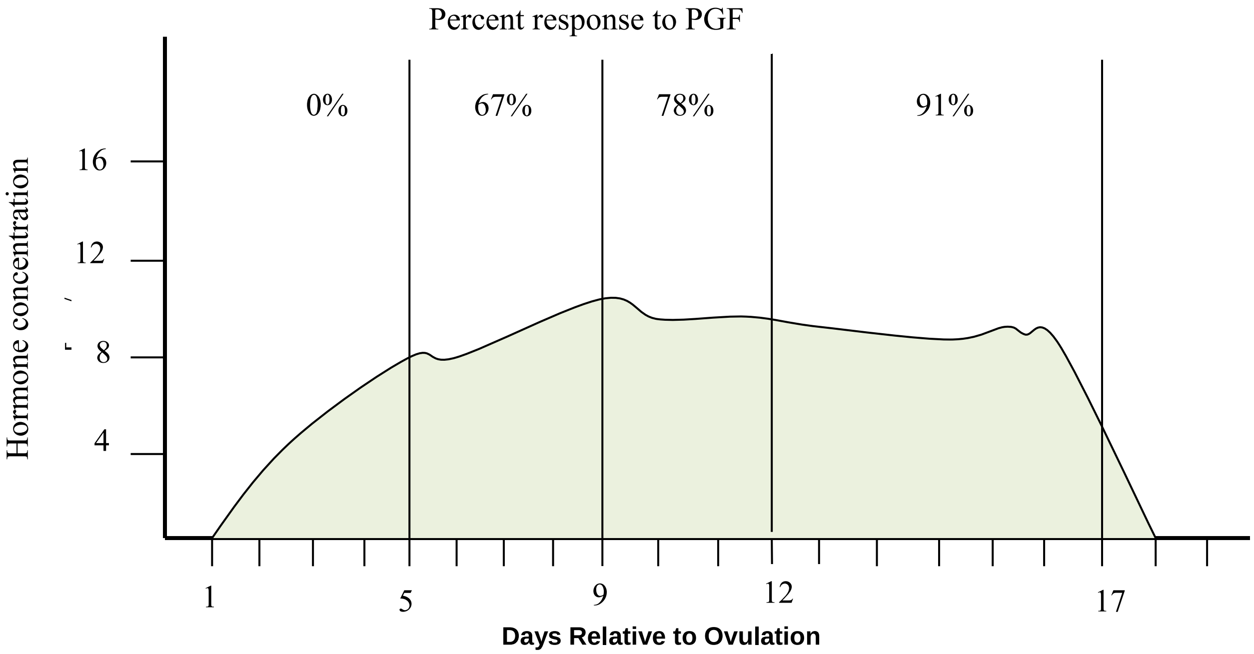

Figure 1. Stage of the Estrous Cycle and CL Regression with PGF2α

Source: Day and Geary, (2007)

PGF2α doesn‟t regress an immature CL (Days 1 to 5), and the effectiveness of this treatment increases with each subsequent day of the cycle after Day 5. Anestrous cows and pre-pubertal heifers do not respond to an injection of PGF2α since no CL exists. PGF2α programs to be used to synchronize estrus in cattle can be in single or double injection and/or other combinations to increase effectiveness of the estrous synchrony (Looney *et al.,* 2005; Bo et al., 2006). The single injection program is accomplished with insemination after PGF2α, and ~70% of the cyclic cows (those on Day 6 or greater of the cycle at injection) would be expected to display heat during the next four to five days, and get inseminated (Day and Geary, 2007).

The two injection programs for synchronization with PGF2α are designed to increase the proportion of females with a CL that is responsive to regression with PGF2α. The two injections of PGF2α administered 11 days apart, but recent data suggest that a 14-day interval is more effective (Mapletoft *et al.,* 2009). Those animals that were not responsive to the first injection (i.e., on Days 1 to 5 of the cycle at the first injection) would respond to the second injection and provide another opportunity for AI (Lamb, 2010). Both of these protocols require detection of estrus for the insemination.

The other technique for estrous synchronization in cattle is the integrated use of PGF2α and progestin. The addition of progestin has two advantages: induce some pre-pubertal heifers and anestrous cows to being cycling and to have a normal-length estrous cycle following the first ovulation; and progestin prevent animals that are cyclic from showing heat and typically increase the proportion of animals with CL that are responsive to PGF2α at the time this hormone is injected (Day and Geary, 2007). The disadvantage of progestin is that abnormal follicular growth occurs when estrous is inhibited by progestin in the absence of a CL and the progesterone it normally produces. In this situation, the dominant follicle does not undergo atresia and persists on the ovary (persistent follicle). When the progestin is withdrawn, the persistent follicle ovulates, and the ovum released is of lowered fertility (Lamb, 2010).

In systems that use progestin, three approaches are taken to avoid this reduction in fertility. The first approach is to avoid the problem and wait until the next heat period to inseminate. The second option is to use a short period of progestin exposure to minimize the reduction in fertility. The third method is to administer a treatment at the start of the synchronization treatment that reset follicular growth and prevent the development of persistent follicles (Day and Geary, 2007).

The most commonly used progestin-PGF2α system (Fig 2) involves the use of progesterone delivered by means of a CIDR and PGF2α. Progestin alters ovarian function in cattle by suppressing estrus and preventing ovulation by suppressing LH release which in turn suppresses growth of the dominant follicle for estrous synchronization (Mapletoft *et al.,* 2003). With this system, the CIDR is inserted into the vagina of the female for seven days. Injections of PGF2α is given either one day before or on the day of CIDR withdrawal, and get inseminated based upon detection of estrus during the three- to five-day period following treatment (Bo *et al.,* 2006; Lamb, 2010; Mapletoft *et al.,* 2013). This system effectively synchronize estrus in a large proportion of cyclic females since treatment for seven days with the CIDR prevents animals from being in the early stage of the cycle and unresponsive to regression of the CL by PGF2α. Fertility is normal at the synchronized estrus in yearling heifers or postpartum cows receiving this synchrony system (Day and Geary, 2007). Re-use of CIDRs in this system for a second or third time delivers progesterone at a lower level and causes development of persistent follicles in some females.

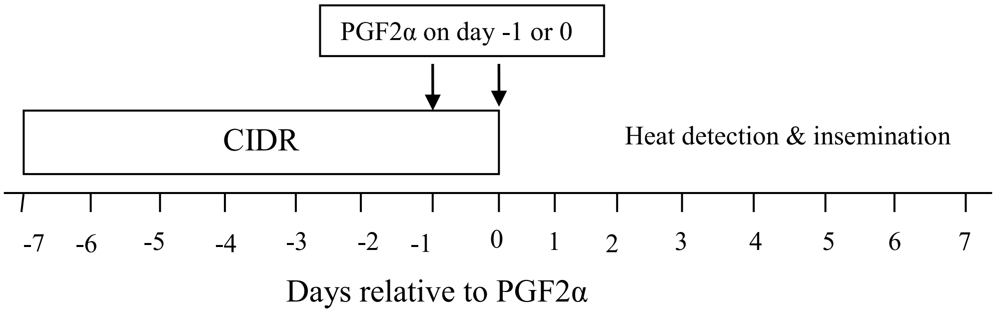

Figure 2. CIDR and PGF2α synchronization system

Alternatively estradiol treatments had been used for estrous synchronization at the commencement of progesterone based treatment (CIDR or PRID) to suppress FSH and allow FSH to rebound to stimulate follicle wave emergence 48-72 h after administration of estradiol. However, use of estradiol as part of an estrous synchronization program in cattle is now not permitted in many countries (Lane *et al.,* 2008).

None of the systems described up to this point have included treatments that address the physiological requirement of managing follicle growth to ensure that follicles ovulate ova of normal fertility and to standardized stage of follicular growth to cause the timing of estrus to be more precise and/or synchronizing ovulation for timed AI. Each of the preceding systems permits the females to show heat based upon spontaneous follicle growth, and they are inseminated on detection of estrous. However, the use of GnRH (OvSynch and Co-Synch; Figure 3) provides a means to standardize the pattern of follicular growth in a majority of animals with the advantage of avoiding the development of persistent follicles, and synchrony of the time of ovulation for timed AI (Bo *et al.,* 2006; Mapletoft *et al.,* 2009; Crowe and Mullen, 2013). The GnRH in the OvSynch protocol with PGF2α (GnRH 100 μg and 25 mg PGF2α 7 days later) eliminates the large follicles by ovulation or atresia and induces emergence of a new follicular wave within 3-4 days after treatment during any stage of the estrous cycle (Looney *et al.,* 2005). By the addition of CIDR with twice GnRH in the GnRH program (CO-

Synch) the additive effect of progesterone along with the ability to initiate a new follicle wave with the GnRH will be utilized.

The GnRH is given the first day of synchronization to program emergence and growth of the subsequent wave of follicles through inducing luteinization and/or ovulation of the existing dominant follicles in the ovary. A second GnRH helps to synchronize ovulation at a chosen time for timed AI (Lamb, 2010).

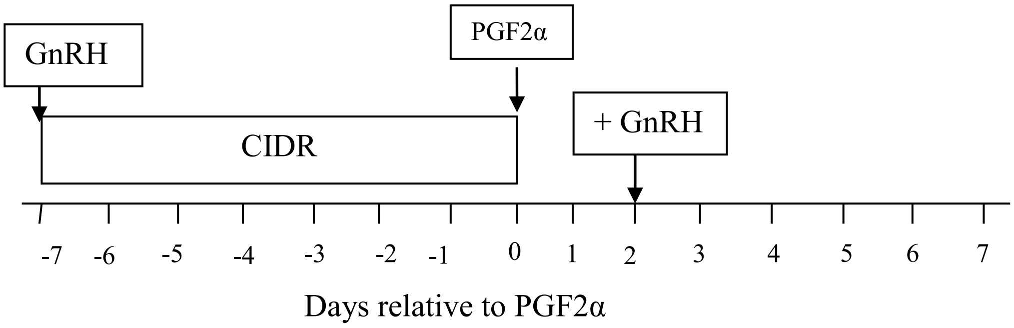

Figure 3. GnRH-CIDR-PGF2α based estrous synchronization for fixed time AI (FTAI)

This system is the most effective if used on cyclic cows, or anestrous cows that would be expected to spontaneously resume estrous cycles within the first couple of weeks of the breeding season. The initial GnRH injection (Day 7) is used to program follicle growth in cyclic females and to induce ovulation (to provide progestin pre-exposure) in anestrous females. The PGF2α (Day 0) induces regression of existing CL to induce a decline in progesterone. The second GnRH given on Day 2 to Day 3 (+GnRH) induces ovulation of dominant follicles that have been pre-programmed by the first GnRH treatment (Day and Geary, 2007; Lamb, 2010).

2.4.3 Superovulation in cattle

Superovulation is the process of maximizing the number of fertilized and transferable embryo with a high probability of producing pregnancy using exogenous gonadotropin hormones. Three different types of gonadotropin preparations have been used to induce superovulation in the cow: gonadotropins from extracts of porcine or other domestic animals pituitaries, equine chorionic gonadotropins, and human menopausal gonadotropins (Mapletoft and Bo, 2015). With the allied use of PGF2α it became possible to initiate gonadotropin treatments at predetermined time during the estrous cycle (FSH during mid-cycle, i.e. 8-12 days after estrous). However, individual variation among females in response to superovulation remains the largest and least understood event. Some females consistently produce large numbers of embryos in response to superovulation, while other females of similar age, breed, management, etc. perform poorly (Mapletoft and Bo, 2015). General factors responsible for the variability of superovulation response can be either intrinsic or extrinsic to the donor (Kafi and McGowan, 1997). The intrinsic factors include genetics (breeds and individual animals that are more or less sensitive to gonadotrophin), physiological characteristics (including age, ovary conditions or follicular dominance, and the population of follicles at the time of superovulation), nutritional status (body condition and deficits or excesses of nutrients) and sanitary conditions (ovary, uterus and oviduct pathologies). The extrinsic factors include the use of different commercial preparations of FSH (recombinant or pituitary-derived FSH with varying amounts of LH, equine chorionic gonadotrophin (eCG) and human menopausal gonadotrophin (hMG) and inhibin), dosage, route of application, season, and farm management (Kafi and McGowan, 1997; Reuben *et al.,* 2002; Silva *et al.,* 2009).

Gonadotropin sources and preparations

Three different types of gonadotrophins have been mostly used to induce superovulation in the cow: gonadotrophins from extracts of porcine or other domestic animal pituitaries, eCG and hMG (with an estimated 5 h or less biological half-life of FSH in the cow) (Reuben *et al.,* 2002).

Equine chorionic gonadotrophin is a complex glycoprotein with both FSH and LH activity having a half-life of 40 h in the cow and known to persist for up to 10 days in bovine circulation, and hence, it is normally injected once (doses range from 1500 to 3000 IU). One problem with eCG is that the prolonged half-life can mean that residual FSH activity continues to stimulate follicle growth after ovulation occurs with a negative effect on fertilization rate and/or zygote development (likely due to the high estradiol concentrations from these additional growing follicles) (Crowe and Mullen, 2013). The long half-life of eCG has a consequences of continued ovarian stimulation, unovulated follicles, abnormal endocrine profiles and reduced embryo quality, and counteracted by the intravenous injection of antibodies to eCG at the time of the first insemination (12 to 18 h after the onset of estrus) (Dieleman *et al.,* 1993; Gonzalez et al., 1994).

Although folliculogenesis in mammals requires both FSH and LH, there is a considerable variability in FSH and LH content of crude gonadotrophin preparations. Previous research reports have shown that purified pituitary extracts with low LH contamination have been reported to improve the superovulatory response in cattle (Chupin *et al.,* 1984; Reuben *et al.,* 2002). It has been suggested that embryo quality may be adversely influenced by high LH levels during super stimulation due to the premature activation of the oocyte. On the contrary, doubling the dose of LH-rich preparations (FSH-P or hMG) resulted in significantly reduced fertilization rates and percentages of transferable embryos, and hence, the detrimental effects of high doses of pituitary gonadotrophins on ova/embryo quality is due to an excess of LH (Alkemade *et al.,* 1993).

Even if it is generally believed that LH is required for successful superovulation, some argue that endogenous LH levels may be adequate. Looney *et al.* (1988) reported that recombinant bFSH induced high superovulatory responses without the addition of exogenous LH (fertilization rates exceeded 95% and viable embryo rates exceeded 85%). The very high fertilization rates and transferable embryo rates in the absence of exogenous LH revealed that the administration of LH, at any dose, may be detrimental to embryo quality. An experiment done by Willmott *et al.,* (1990) that have shown that more ovulations, ova/embryos collected, and more fertilized ova in the two treatment groups with the least LH level (16% LH – equivalent to Folltropin®-V; and preparation with 98% LH removed (Pure FSH) in *Bos taurus* breed substantiate the idea (Reuben *et al.,* 2002). Another experiment done on Brahman-cross (*Bos indicus*) heifers by Tribulo *et al.,* (1991) showed that the most purified preparation induced more CL, ova/embryos and fertilized ova when compared to the least purified preparation, and corroborate the previous findings.

One way to circumvent the difficulty due to variations in the content of contaminating LH in LH: FSH ratio in commercially available FSH preparations isolated from pituitary glands is the isolation of pig FSH to high purity. However, superovulation of cattle with purified pig FSH led to varied responses from a complete lack of ovulation to a normal ovulation rate but with a small number of embryos to a normal number of transferable embryos (Takagi *et al.,* 2001). This problem has been resolved by use of recombinant DNA technology (recombinant human FSH (rhFSH), recombinant bovine FSH (rbFSH) and recombinant pig FSH (rpFSH), which enables production of FSH in the complete absence of contaminating LH. It was also indicated that recombinant FSH has also been used for superovulation in cattle; rbFSH resulted in normal production of viable embryos, indicating that exogenous LH activity is not necessary for follicular maturation and ovulation in gonadotrophin-stimulated cattle (Takagi *et al.,* 2001).

Route of administration

The route of administration of superovulatory agent is indicated to be one of the factors among the multiple factors contribute to the variability of superovulatory response. For instance, twice daily intramuscular injections of Folltropin®-V resulted in a significantly higher superovulatory response than twice daily subcutaneous injections (Reuben *et al.,* 2002).

2.4.4 *In vivo* embryo production and management

ET is the safest and most cost effective reproductive biotechnology that facilitates rapid genetic improvement (increased selection intensity and reduced generation intervals) in the dairy and beef cattle industries, increases the number of offspring obtained from donors with high genetic value, and used to disseminate desirable genetics around the world (Baruselli *et al.,* 2006; Jahnke *et al.,* 2015). Since the advent of ET numerous technologies have been introduced and adopted by the dairy industry including non-surgical embryo recovery and transfer, closely timed hormonal synchronization of donor and recipient estrous cycles, reliable cryopreservation of embryos and direct transfer following thawing, embryo splitting, embryo sexing, production of in vitro derived embryos, and cloning.

The commercial bovine embryo transfer industry arose in North America in the early 1970"s to overcome international health and trade restrictions on live animal (Betteridge, 2003; Mapletoft, 2012). The successive improvement in implementation protocols such as MOET program and nonsurgical embryo recovery has brought about substantial contribution of the technology in increasing the number of offspring obtained from donors with high genetic value (Baruselli *et al.,* 2006). However, sizeable number of research findings evidenced that many environmental and genetic factors control the success of MOET technology. Breed difference is the one to mention at the forefront. The physiology and reproductive behavior differences between *Bos indicus* and *Bos taurus* cattle are crucial, and includes greater sensitivity to gonadotropins, a shorter duration of estrus, smaller diameter follicles at deviation and at the time of ovulatory capability, and higher circulating concentrations of hormones (estradiol, progesterone, insulin and IGF-I) despite having smaller ovulatory follicle size and corpora lutea in *Bos indicus* (Baruselli *et al.,* 2006; Sartori *et al.*, 2011). Contrary to this conclusion Tegegne *et al.,* (1997) showed similarity or slight difference between Boran (*Bos indicus*) and Boran*Holstein in terms of numbers of palpable corpora lutea (ovulation rate) and large anovulated follicles, number of embryos/ova recovered per superovulation, and number of transferable embryos per superovulation. Indeed the average embryo production per super-ovulation is a good indication of the ability of a breed of cows/heifers to produce embryos.

Besides the contribution of ET in genetic improvement and reducing risk of disease transmission in global germplasm exchange, it has and economic advantage in intercontinental transport of live animals whereby an entire herd can be transported in the form of frozen embryos (Mapletoft, 2012). Accordingly the development of effective methods of freezing embryos has made embryo transfer a much more efficient technology. Consequently embryos were frozen very successfully in glycerol for many years. However, glycerol being cryoprotectant that penetrate cell membranes slowly and has to be removed slowly after thawing necessitate microscopic examination and time for dilutions in the laboratory. Recently, the use of highly permeating cryoprotectants such as ethylene glycol has allowed the direct transfer of bovine embryos thawed in water-bath and directly deposited into the uterus of the recipient much like AI without causing osmotic stress and avoiding complicated procedures with use of glycerol (Mapletoft, 2012). The survival rate of cryopreserved/thawed embryos in this approach is nearly comparable with fresh embryos (Shaw *et al.,* 2000a). Nevertheless, despite the wider use of cryopreservation of embryos in commercial embryo transfer conventional cryopreservation is a slow procedure which exposes the embryo at various phases of freezing to the action of many physical, chemical and biological factors. These factors can cause disruption of the zona pellucida, cell membranes or cytoskeleton and metabolic disturbances. Such cell damage leads to loss of self-control of the cell and eventually to its death by apoptosis or necrosis (Baguisi *et al.,* 1999; Mapletoft, 2012).

The success of embryo transfer depends not only on many factors associated with the embryo donors (genetics, nutrition, ovarian status, response to gonadotrophin super stimulation discussed elsewhere in the manuscript), embryo and recipient factors, but also on the embryo management from the time of flushing to deposition in the recipient (Lamb, 2005). This is because the procedure of removing an embryo from its natural uterine environment, freezing and thawing increases the level of stress experienced by that embryo and could result in decreased survival rate of the embryos. Conventional cryopreservation methods consist of several steps:

(1) Pre-equilibration: embryos are exposed to a simple salt solution containing a permeable cryoprotectant (1, 2-propanediol, DMSO, glycerol, ethylene glycol, etc.) and usually a low concentration of non-permeable cryoprotectant (sucrose).

(2) Cooling: after a brief time of exposure to allow uptake of cryoprotectant and initial dehydration, the cells are cooled rapidly to a temperature slightly below the melting point of the solution (usually around -7^{0}C).

(3) Seeding: at this point the container with the cells is super-cooled in a process known as „seeding" so that ice forms in the extracellular solution.

(4) Slow cooling: upon ice formation and further cooling at a slow rate (usually -1^{0}C/min to below -30^{0}C), the osmolarity of the extracellular solution increases as water freezes to ice, causing the cells to dehydrate with the increasing tonicity.

(5) Plunging/vitrification: dehydration continues during slow-cooling until the cells are plunged into liquid nitrogen, usually at a temperature below -30^{0}C. At this point the intracellular cryoprotectant concentration is high enough so that the remaining intracellular water vitrify, preventing Intracellular ice formation (IIF).

(6) Thawing and rehydration: during thawing, the dehydrated cells are exposed to hypotonic conditions and rehydrate as the cryoprotectant is removed (Stachecki and Wiemer, 2007).

In the process of freezing and thawing of embryos two major cryopreservation protocols were being commonly used: conventional slow cooling and vitrification. Regarding the physico-chemical processes, they both aim to protect the cells from chilling injuries, intracellular ice formation, dehydration and toxic effects. Highly efficient methods have been developed for in vivo-produced bovine embryos, using different cryopreservation protocols. Nevertheless, the outcome is mainly influenced by many factors including the physiological properties of embryos directly related to their chilling sensitivity.

Cryopreservation exposes embryos to numerous types of stress, and damage can occur at any time throughout the cryopreservation process, and may be manifested in different ways and at different times. Extreme types of damage such as IIF and cell fracture lead to overtly blatant effects such as the cell lysis and death, and/or a variety of effects on embryo morphology, ranging from subtle damage on intracellular organelles, cytoplasm, or processes that can negatively affect normal cell development, and is more difficult to diagnose. Many underlying factors can influence the outcome of cryopreservation: the quality of the embryo, type of freezing protocol used (slow-cooling or vitrification), the concentration and type of cryoprotectant(s) affect subsequent morphology (Smith and Silva, 2004). Some of these factors are discussed as follows.

Intra- and interspecies differences

The effects of cryopreservation on intracellular organelles may not be limited to *in vitro* produced embryos. A recent study suggests that high quality cattle embryos from different breeds appeared morphologically similar to their non-frozen counterparts. Furthermore, embryos from *Bos tarus* had higher amounts of intracellular lipids than embryos from *Bos indicus*, and notably embryos from *Bos tarus* had a slightly better morphological appearance than their *Bos indicus* counterparts (Smith and Silva, 2004). The *in vivo* development of cryopreserved embryos was also influenced by genotype and cryopreservation method (slow-cooling *vs* vitrification). In other words, differences in the developmental ability of embryos of different genotypes became apparent only after cryopreservation (Stachecki and Wiemer, 2007).

Embryo origin

The origin of the embryo itself may have a profound impact on the survival of embryos following cryopreservation. In cattle *in vivo* developed embryos commonly have less irregularity at the morula stage compaction, have uniform blastomeres. Expanded blastocysts also contain distinct inner cell masses that are not dark in appearance, and hence, less sensitive to cryopreservation than their *in vitro* counter parts (Hasler, 2001). Some of the inherent differences may be due to suboptimal maturation and culture systems, inability to adapt cryopreservation methodologies to suit the kinetics of *in vitro* embryos, and inabilities to manipulate energy substrates or biochemical pathways following thawing (Hasler *et al.,* 1997; Hasler, 2001).

Embryo quality

The initial quality of embryos prior to cryopreservation is also a determining factor in their resistance to the freezing process. In a recent study, cattle embryos of differing quality were cryopreserved, thawed and evaluated has shown that the degree of apoptosis was directly related to the initial morphology of the embryo prior to freezing: embryos of good morphology had significantly lower degrees of apoptosis than embryos of poor morphology (Marquez *et al.,* 2005). In higher quality blastocysts apoptotic cells were more prevalent within the ICM, whereas in blastocysts of lesser quality apoptotic cells were randomly distributed. It was suggested that embryos of higher quality (based on their appearance) have better resistance to the damage often associated with cryopreservation. Seasonal effects were also noted and embryos collected during the seasonally stressful time, when natural forage is less abundant, had a higher proportion of lyzed cells following cryopreservation than embryos produced during more ideal conditions (Stachecki and Wiemer, 2007).

In cattle embryos the quality of embryos prior to cryopreservation has a significant effect on the morphology following thawing due to the osmotic effects associated with the introduction and removal of cryoprotectants (Wiemer *et al.,* 1995). In cattle, there was evidence of vacuolization of the nucleus, apoptotic cells were present within the inner cell mass of high quality blastocysts, or throughout the cells of lower quality cattle embryos (Visintin *et al.,* 2002; Marquez *et al.* 2005). In general, only embryos of the highest quality are frozen, due to the stresses involved with cryopreservation, which can further reduce an embryo‟s chance of full development. Embryos of suboptimal morphological quality sometimes, but not always, have poorer survival rates and significantly higher proportion of lyzed blastomeres, and birth rate was three times higher after the transfer of fully intact embryos, when compared with the transfer of damaged embryos (van den Abbeel *et al.,* 1997).

Embryo ultra-structure

Cryopreservation can have effects on the zona pellucida and cell membrane, in addition to intracellular components. For example, the zona can be damaged by cracking, splitting, elongation, and distortion, caused by different stresses during freezing and/or thawing. However, the zona is not always damaged during cryopreservation, or it may be damaged to differing degrees, some of which may not be microscopically apparent. In the case of frozen–thawed blastocysts, distinctive cell types within the embryos themselves may be affected differently by this process. Morulae that were frozen, thawed, and cultured prior to fixation often produced blastocysts that were delayed in development, with reduced cell numbers within the trophectoderm and the ICM (Iwasaki et al., 1994).

Freezing methods

The problems that can occur during cryopreservation differ in association with slow cooling and with vitrification. Problems associated with slow cooling that have been described include IIF and osmotic effects, whereas chemical toxicity is a major obstacle with current vitrification techniques. The two major category of cryoprotectants include propanediol, glycerol, DMSO, and ethylene glycol (permeable) and sucrose and trehalose (impermeable). All of these cryoprotectants cause the embryo to undergo changes in osmolarity. During pre-equilibration, the cell is exposed to a hyperosmotic environment that allows some dehydration, along with uptake of permeable cryoprotectant(s). If the dehydration is too severe, membrane damage can occur, sometimes resulting in blebbing of the membrane. The cryoprotectant can be toxic to the cell if the concentration is too high (Stachecki and Wiemer, 2007). The toxicity of cryoprotectants is also related to the temperature at which they are used: the higher the temperature, the greater the toxicity. Cryoprotectant toxicity may result in immediate cell lysis, or lysis after thawing. The pre-equilibration process is usually performed in a series of steps, in order to reduce the stress of dehydration and facilitate cryoprotectant uptake. During initial cooling, temperature shock may occur and damage the cell, resulting in lysis or degeneration following thawing. Temperature shock is most likely to occur in species that are „chill-sensitive‟, including bovine and porcine (Martino *et al.,* 1996).

The process of seeding can also cause cell damage. Seeding is usually done by touching the side of a cryovial or straw with liquid nitrogen-cooled forceps, and if the cooled area is too close to the cells they may freeze and then die upon thawing. During slow cooling, the remaining solutes become more concentrated as water freezes, and this exerts hypertonic pressure on the cell, resulting in its further dehydration and osmotic stress. At this time, the concentration of cryoprotectant increases to potentially lethal levels. If the cell is not sufficiently dehydrated during the slow cooling process and the intracellular concentration of cryoprotectant is not sufficient to intercalate with the residual water inside the cell, intracellular ice may form and kill the cell. If the cell is excessively dehydrated, it may be incapable of rehydrating sufficiently to resume normal function after thawing (Mazur 1990).

Intracellular component

Embryos may be adversely affected by cryopreservation at the intracellular level with the potential of altering the function of the intracellular organelles and cytoplasm, protein structure and function, and metabolism. As a result embryos require a period of „recovery" following cryopreservation, before they are able to continue normal intracellular function. Embryos that are able to compensate for lost or decreased function are the ones most likely subsequently implant to and develop further (Stachecki and Wiemer, 2007).

The effects of cryopreservation on cellular metabolism are difficult to assess morphologically, but this important aspect must also be considered. Disruption of mitochondrial membranes leads to loss of protons and reduces the oxidative potential for ATP production. Rieger *et al.,* (1992) noted that cryopreservation of horse and cattle embryos caused an increase in glutamine production, possibly due to a disruption in mitochondrial ATP production and therefore a flux in the Krebs cycle. Gardner *et al.,* (1996) also noted that the process of freezing and thawing on IVF-produced bovine blastocysts had a significant effect on nutrient uptake and utilization. The freeze–thaw process had a negative impact on the rate of glucose and pyruvate uptake as well as lactate production, and the viable embryos do not recover the metabolic activity recorded prior to freezing. The same author noted that damage to the mitochondria may have resulted in changes in oxidative phosphorylation, thus increasing oxygen consumption.

Zona hardening due to both cortical granule releases (occurs naturally during egg activation and/or fertilization) and *in vitro* culture may impair the embryo"s ability to hatch during the time of implantation. Cryopreservation may also contribute to zona hardening over and above what would occur naturally, and this may affect blastocyst hatching (Stachecki and Wiemer, 2007).

Intracellular lipids

It is well known that embryos from certain species including cow are substantially more sensitive to injury from a reduction in temperature than are mouse or human embryos. *In vivo* embryos sink in 2.35 M sucrose solutions, while their *in vitro* counterparts float in solutions containing more than 1.6 M sucrose (Leibo and Loskutoff, 1993). This may be due to altered ratios of lipids to proteins found in cell membranes. *In vitro* produced bovine embryos have higher lipid content, making them less buoyant, and this lower buoyancy renders the embryo more sensitive to chilling and freezing when compared with its *in vivo* counterpart. Furthermore, differences in lipid phase transition (the progression from one phase or physical state to another) were observed between *in vivo* and *in vitro* matured oocytes. This suggests that alteration of membrane composition affects chill sensitivity, and subsequently survival following freezing (Arav *et al.,* 1996; Stachecki and Wiemer, 2007).

Thawing

Numerous types of injury can occur during thawing. The process of taking a cell from a resting temperature of -196^{0}C and warming it to 0^{0}C or higher over the period of a minute or less, is an extreme temperature change. For example, a straw is usually held in room temperature air for a period of time before further warming in a water bath (usually 30^{0}C). Thawing that is too rapid can result in large and/ or small fractures in the zona pellucida or the cell itself, as described previously. These fractures are caused by a non-uniform change in the volume of the medium during rapid phase changes that occur during thawing (Rall and Meyer, 1989). If the cell membrane fractures it lyzes immediately, but even if the cell membrane does not fracture intracellular components may be fractured or damaged leading to the eventual demise of the cell. During re-warming, ice can form once again if the vitrified solution warms at a rate that permits the process of devitrification. This occurs when the temperature reaches a point where the molecular mobility of water increases so that water molecules can move and rearrange themselves from a disorderly amorphous vitrified position to an orderly crystalline position. This happens well below the melting point, and is therefore a potentially lethal problem (Luyet, 1970; Stachecki and Wiemer, 2007). IIF at this stage can lead to cell lysis and/or damage to organelles or other intracellular components. Damage of this type may not be apparent from simple observation of morphology. During the final step of thawing, the previously frozen cell may be very dehydrated and must undergo rehydration and removal of cryoprotectant(s) in order to continue development. Because water permeates more rapidly than does cryoprotectant, the cell may swell and lyze in the process of trying to remove the cryoprotectant. Sucrose is usually used in the step-out process in order to reduce the osmotic effects (Stachecki and Wiemer, 2007). Damage at this stage or during the next several hours of culture may be manifested as cell expansion and rupture, lysis, or a darkening of the cytoplasm and cell death. Sometimes the cell appears to have survived thawing, but after culture fails to develop, lyzes, or degenerates.

Recently embryo freezing procedures is being replaced by a relatively simple procedure called vitrification (use of high concentrations of cryoprotectants) and placement of the embryo in its cryoprotectant solution directly into liquid nitrogen (Mapletoft, 2012). Normally formation of intracellular ice crystals is considered the most harmful factor which can occur under specific conditions of freezing and thawing with negative effect on recuperation and survival of embryonic cells. Because of the high concentration of cryoprotectants and the ultra-rapid freezing rate used, ice crystals do not form; the frozen solution forms a “glass”. Since ice crystal formation is one of the most damaging processes in freezing, vitrification has much to offer in the cryopreservation of oocytes, IVEP embryos and biopsied embryos (Mapletoft, 2012).

2.5.5 *In vitro* embryo production (IVEP) and management

The IVEP is a useful tool to multiply improved genotypes and is an alternative to conventional embryo transfer encompasses three major steps: *in vitro* maturation (IVM); *in vitro* fertilization (IVF) and *in vitro* culture (IVC). Naturally the dormant primordial follicles become active and undergo progressive development at regular intervals from the late fetal stage to the adulthood. Once activated, follicles and oocytes in a cohort either grow to maturation and ovulation (estimated to take 100 days) or undergo atresia again and again until the pool in the ovaries get depleted (Britt, 2008).

The primary method of collecting oocytes from live cattle, called ovum-pick-up (OPU), is aspiration of ovaries imaged per rectum by a vaginally inserted ultrasound probe and needle. All follicles between 3 and 8 mm in diameter can be aspirated and the collected oocytes can be used to generate in vitro developed embryos. Nevertheless, numerous factors such as the vacuum pressure used to remove the oocyte from the follicle, the gauge of the needle and the length of the bevel on the needle, and the frequency of collection, breed of cattle, age of the donor affect the number of intact cumulus-oocyte complexes collected (Hasler and Barfield, 2015).

IVM of mammalian oocytes, nuclear and cytoplasmic maturation, is defined as the sequence of events occurring from the germinal vesicle stage to completion of the second meiotic division (Blanco *et al.,* 2011). The nuclear maturation is characterized by the oocyte's ability to resume meiotic division up to metaphase II during *in vitro* maturation, and can be visualized by the extrusion of the first polar body and the appearance of the second metaphase plate (Blanco *et al.,* 2011). In bovine cumulus cells are able to secrete estradiol and progesterone in culture systems for IVM. The FSH hormone activates cumulus cells expansion *in vitro* in a similar manner as the LH surge acts in vivo (Blanco *et al.,* 2011). And hence, removal of an oocyte from the inhibitory follicular environment followed by favorable culture conditions (with the addition of hormones including LH, FSH, estradiol 17β & epidermal growth factor) results in spontaneous maturation and morphological changes (e.g. expansion of cumulus cells) without the physiological serious of events that occur *in vivo* (Hasler and Barfield, 2015). However, the oocytes maturation status dictates how they must be handled after collection. Immature oocytes can be collected from unstimulated cows undergoing OPU every 3-7 days, irrespective of the stage of their estrous cycle; however, it is also possible to time aspiration after ovarian stimulation and injection of LH or gonadotropin-releasing hormone (GnRH) so that one collects mature oocytes (Hasler and Barfield, 2015). These *in vivo* matured oocytes do not require further maturation *in vitro* through some hours in maturation medium prior to fertilization may be beneficial.

IVF is a technique where sperm is co-incubated for and fertilize an ovum in a laboratory dish or test tube. The presence of cumulus cells, the duration of sperm–oocyte co-incubation (mostly 12-18 hours), and the concentration of sperm are major factors for successful *in vitro* fertilization. Furthermore, for the proper fertilization the spermatozoa must undergo the changes that enable it to fertilize, called capacitation. Cumulus cells, their conditioned medium and hyaluronic acid, which are present in the cumulus mass and oviductal fluids, induce sperm capacitation, an acrosome reaction that enhances the fertilization rate (Iwata *et al.,* 2008). Temperature is also a critical factor for the successful capacitation, and in cow it is typically done at 38.5^{0}C (Hasler and Barfield, 2015).

The primary capacitation agent added to fertilization media is heparin, which is thought to assist in the removal of seminal plasma components from the sperm surface by binding to proteins and stimulating the efflux of cholesterol and phospholipids. Other capacitation agents include caffeine, an inhibitor of phosphodiesterase‟s; bicarbonate which induce protein kinase A-dependent changes in lipid architecture; adenosine which interacts with membrane receptors and increases intracellular cyclic Adenosine monophosphate (cAMP); and reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and nitric oxide which activate membrane targets to trigger intracellular mechanisms including protein tyrosine phosphorylation (Hasler and Barfield, 2015).

In vitro culture (IVC) of bovine embryos is the last step in the IVEP process and involves approximately 6 days of culture from the 1-cell zygote stage following IVF to the blastocyst stage endpoint. The synthetic oviductal fluid (SOF)-based culture may involve one medium formulation for the duration of IVC or a two or three step sequential system in which the medium formulation changes to certain points in the culture period (Hasler and Barfield, 2015). Sequential system are based in an attempt to mimic the physiological changes that *in vivo* zygotes encounter as they move down the oviducts and in to the uterus during the first 6 or 7 days of development. The volume of the medium used in IVC system also varies, with some laboratories culturing embryos in micro drops as small as 25µL under a layer of oil, ranging up to 500 µL with or without an oil layer. Some IVC systems are conducted in an incubator atmosphere of 5% CO_2 in air, however, there is significant evidence that in the absence of coculture, bovine embryos develop better in a 5% CO_2, 5% O_2 and 90% N_2 atmosphere (Hasler and Barfield, 2015).

Although the intrinsic quality of oocytes determines embryo quality, the microenvironment of the *in vitro* production system /culture systems largely influences the development and quality of embryos. There are evidences that IVP embryos have fewer cells and lower proportions of ICM cells to trophoblast cells than *in vivo* embryos at the same stage of development and this variation can be related to different culture system.

2.5 Ultrasonography in bovine gynecological examination

In contrast to therapeutic ultrasound (physiotherapy) and lithotripsy (calculi disintegration), diagnostic ultrasound is considered to be safe for uses associated with reproductive management (Lamb, 2005). Previous studies have shown that sequential ultrasonography was used to identify whether follicles were growing (increasing in size), static (no change in size), or regressing /decreasing in size (Ginther, 1989b).

From a research standpoint, ultrasound has given us the ability to visually characterize the uterus, fetus, ovary, CL, and follicles in a less invasive ways and more effective in identifying follicles <5 mm in diameter than rectal palpation (Hanzen *et al*., 2000). Ultrasonographically a cross-sectional view of the uterus is displayed as a “rosette” and is easily distinguished from other peripheral tissues. Pregnancy diagnosis via ultrasound during days 18 to 22 of gestation yields excellent results. Furthermore, pathological applications for ultrasound technology have extended to identifying endometritis, pyometra, mucometra and hydrometra (Perry *et al.,* 1990). Today the application of ultrasound has impacted the monitoring of follicular dynamics associated with wave emergence, follicular atresia, and follicular dominance. An efficient exploitation of the female gamete pool without compromising donor fertility became possible by harvesting developmentally competent cumulus oocyte complexes (COC) for IVF (Lamb, 2005). Ultrasound-guided technology provides an excellent alternative for the aspiration of small and preovulatory follicles from clinically infertile animals, cows in their first trimester of pregnancy, and from cows stimulated biweekly with Pregnant mare serum gonadotropin (PMSG) or FSH to enhance follicle numbers (Singh *et al.,* (1997; Singh *et al.,* 1998).

Although the ultrasound frequencies commonly used for diagnostic purpose ranges from 1.0 to 20 megahertz (MHz), the most commonly used transducers for gynecological examination operates between 3.0 and 7.5 MHz. For most clinical bovine reproductive applications a 5-MHz linear array transducer are the most versatile and effective. That unit performs adequately on early pregnancy examinations; fetal sexing; pathologic ovaries; and, in general, most all reproductive uses. A 7.5-MHz linear transducer may be more practical if the ultrasonographer intends to do research on follicular dynamics. For transvaginal oocyte recoveries for *in vitro* embryo production, a convex linear transducer gives the technician much more flexibility in gaining access to the hard-to-reach follicles as compared with a linear transducer (Stroud, 2005).

Computer-assisted analyses of echotexture (pixel heterogeneity) characteristics of high-resolution ultrasound images of the follicle antrum and wall were correlated with the functional and endocrine status (estradiol or the estradiol: progesterone ratio) of the follicle (Singh *et al.,* 1998; Tom *et al.,* 1998b). The mean pixel value (obtained by spot analysis of randomly selected spherical area) and heterogeneity (a measure of the variation in gray-scale value of pixels falling under the measuring circle) of the antrum and wall were low in preovulatory follicles as well as in growing and early-static phase anovulatory dominant follicles. In late-static and regressing phase subordinate follicles pixel heterogeneity was high (Singh *et al.,* 1997; Singh *et al.,* 1998; Tom *et al.,* 1998b). Singh *et al.,* (1997) and Tom *et al.* (1998a) found that pixel values of ultrasound images, obtained by quantitative echotexture analysis, decreased from metestrus to mid-diestrus, and increased during proestrus. Pixel values of ultrasound images were highly correlated to plasma and luteal tissue progesterone concentrations and volume densities of luteal cells and stroma (Lamb, 2005).

CHAPTER 3

3. MATERIALS AND METHODS PAGES 44-50

3.1 Location

The experiment was carriedout at Ethiopian Institute of Agricultural Research, Debre Zeit Agricultural Research Center (DZARC). DZARC is located about 45 km east of Addis Ababa, the capital city of Ethiopia (8°46′13.57"N, 38°59′50.45"E) at an altitude of 1920 masl. The average annual temperature for the last five years was 18.5°C with an average annual rainfall of 757.05 mm (DZARC, 2015). DZARC has a dairy cattle improvement farm where a total of 160 dairy cattle (Boran and Boran*Holstein crosses) are reared. The farm has a forage production area where alfa alfa and other green forages are produced. Further, the farm has different holding areas for lactating animals, calves and heifers. As a research center, DZARC also has facilities for other livestock, crops and various specialized laboratories.

3.2 Experimental animals

Primiparous pure Boran and Boran*Holstein cows were used for this study. Breeding and clinical records of all cows were reviewed, and only cows without any history of reproductive problems were considered for the study. All cows underwent a thorough reproductive examination, and only cows free of reproductive abnormalities were included. Selected cows had a mean body condition score (BCS) of 7 (on a scale of 1 to 9; 1= emaciated; 9 = obese). Cow BCS was recorded at the beginning, middle and end of the study. All cows were managed under uniform management and housing system. They were provided with feed of different mix: *tef* straw (*Eragrostis tef)* and grass hay (*Andropogon abyssinicus*) as a basal diet and supplemented with commercially prepared concentrate, mineral salts and alfalfa green fodder up to a limit of 3% life body weight per cow.

3.3 Follicular dynamics

Boran (n=9) and Boran*Holstein (n=8) crossbred cows aged between 6 to 8 years were selected for this study after thorough clinical and gynecological examinations. The mean BCS for study cow in this experiment was 7 (on a scale of 1 to 9; 1 = emaciated; 9 = obese). All cows were serially scanned for 61 days using a real time B-mode ultrasound (CTS 3300, Altay Scientific, Rome, Italy) with a 5.0-7.5 MHz linear array rectal transducer to monitor ovarian follicular dynamics. At each scanning the number of ovarian follicles and the number of CL were counted on both ovaries; the diameters of the three largest ovarian follicles and the CL were also measured using the internal electronic caliper. Date of disappearance of a dominant follicle and CL formation were also recorded as date of ovulation. Retrospectively, the number of waves of ovarian follicular growth was identified from recorded data. The mean inter-ovulatory interval (IOI; days between two consecutive ovulations) in days, the date of wave emergence and date of divergence/deviation were determined for each ovarian cycle. The first day of detection of the disappearance of dominant follicle was defined as the day of wave emergence on each wave (day zero). The numbers of follicular waves were determined by the non-identity technique of ultrasonic data management by scanning the three large follicles on everyday scanning of the ovaries.

About 10 ml of blood was collected from all cows via jugular venipuncture every other day through the 61-days experiment to determine the progesterone profile. Samples were submitted to the Ethiopian Institute of Public Health, Addis Ababa for analysis. Serum progesterone concentrations were determined by an Elecsys Progesterone II assay system using „Elecsys and cobas e immunoassay analyzers‟ (Cobas®, Roche Diagnostics GmbH, Mannheim, Germany) with the sensitivity of 0.030‑60.0 ηg/mL (lower and upper detection limits, respectively), and 5.4 and 2.9 intra assay and inter assay CV of the test.

3.4 Superovulation experiment

A total of 36 apparently healthy primiparous and multiparous cows (25 Boran and 11 Boran x Holstein crossbreds) between 6 and 8 years of age with an average BCS of 7.0 were selected for this experiment. The superovulation treatment regime is illustrated in Figure 4. Cows of both genotypes were implanted with a CIDR (Progesterone 1.38 gm, Hamilton, New Zealand) for seven days (Day 0 = date of CIDR insertion). On Day 4 cows were randomly allocated into one of the three FSH treatment groups: 300 IU, 250 IU, or 200 IU (Pluset®, Barcelona, Spain). FSH was administered in a series of eight intramuscular injections given over four days in a decreasing dose regimen (Table 2).

Table 2. Three FSH dose treatment groups for superovulation of Boran and Boran*Holstein cross cows

Group	No of cows	Day 4		Day 5		Day 6		Day 7	
		Am dose	Pm dose	Am dose	Pm dose	Am dose	Pm dose	Am dose	Pm dose
300 IU	14	60IU	60IU	45IU	45IU	30IU	30IU	15IU	15IU
250 IU	8	50 IU	50 IU	35 IU	35 IU	25 IU	25 IU	25 IU	25 IU
200 IU	14	40 IU	40 IU	30 IU	30 IU	20 IU	20 IU	10 IU	10 IU

All cows received an intramuscular injection of a prostaglandin F2α analogue (Estrumate®, Intervet Shering-plough, Beaucouze, France) on Day 6, and the CIDR was withdrawn on Day 7 at 6 PM concurrent with the last (8th) injection of FSH. All cows were meticulously monitored to determine the onset and duration of estrus until the last cow exhibited estrus. Cows were inseminated twice (in the morning and afternoon) based on the manifestation of standing heat to capture the possibility of extended ovulation. Cows were subjected to ultrasonic evaluation to determine the number of CL, and presence of unovulated follicle/s. Superovulatory response was determined based on time from CIDR removal to onset of estrus, duration of estrus, and the total number of CL present on both ovaries. Cows were manually palpated to determine the number of CL before they were ultrasonically (CTS 3300, Altay Scientific, Rome, Italy) evaluated.

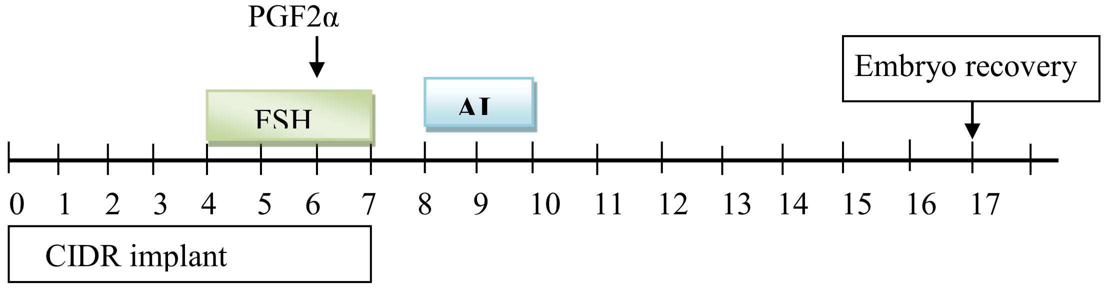

Figure 4. Superovulation and embryo recovery protocol used in Boran and Boran*Holstein cows

Before embryo recovery was attempted on Day 17 the number of corporalutea and the number of unovulated follicles was determined. Non-surgical embryo recovery was performed seven days after the second AI with commercially prepared flushing medium (ViGRO™, Bioniche Animal Health, Manhattan, USA). A two way bovine Foley catheter (Silicon Foley catheter: 18 Fr 650 mm length; Disposable Y- junction connector and MiniFlush embryo recovery filter; MOFA®, Canada) with a standard closed gravity flow method was used to collect the embryos.

3.5 Evaluation and grading of embryos

Harvested unfertilized ova and/or embryos were transferred from the embryo filter to a search dish containing holding medium. Searching was carried out under a stereo-microscope (Motis SMZ 140/143®, Roanoke, USA), when found, recovered specimens were sorted into two groups: embryos and UFO. The embryos were graded for their developmental stage (from stage 1 = 1-cell to stage 9 = expanded hatched blastocyst) and quality (from quality 1 = excellent to quality 4 = degenerate) according to the standards given by the International Embryo Transfer Society (IETS) as described and illustrated by Bó and Mapletoft, (2013) and Jahnke *et al.* (2015) (Annex 1). IETS developmental stages 4- 8 with a quality grade of 1 or 2 were considered transferable; all others were considered non-viable.

3.6 Embryo cryopreservation

Viable embryos were prepared for cryopreservation using the traditional slow-cooling/rapid-thawing equilibrium method of embryo freezing (Youngs, 2011). Embryos were placed for 10 minutes into a commercially prepared embryo freezing medium containing 1.5 Molar ethylene glycol and 0.3 Molar sucrose (BoviPRO™, MOFA®, Canada) before being loaded individually in a 0.25 ml straw. All straws were placed into the heat transfer chamber of a portable embryo freezing machine (EFT-3002, BELTRON Instruments®, Colorado, USA) filled with 90% ethanol at a temperature of -6.5 °C. Embryo loaded straws were seeded after 2 minutes, held for an additional 8 minutes, and then slowly cooled at a rate of 0.6 ^{0}C per minute to -32 ^{O}C. After a 10-minute hold at -32 °C, straws containing the embryos were plunged into a liquid nitrogen (-196^{O}C) for a final storage. Some embryos were fresh transferred to synchronized recipients. Recipients were checked for establishment of pregnancy on day 28 of the transfer by using ultrasound. The presence of fluid in the uterus detected by ultrasound and beating heart of the conceptus observed was recorded as positive result for pregnancy establishment.

3.7 Thawing, incubation and post-thaw evaluation of Boran embryos

Embryos were thawed 30 days after freezing by removing an individual straw from the liquid nitrogen Dewar. The straws were held at room temperature for 3-5 seconds, and placed into a water bath at +37°C for 25-30 seconds. After blot drying the straw, the heat-sealed end of the straw was cut off and the contents of the straw were emptied into a 4-well culture plate containing a commercial thawing medium (BoviPRO™ Thawing Medium, MOFA Global, Wisconsin, USA) pre-warmed to 37 °C in an incubator. Embryos were then morphologically evaluated under a stereomicroscope and were assigned an IETS rating for their stage of development and quality. The thawed embryos were then transferred into a culture medium (TCM199; Sigma-Aldrich, Missouri, USA) supplemented with 10% bovine serum albumin and incubated at 37^0C for 48 hours. All embryos were evaluated for their developmental stage and quality after 24 and 48 hours.

3.8 Data management and analysis

The Non-Identity Method methods were used to characterize changes in follicle diameter during the IOI (Ginther, 1993). The diameters of the follicles occupying the largest, second largest, third largest categories, and total count of follicles >4mm were then plotted daily during the IOI, regardless of individual identity. Follicle number data were combined for both ovaries. For statistical and illustrative purposes, folliclar diameter and number data were normalized and centralized to the number of follicle waves observed. Profiles of the mean diameter of the largest three follicles throughout the IOI, as determined by the Non-Identity Method, were normalized to the mean IOI for cows exhibiting two or three waves of follicle development during the cycle to 20 days and 23 days IOI for two and three waves, respectively, according to Mapletoft et al., (2009). Normalized and centralized follicle diameter and follicle number data were then truncated to day 20 (i.e., day 0 - first ovulation) for the data analyses.

For each wave a descriptive analysis (Mean ± SEM) was performed for the following characteristics: Maximum diameter of the DF [mm], Onset of atresia of the DF [day], Growth rate of the DF [mm/day], Wave length [days] and Inter-wave interval [IWI, days]. The

PROCMixed model of the SAS (SAS, 2004) was used to compare follicular dynamics of the two breeds (n = 1037 ovarian scanning) which showed genotype differences. Ultrasonic follicular data was managed using a non-identity (Mathetmatical) technique previously described by Ginther, (1995). One way ANOVA was used to analyze the IOI in days, size of preovulatory and second and third largest follicles. Descriptive statistics and univariate analysis of the SAS were used to analyze the genotype response to FSH treatments. Frequency distribution, chi-square test and one way ANOVA were used to analyze the data for rate of embryo recovery and results of laboratory evaluation. The level of significance was held at $P<0.05$.

CHAPTER 4

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4.1 Ovarian follicular dynamics

The overall mean (±SEM) interovulatory interval (IOI) was 19.8 ± 0.21 days, whereas IOI for Boran and Boran*Holstein crosses was 19.4±0.34 and 20.1±5.2 days, respectively, and was not affected by genotype. Table 3 shows the IOI and the diameters of the three largest ovarian follicles. The mode (maimum repeated value) IOI for Boran cows was 20 days (44.4% of cows) while 22.2% had 18 or 19 days; and 11.1% had 21 days). The mode IOI for Boran*Holstein cows was 21 days exhibited by 37.5% of cows.

Table 3. IOI [days], number of follicular waves, size of the dominant and subordinate follicles [mm] and rate of follicular growth of dominant follicle [mm/day] after in Boran and Boran*HF cross cows

Parameter	Genotype	N	Mean (±SEM)	Range
Inter-ovulatory Interval (IOI) [days]	Boran	18	19.44 ± 0.23	18-21
	Boran*HF cross	16	20.13 ± 0.35	18-22
	Total	34	19.76 ± 0.30	18-22
Size of pre-ovulatory follicle [mm]	Boran	18	16.22 ± 0.47	14-27
	Boran*HF cross	16	17.13 ± 0.52	15-20
	Total	34	16.65 ± 0.35	14-27
Follicular growth rate [mm/day]	Boran	18	1.14 ± 0.15	0.5-3.2
	Boran*HF cross	16	1.46 ± 0.12	0.7-2.3
	Total	34	1.29 ± 0.10	0.5-3.2
Size of second large follicle [mm]	Boran	18	9.22 ± 0.36	7-16
	Boran*HF cross	16	9.88 ± 0.58	6-11
	Total	34	9.53 ± 0.33	6-16
Size of third large follicle [mm]	Boran	18	7.39 ± 0.29	6-10
	Boran*HF cross	16	7.44 ± 0.22	5-8
	Total	34	7.41 ± 0.19	5-8

All cows exhibited either two or three ovarian follicular waves during the IOI (Figure 5). All Boran and 56.2% of the Boran*Holstein crosses had two follicular waves per cycle, and this was significantly different (P=0.002) from the proportion of cows with three waves. The mean (±SEM) IOI for the cows with two waves was 19.48 ± 0.94 days (Figure 6) and 20.86 ± 2.14 days for cows with three waves. The length of the IOI was significantly shorter (P=0.001) for cows with two waves than for cows with three waves.

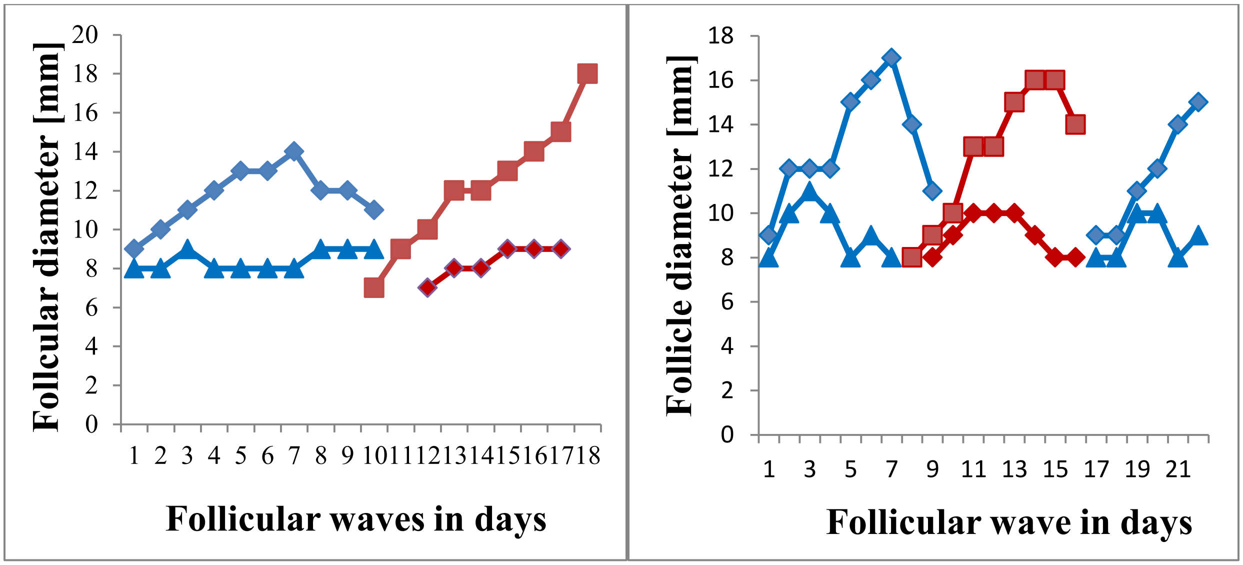


Figure 5. Follicular dynamics with 2 waves (left panel) of 18 days IOI and 3 waves (right panel) in 22 days IOI in Boran & Boran*Holstein cross cows

The preovulatory follicle reached a mean (±SEM) maximum diameter of 16.97 ± 2.88 mm, and this diameter was not affected by genotype (16.89 ± 1.48 mm for Boran and 17.06 ± 2.93 mm for Boran*Holstein crosses). There was, however a genotype effect (P=0.001) in the number of ovarian follicles (Table 4; Figure 6). The total number of ovarian follicles was 11-28 for Boran and 10-28 for crosses with no significant breed difference. However, more follicles were found on the right than left ovaries in both breeds. Clear differences among sonograms of the preovulatory, growing follicles and CL were diameter and echotexture (Plate 1).

Table 4. Total number of follicles ≥ 4 mm diameter in Boran and Boran*Holstein cross cows

Breed	N	Total follicles ≥ 4 mm on Right ovary Mean (±SEM)	Total follicles ≥ 4 mm on Left ovary Mean (±SEM)	Total follicles ≥ 4 mm on Both ovaries Mean (±SEM)
Boran	9	9.14±0.091 (Range=4-17)	9.20±0.083 (Range=5-14)	18.34±0.145 (Range=11-28)
Boran*Holstein Friesian cross	8	8.69±0.089 (Range=5-17)	8.87±0.084 (Range=4-14)	17.56±0.141 (Range=10-28)
Total	17	8.93±0.064 (Range=4-17)	9.05±0.059 (Range=4-14)	17.98±0.102 (Range=10-28)

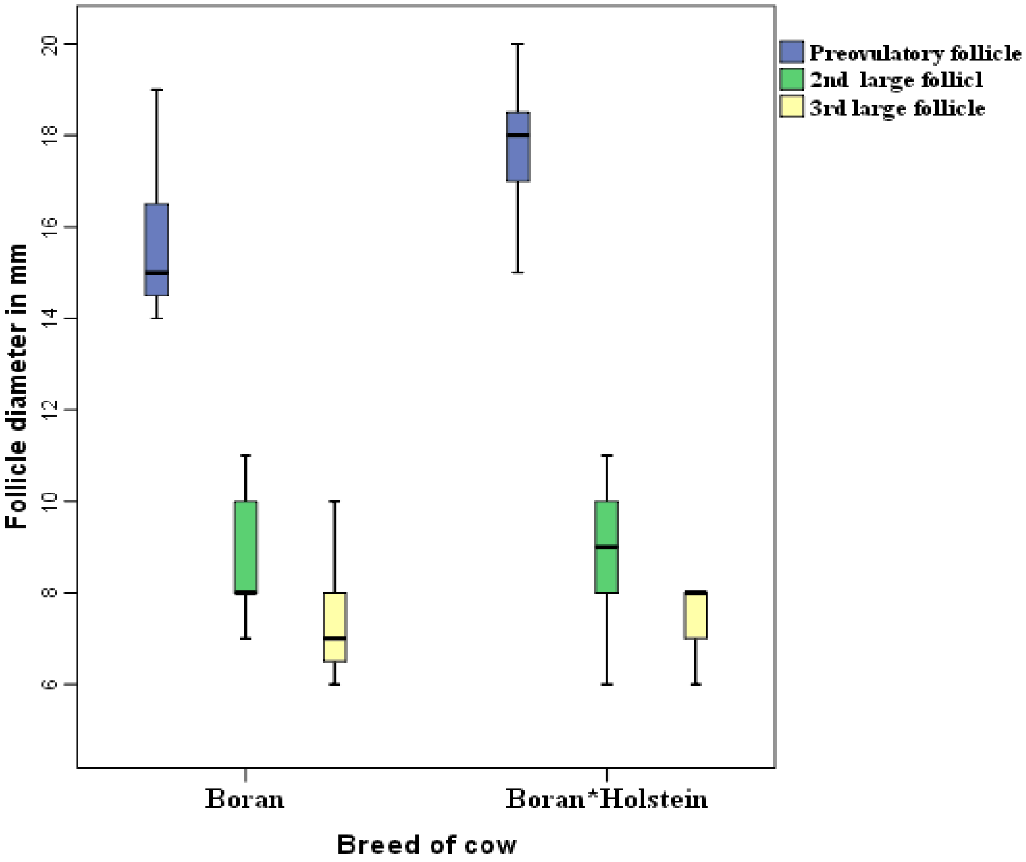


Figure 6. The diameter [mm] of the preovulatory/DF/ follicle and the second largest and third largest (subordinate) follicles in Boran and Boran*Holstein cross

Serum progesterone concentration and diameter of the corpus luteum (CL) was affected (P=0.001) by genotype (Table 5). The mean (±SEM) serum progesterone concentration in purebred Boran cows was 2.01 ng/ml versus 0.88 ng/ml in Boran*Holstein cows (Figure 7). Contrarily, mean (±SEM) CL diameter was higher (p=0.001) in Boran*Holstein crossbred cows (20.68 mm) than in purebred Boran cows (19.33 mm).

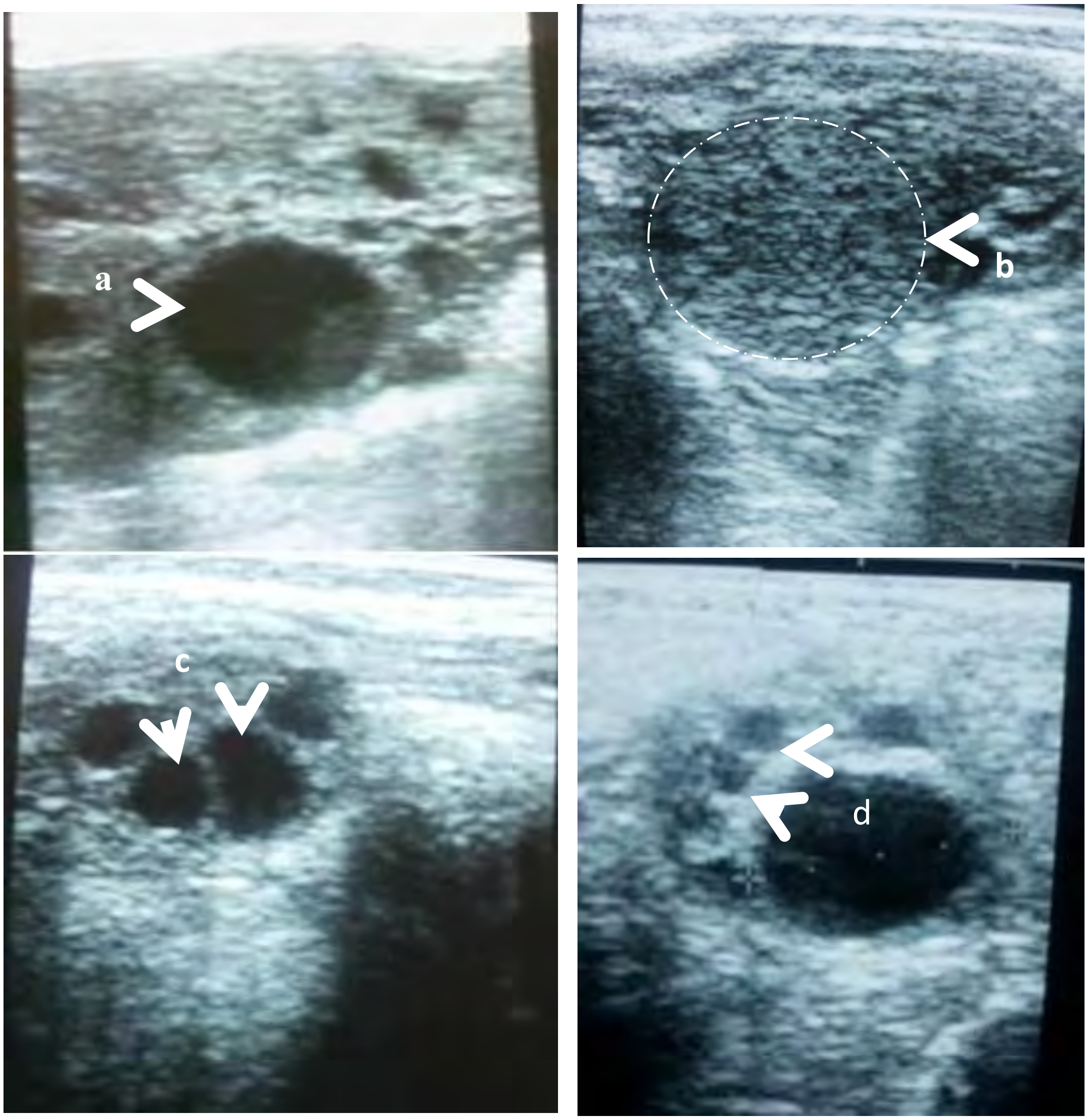


Plate 1. A sonogram of CL and ovarian follicles from Boran cows: a= DF; b= Mature CL; c= medium and large sized follicles; d= Small follicles;

Table 5. Mean (±SEM) serum P4 level [ηg/ml] and diameter of corpus luteum in Boran and Boran*Holstein crossbred cows

		N	Mean±SEM	Range
Serum Progesterone level [ηg/ml]	Boran	243	2.01±0.11	0.03 – 8.02
	Boran*Holstein crosses	216	0.88±0.07	0.03 – 5.22
	Total	459	1.48±0.08	0.03 – 8.02
CL size [mm]	Boran	226	19.33±0.12	16.0 – 24.0
	Boran*Holstein crosses	216	20.68±0.17	15.0 – 27.0
	Total	442	19.99±0.11	15.0 – 27.0

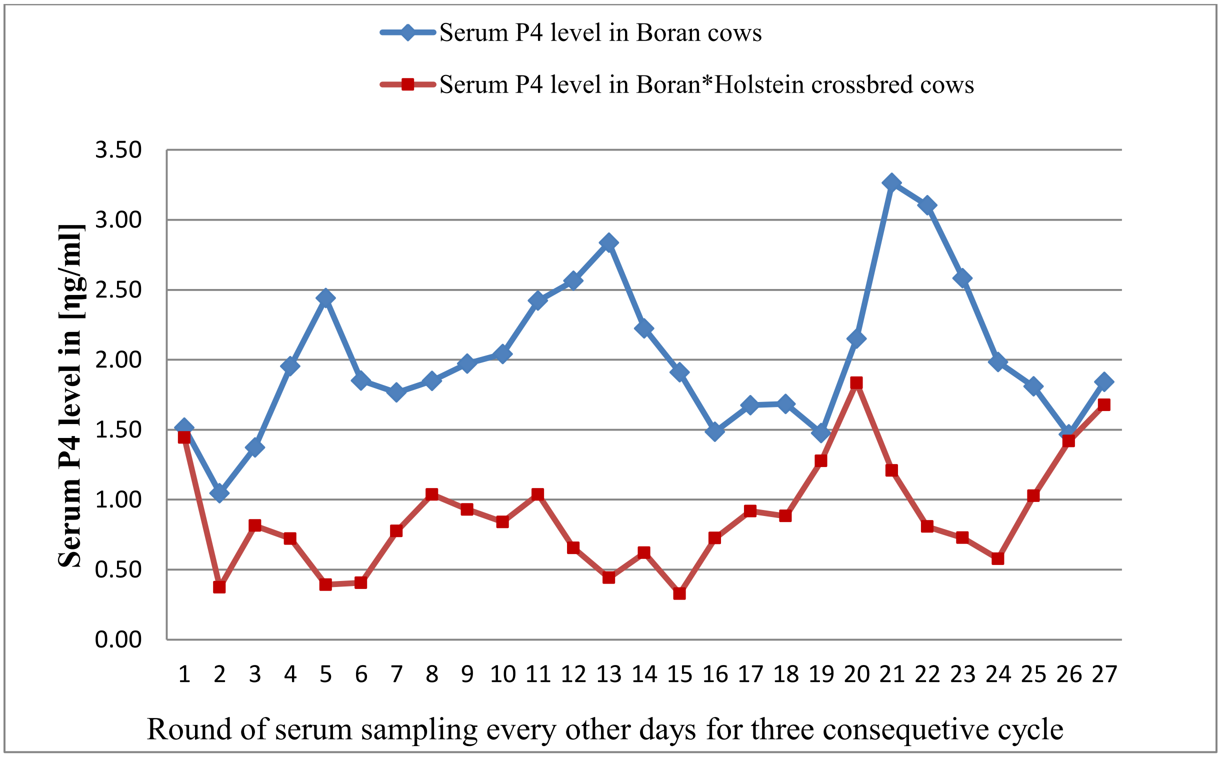


Figure 7. Serum progesterone concentration [ηg/ml] in cycling Boran and Boran*Holstein crossbred cows

4.2 Superovulatory response

The overall response to superovulatory treatment was 76.6%, but response was higher (92.6%) in Boran (χ^2 = 9.057; p=0.003) than the 55.0% in Boran x Holstein crossbred cows. The mean (±SEM) time interval from withdrawal of the CIDR to onset of estrus was 20.4± 1.8 hours, and this was not affected by genotype (18.6± 2.10 hours in Boran and 19.75 ± 2.88 hours in Boran x Holstein; Table 6). However, the interval from CIDR removal to onset of estrus was shorter (p=0.01) in cows treated with 250 IU FSH (10.75 ± 3.3 hours) than in cows receiving either 200 or 300 IU.

Table 6. Time interval from CIDR withdrawal to onset of estrus [hrs.] and duration of estrus [hrs.] in Boran and Boran*Holstein crosses treated at different doses of Pluset

Breed of cows and dose of FSH	N	FSH dose [IU]	Mean (±SEM) Interval (hrs.)	P - value	Mean(±SEM) Duration of estrus (hrs.)	P - value
Boran	25		18.6±2.1	NS	21.8±1.4	NS
Holstein	11		19.8±2.9		22.8±1.9	
	14	200*	22.6±3.1		26.1±1.9[a]	
FSH	8	250**	10.8±3.3		16.8±2.1	
	14	300**	24.2±2.8		24.0±1.8[b]	
	11	200	23.2±2.9	NS	24.9±1.7	NS
Boran	4	250	10.8±4.7		15.5±2.9	
	10	300	21.8±2.9		24.4±1.8	
	3	200	22.0±5.5		27.3±3.4	
Boran x Holstein	4	250	10.8±4.7		17.0±2.9	
	4	300	26.5±4.7		24.0±2.9	

* The mean difference was significant at p=0.007; ** the mean difference was significant at p=0.006; [a] the mean difference was significant at p=0.003; [b] the mean difference was significant at p=0.01

The mean duration of estrus was 22.9 ± 1.1 hours, and duration of estrus was shorter (p=0.005) for cows treated with 250 IU FSH than for cows receiving 200 IU or 300 IU.

The mean (±SEM) number of CL counted manually and ultrasonographically was 8.5 ± 0.9 and 8.6 ± 0.7, respectively, and this did not differ by method of counting. The maximum number of CL counted (17 CL) was detected in a Boran cow treated with 250 IU. Table 7 shows the number of CL by genotype and by pluset dose.

Table 7.The number of CL counted manually and by ultrasound for purebred Boran and Boran*Holstein crossbred cows receiving different doses of exogenous FSH

Breed of cows		N	No of CL by Manual		No of CL by Ultrasound	
			Mean±SEM	P-value	Mean±SEM	P-value
Boran		25	10.1±0.8	0.03	10.1±0.7	0.01
Holstein cross		11	7.2±1.1		7.2±0.9	
FSH level (IU)	200	14	6.8±1.2 [a]	0.03	7.0±0.9	0.05
	250	8	10.9±1.3 [b]		10.6±1.1	
	300	14	8.1±1.1 [b]	NS	8.3±0.9	NS
Boran	200	11	7.6±1.1	NS	8.0±0.9	NS
	250	4	14.0±1.8	NS	13.8±1.5	NS
	300	10	8.5±1.1	NS	8.6±0.9	NS
Boran x Holstein crosses	200	3	6.0±2.1	NS	6.0±1.7	NS
	250	4	7.8±1.8	NS	7.5±1.5	NS
	300	4	7.8±1.8	NS	8.0±1.5	NS

4.3 *In vivo* embryo production

A total of 39 flushings were made on 10 Boran and 10 Boran*Holstein with a total yield of 118 embryo/ovas. Boran cows were flushed 1-4 time while Boran*Holistein were flushed 1-2 times (Annex 2). Sixty five percent of (N = 25) of flushing was done on Boran cows yielding 76.8% transferable embryo and 49 UFO while it yielded 23.2% of the transferable embryo (Plate 2a and b) and no UFO from Boran*Holstein crosses. The total collection of embryo/ova from Boran and Boran8Holstein crosses was 5.36±0.86 embryo/ova per cows (Table 8). Significant (p=0.001) genotype effect was seen on the total collection. There was a higher number of embryo/ova (P=0.001) recoveries from Boran than from Boran*Holstein cows. More UFO (Plate 3) were collected from Boran cows (mean ± SEM = 8.17 ± 1.82) than from Boran*Holstein cows (no UFO recovered).

All collected embryos were light amber colored cytoplasm, round shape, distinguishable blastomeres, wider per vitelline space, well defined inner cell mass (in blastocyst stage embryos), and clear and transparent zona pellucida which is an evident in the top quality embryos.The mean number of transferable quality embryos (TQE) produced was 3.45 ± 0.50, and this was not different between the genotypes (3.79 ± 0.57) for Boran, and 2.67 ± 0.99 for Boran*Holstein). Though it was not statistically significant, the mean number of unovulated follicles tended to be higher in Boran*Holstein crossbred cows (2.41 ± 0.59) than in purebred Boran cows (1.59 ± 0.28).

Among the TQE 71.4% were quality grade 1 whereas the remaining 28.6% were quality grade 2 (Plates 2, 4 & 5). Embryonic stages of development of harvested TQE were stage 4, 6 and 7 (Plate 2a & b). Some irregularities/deformities were observed in the zona pellucida of some embryos (Plate 5). A total of 6 fresh embryos (4 stage 4 and quality grade 1, and 2 stage 7 and quality grade 1) were transferred to a synchronized recipients of which three pregnancy were proved to be established (Plate 6).

Table 8. Mean number of embryos/UFO and transferable embryos flushed from Boran and Boran*Holstein cross cows

Breed	Counts	N	Mean±SEM	Range
Boran	CL number	25	**9.80±0.57**	3 - 17
	Embryo yield	14	**3.79±0.57**	1 - 8
	UFO	6	**8.17±1.82**	3 - 14
	Total collection	16	**6.38±1.03****	1 - 16
Boran*Holstein cross	CL number	14	**5.79±0.90**	1 - 11
	Embryo yield	6	**2.67±0.99**	1 - 7
	UFO		**0.0**	0
	Total collection	6	**2.67±0.99***	1 - 7
Total	CL number	39	8.38±0.58	1 - 17
	Embryo yield	20	3.45±0.50	1 - 8
	UFO	6	8.17±1.82	3-14
	Total collection	22	5.36±0.86	1 - 16

** Significantly different by genotype (p=0.01)

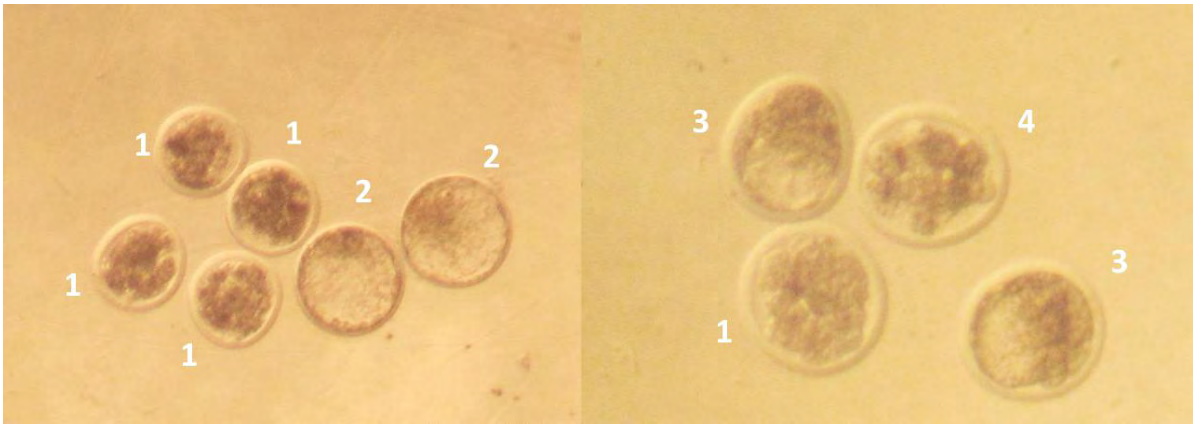


Plate 2a. Development stage & quality of in vivo produced Boran: 1 = Stage 4 (Compacted morulla) quality1; 2 = Stage 7 (Expanded blastocyst) quality 1; 3 = Stage 6 (blastocyst) quality 1; 4 = stage 4 (compacted morulla) quality 2

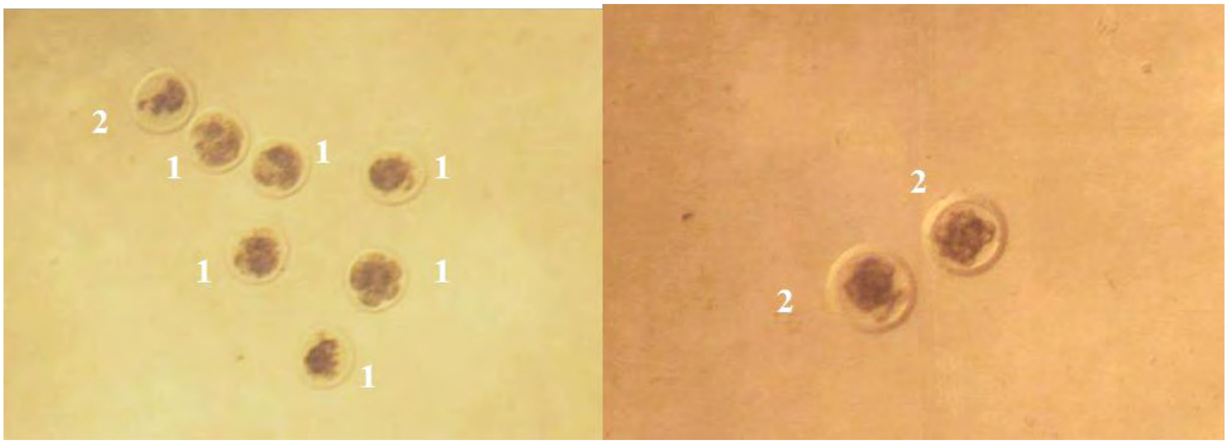


Plate 2b. Development stage & quality of in vivo produced Boran*Holstein embryos: 1 = Stage 4 (Compacted morulla) quality 1; 2 = stage 4 (compacted morulla) quality 2

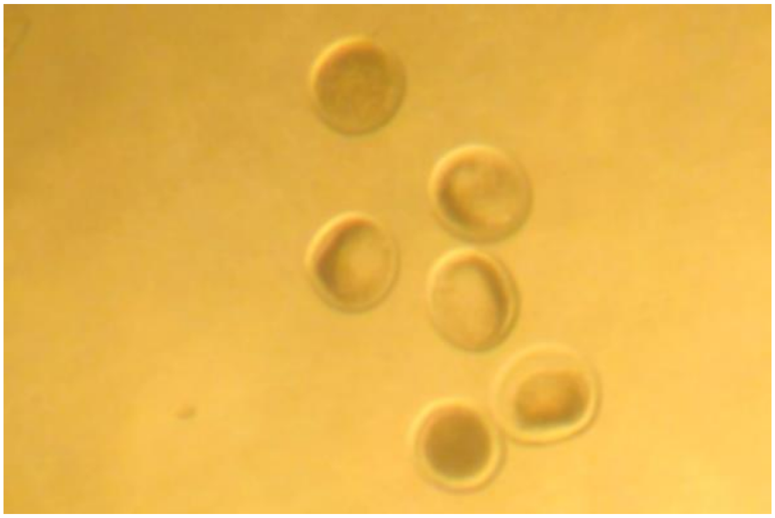


Plate 3. Unfertilized ovum collected from Boran

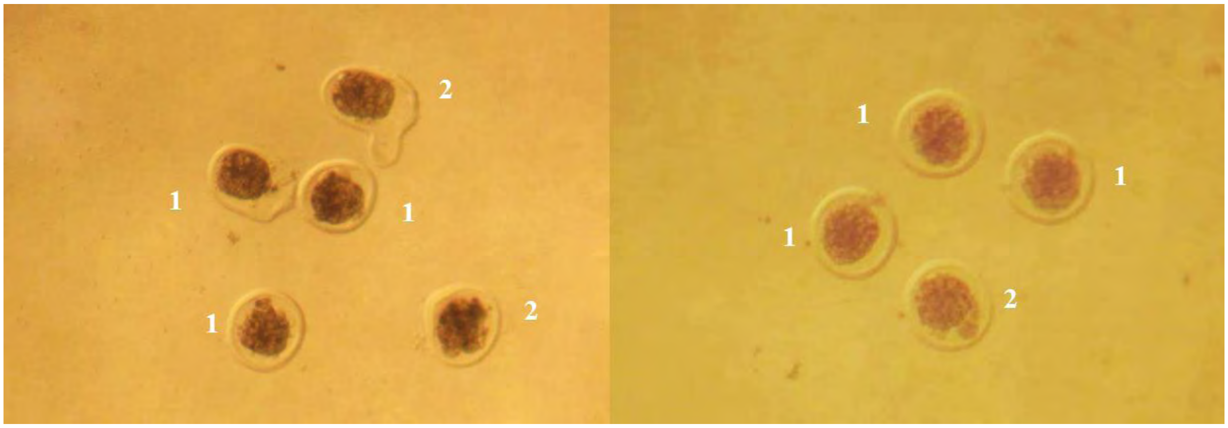


Plate 4. Embryos at the same development stage but different quality grade: 1 = Stage 4 (compact morula), Quality grade 1; 2 = Stage 4 (compact morula), Quality grade 2

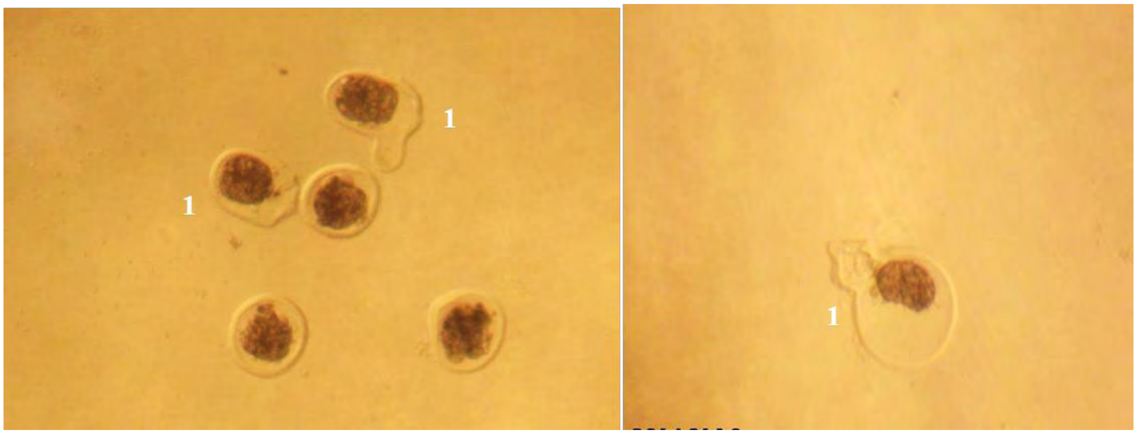
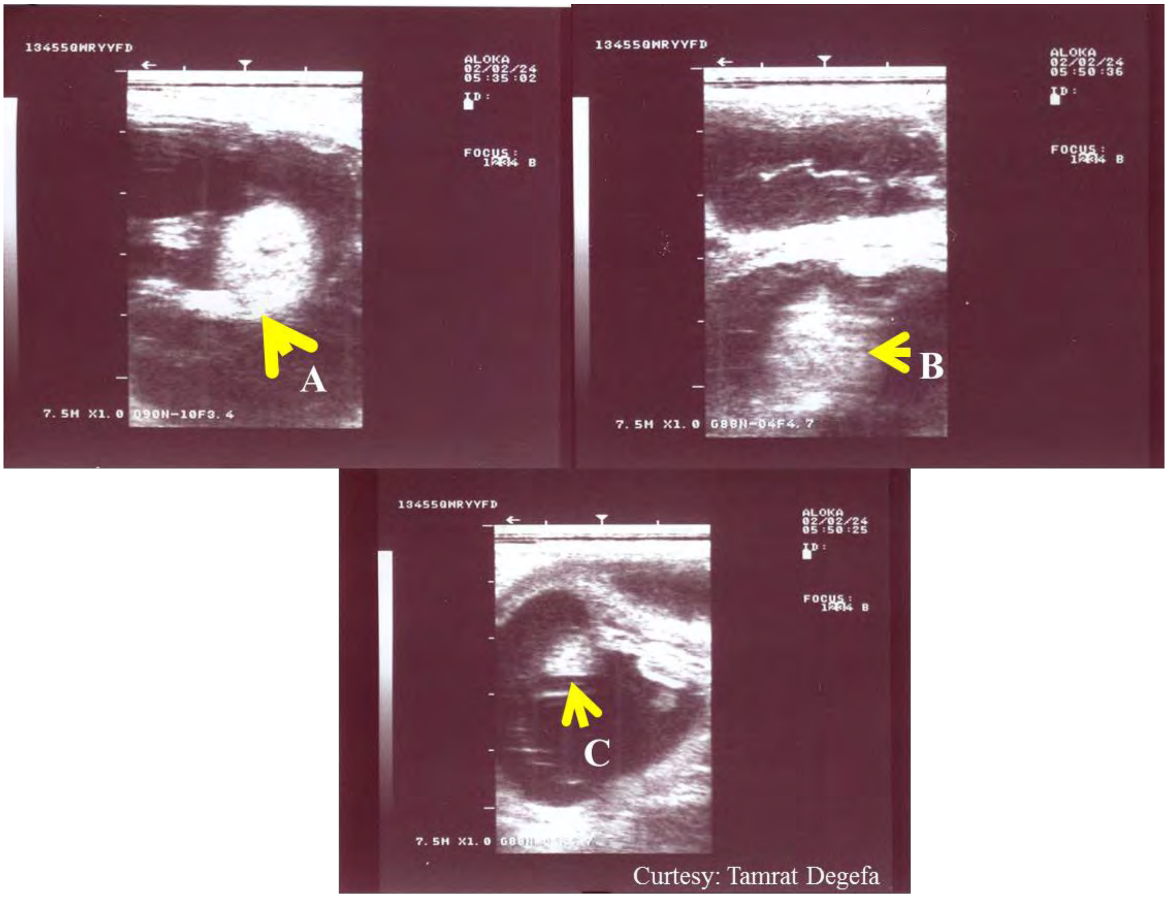


Plate 5. Embryos with stage 4 (compact morula) with deformed zona pellucida



Curtesy: Tamrat Degefa

Plate 6. Sonogram of early pregnancy establishment from fresh embryo transfer (A, B, C)

4.4 Post incubation changes in embryo developmental stage and quality

A total of 35 embryos were frozen and thawed. At the immediate post-thaw evaluation (prior to *in vitro* culture), 94% of embryos (92% of Boran embryos and 100% of Boran*Holstein embryos) were at stage 4 (compact morula) and 88.6% of the embryos (92% of Boran embryos and 80% of Boran*Holstein embryos) were quality grade 2. The difference in development and quality grade of frozen-thawed embryo was insignificant when compared with the pre-incubation recorded stage and quality. After 24 hours of incubation at 37^0C, approximately 26% of embryos had developed to stage 5 (early blastocyst) and without a change in embryo quality grade: 28.6% and 20% of incubated embryos of Boran and Boran*Holstein crossbred cows, respectively (Table 9). Developmental stage changes were observed on 24% and 40% of the incubated Boran and Boran*Holstein embryos, respectively, and affected by genotype (p= 0.009). Boran*Holstein embryos showed relatively advanced development than Boran embryos. One Boran embryo degenerated, but none of the Boran*Holstein embryos did. After an additional 24 hours of incubation (48 hours of total incubation time), all embryos were retained the same developmental stage as had been observed at 24 hours, and slight improvement in quality grade were observed.

Table 9. Freeze-thawed embryos‟ developmental progress at 24 and 48 hours incubation

Stage of embryo at incubation	**N**	**Percent**	**Quality**	**N**	**Percent**
4	33	94.3	1	3	8.6
5	1	2.9	2	12	34.3
6	1	2.9	3	15	42.9
Total	35	100.0	4	5	14.3
			Total	35	100.0
Embryo Stage and Quality after 24 hours incubation					
4	21	67.7	1	2	6.5
5	8	25.8	2	12	38.7
6	2	6.5	3	10	32.3
Total	35	100.0	4	7	22.6
			Total	35	100.0

CHAPTER 5

5. DISCUSSION PAGES 64-70

5.1 Follicular dynamics

The Ethiopian pure Boran had two follicular waves unlike the Boran*Holstein crossbred counterparts, and is in accordance with earlier reports for zebu - Nilore, Gir and Brahman (Zeitoun *et al.* 1996; Figueiredo *et al.* 1997; Gambini *et al.* 1998; Alvarez *et al.* 2000; Viana *et al.* 2000; Mollo *et al.* 2007). However, they differ from the Kenyan Boran (Muraya, 2013). This similarity may reflect the long-lasting adaptation of the breed in the tropical region. The IOI was shorter than previous report for *Bos indicus* (Bó *et al.* 2003), and Holstein heifers and lactating cows (Sartori *et al.* 2004) but similar to the result reported for *Bos indicus* cattle (Anivaldo *et al.* 2012; Muraya, 2013). It is also shorter than those with three follicular waves (Savio *et al.,* 1988; Sirois and Fortune, 1988; Savio *et al.*, 1990; Alvarez *et al.,* 2000; Viana *et al.,*2000; Townson *et al.,* 2002; Sartori *et al.,* 2004; Muraya, 2013). The short length of the IOI observed may also be due to the favorable climatic condition which was continuously warmer and differ from the seasonal extremities often observed in the temperate regions. This similarity and fewer follicular waves give better opportunity for the application of superovulatory treatments about day 10 -11 (the emergence of the second follicular waves) and estrus synchrony. Previous report by Figueiredo *et al.,* (1997) showed follicular wave patterns in Zebu cows can be influenced by parity. This situation was not observed in this study since the experimental cows included were either in their 1st or 2nd parity.

The diameter of the preovulatory follicle found in the pure Boran cows was comparable to the size previously reported for *Bos taurus* cattle (Fortune and Quirk, 1988; Savio *et al.* 1988; Ginther *et al.* 1989b) but it was larger than commonly reported for *Bos indicus* cattle elsewhere (Ginther *et al.,* 1989a; Figueiredo *et al.,* 1997; Sartorelli *et al.,* 2005). The follicular diameter at the point of deviation with Boran cattle was also much larger than that reported for *Bos taurus* breeds (Ginther *et al.,* 1996; Sartori *et al.,* 2001) and for Zebu cattle (Figueiredo *et al.,* 1997; Sartorelli *et al.,* 2005; Castilho *et al.,* 2007; Gimenes *et al.,* 2008). This could possibly be attributed to the large number of antral follicles at the time of selection which contribute to the circulating level of ostradiol that enhance the final development of preovulatory follicle. The larger size of the preovulatory follicle in Boran should give an advantage over the production of higher concentrations of circulating estradiol, and could be the reason for the manifestation of pronounced behavioral estrus unlike the previous report for short duration or silent estrus in zebu.

The total number of ovarian follicles greater than 4 mm was higher in the pure Boran than the Boran*Holstein crosses but it is comparable to the one reported in *Bos taurus* (Ginther *et al.,* 1996; Alvarez *et al.*, 2000; Carvalho *et al.,* 2008; Gimenes *et al.,* 2009; Bastos *et al.,* 2010). Nellore, Brahman and Senepol breeds, on the other hand, produced relatively larger number of follicles than the present finding (Alvarez *et al.,* 2000; Buratini *et al.,* 2000; Carvalho *et al.,*2008; Gimenes *et al.,* 2009; Bastos *et al.,* 2010). Nevertheless, genetic adaptation of the pure Boran in the tropical condition seems to have influence on the fertility and fecundity compared to the temperate breeds. Furthermore, intensive selection and improvement made on the Nellore and other breeds could be another sources of the difference in the follicular activity. Similar to the previous documentation (Pierson and Ginther, 1987; Muraya, 2013) the right ovaries were more active than the left ovaries in both breed studied.

Despite the similarity in diameter of the preovulatory follicles in the two genotypes, disparity was observed in the size of CL and concentrations of serum progesterone. Even though the difference was not significant, Boran cows had a relatively smaller CL. On the contrary, Boran cows had higher plasma P4 concentrations than their Holstein crosses. This finding is in complete agreement with the earlier studies (Ginther *et al.* 1989; Rhodes *et al.* 1995; Figueiredo et *al.* 1997; Bo *et al.* 2003; Mutha Rao *et al.*2005; Singh *et al.*2006; Carvalho *et al.* 2008; Sartori *et al.* 2010; Naik *et al.* 2013) on various tropical breeds. Nevertheless, there is a controversy on the importance of higher concentration of P4 in non-pregnant luteal phases. Some researchers found no difference in P4 concentrations during embryo transfer from recipients that became pregnant and non-pregnant with values of 2.51 and 2.42 ng/ml, respectively (Mann et al., 1995; Marques et al. 2003). Other studies also confirm that P4 produced by the CL is essential for the establishment and maintenance of pregnancy in cattle and larger CL in recipients was correlated with increased pregnancy rates in embryo transfer programs (Binelli et al. 2001; Ériklis *et al.,* 2012).. Lower conception rates and early embryonic mortality were rported to be attributed to inadequate luteal function exhibited by smaller CL size and low P4 secretion (Vasconcelos *et al.* 2001; Silva *et al.*, 2002; Bilodeau-Goeseels and Kastelic, 2003). Looney *et al.* (2006) also indicated that CL of at least 10 mm in diameter is acceptable for embryo transfer in recipient female which is by far smaller than the CL size recorded in Boran. The existing evidence on CL size and circulating P4 level is a proof of liberty of Boran cattle from the consequence of inadequate luteal function or low P4 level. Nevertheless, the cases of development of persistent ovarian follicles and lower conception rates expected in cows with two follicular waves per estrous cycle (Townson *et al.* 2002) deserves further studies in Boran cattle as they exhibited two follicular waves per estrous cycle. It is hence pragmatic to suggest that pure Boran breeds can be used as recipient of bovine embryos, provided the other preconditions (synchronization of the day of the estrous cycle, age of the embryo, health and nutrition) are met.

5.2 Superovulation response of Boran cows

Genotype was found to be an important factor that influenced superovulatory response. Pure Boran was more sensitive to FSH dose and produced relatively large number of CL. Previous reports in other African cattle breeds such as the Cheurfa breed in Algeria (Ferrouk *et al.,* 2008), Oulmes-Zaer breed in Morocco (Elaidi *et al.*1996a; Elaidi *et al.*, 1996b), Baoulé breed of Central Africa (Bianchi *et al.,* 1986; Chicoteau, 1989), and N"dama breed in west Africa (Jordt *et al.,* 1986; Diop *et al.,* 1994) confirm Boran to be superior. This could further be an indication of better performance of Boran in tropical environment than these African breeds, and greater sensitivity to exogenous gonadotropins. This is again in complete agreement with Kanitz *et al., (*2002) who also studied dose and genotype interaction on the response to superovulation. The number of palpable CL recorded in the present study was substantially higher compared with a previous report on the same breeds treated with higher doses of "Pluset" (500 IU and 1000 IU) (Tegegne *et al.*1994).

Borans produced undesirable result during an effort to increase the number of ovulations by increasing the FSH doses beyond the optimal level for a given breed (Donaldson and Ward 1985; Lerner *et al.* 1986; Escouflaire *et al.* 1989; Murphy *et al.* 1998; Kanitz *et al.* 2002). Further increase in FSH dose did not increase superovulatory response. The same phenomenon was observed in pure Boran treated with 300 IU FSH. Murphy *et al.* (1998) and Kanitz *et al.* (2002) concluded that higher doses of FSH may disturb the process of ovulation by making ovaries too large or by inducing extremely high levels of estradiol synthesis. And hence, this assumption holds true for Boran as well, and optimization of the superovulatory agent has a paramount importance for the wider application of the technology.

5.3 *In vivo* embryo production

Overall product of transferable quality embryo (TQE) from pure Boran were consistent with previous finding by Tegegne *et al.,* (1997) from the same genotypes in Ethiopia, from the Algerian Cheurfa (Ferrouk *et al.* 2008), from the "N‟dama" (Jordt and Lorenzini, 1990), from the Oulmes-Zaer (Diop *et al.* 1994 and Elaidi *et al.*1996a), on African breeds. However, it was much lower than the result reported by Silva *et al.* (2009) for the Nellore breed. The number of TQE produced from Boran was higher than produced by Baoulé breeds (Chicoteau, 1989; and Jordt *et al.* 1986). Although the difference was not significant the TQEs collected from Boran were better in quality than the embryo from the Boran*Holstein crosses by all evaluation characteristics. Light amber colored cytoplasm, round shape, distinguishable blastomeres, wider per vitelline space, well defined inner cell mass (in blastocyst stage embryos), and clear and transparent zona pellucida were evident in the top quality embryos. This finding is in agreement with the defined character of excellent and good quality *in vivo* produced bovine embryos (Holm and Callesen, 1998; Contreras *et al.,* 2008). Hence, pure Boran in vivo embryo production can be implemented at larger scale for genotype multiplication and can also be commercialized. Nevertheless, considerable numbers of UFO were also harvested from Boran that could potentially diminish their status.

Several factors are known to cause collection of UFO from superovulated cows: cow factors such as lactation, seasonal effect, repeat-breeding, insemination at the onset of estrus, suboptimal oocyte quality (Hawk and Tanabe 1986; Dalton *et al*. 2000; Contreras *et al.,* 2008; Peippo *et al*., 2009), and increased doses of gonadotropin (Mapletoft*et al*., 2002); inferior quality semen, disturbances in transport of spermatozoa and/or insemination with sex sorted spermatozoa (Kafi and McGowan, 1997). The most probable reason for the current situation was the inferior semen quality of the pure Boran bull procured from the National AI center. Different copping mechanisms such as repeated insemination (3X) with doubling doses at each insemination, use of well experienced technicians, use of different batches of pure Boran semen and use of semen from Boran*Holstein cross bulls were used; the final option yielded acceptable number of TQEs. Insemination of Boran cows with pure Boran semen for embryo production should be implemented only after proper evaluation of the semen at each AI. Besides, the production and laboratory management of pure Boran semen deserves due revision to solve the problem.

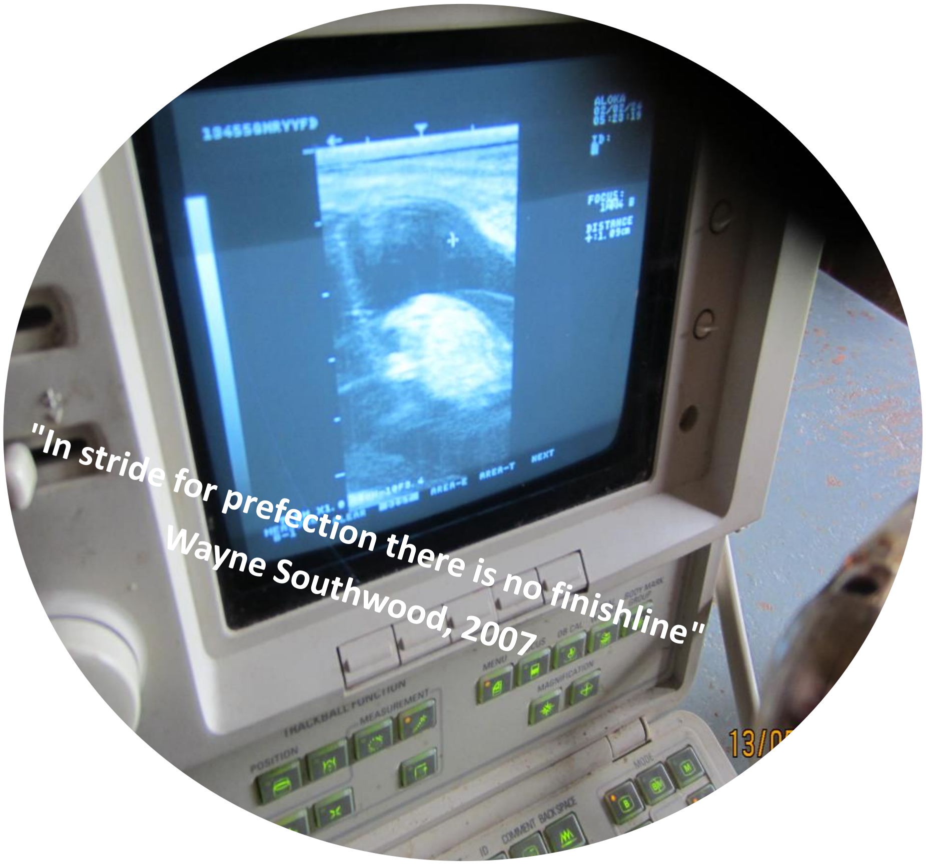
Previous report on the quality of oocytes of cows with two waves showed a decreasing quality than oocytes of cows with three follicular waves. Nevertheless, difference in quality of *in vivo* produced embryo between the two genotypes and/or the good quality of embryos collected from Borans didn‟t show the difference in the current study. However, the effect of two wave‟s cycle on the quality of oocytes as well as the quality and viability of embryo need further investigation. By and again, the higher population of growing follicles >4 mm in Boran clearly indicate the potential of the breed for embryo/oocyte production.

5.4 Freezing, thawing and viability test of *in vivo* produced Boran embryo

The morphological evaluation of embryo quality before and after freezing was performed in a manner consistent with that previously described (Contreras *et al.,* 2008). Contreras *et al*., (2008) reported that embryos of varying quality displayed inconsistent developmental changes with different lengths of time of *in vitro* culture. Good and fair quality embryo did not undergo major detrimental changes in development after 7 h of incubation, whereas poor quality embryos experienced changes as early as 2 h after the start of incubation. Although pronounced progressive changes were not observed during the *in vitro* culture of Boran and Boran*Holstein embryos, the absence of destructive change in cultured embryos confirms their good quality which is in agreement with previous studies by Contreras *et al.,* (2008). Nevertheless, there was a much lower percentage of Boran embryos that advanced in development in the present study when compared with the report by Contreras *et al*., (2008) who also reported embryo growth in 80% of the good quality embryos during 6 hours of culture, and a similar development pattern in 72% of the fair quality embryos. Furthermore, the percentage of embryos with cellular detritus increased from 10% to 36% and 10 to 70% after 24 h of culture in good and poor quality embryos, respectively (Contreras *et al.,* 2008).

Cultured Boran and Boran*Holstein embryos, however, did not suffer significant alterations or structural changes associated with degeneration for a period of 24 hours, thus confirming their good quality. Moreover, Boran embryos showed improvement in quality with slow growth pattern during the culture which could be a typical characteristic of the breed, yet remains to be further verified. For this study, a modified laboratory protocol with commercial media was used (culture without 5% CO_2), and it may be necessary to compare this result with the conventional protocol in which 5% CO_2 is added to see if there are differences in developmental pace.

CHAPTER 6



"In stride for prefection there is no finishline"
Wayne Southwood, 2007

6. CONCLUSION AND RECOMMENDATIONS Pages 71-73

Several research findings have confirmed the influence of many environmental (e.g. donors selection and management) and genetic factors (e.g. breed) on the success of superovulation and *in vivo* embryo production in bovine. Reproductive parameters considered (follicular dynamics, superovulation response, sensitivity to exogenous gonadotropin) for Boran cows signified this fact, and Boran responded to superovulation and embryo recovery differently from their Boran*Holstein counterparts. Subtle physiological differences between Boran (*Bos indicus*) and Boran*Holstein crosses (*Bos indicus**Bos taurus*) were observed with respect to the number and growth rate of ovarian follicles, number of ovarian follicular waves per estrous cycle, response to superovulation, sensitivity to exogenous gonadotropins, and blood concentration of P4. Collectively, these differences suggest crucial and important physiological aspects of the Boran breed that need to be observed during an embryo technology program.

The IOI of the Boran lies within the range already known for other zebu breeds. However, unlike other zebu breeds, the maximum diameter of the preovulatory follicle as well as the size of mature CL was similar to that observed in Boran*Holstein crossbred cows. The similarity of the presence of two waves of ovarian follicular growth per estrous cycle in Boran is a boon on the list of peculiarity of the breed. This could give an additional opportunity for application of biotechnology tools such as synchronization of estrus and superovulation. If the information is an addition to the peculiarity, consistency might be contradictory to this conclusion. The relatively higher number of ovarian follicles observed in Boran than Boran*Holstein crossbred cows affirms that the Boran breed is a breed that can be reproductively exploited through technologies such as conventional embryo transfer (i.e., multiple ovulation and embryo transfer) ovum pick up and *in vitro* embryo production. It seems quite feasible to develop effective and sustainable genetic improvement schemes that are fit for multiplication and/or conservation of this iconic indigenous breed of cattle in Ethiopian.

This experiment on the freezing and thawing of Boran preimplantation embryos yielded encouraging results. Although it is widely accepted that post-thaw viability of embryos obtained from *Bos indicus* breeds is lower than that for embryos from *Bos taurus* breeds, this preliminary results revealed that compact morula stage of Boran embryos can be successfully frozen and thawed. Not all embryonic stages are appropriate for embryo transfer and/or cryopreservation, and additional studies with Boran embryos will enable a more thorough characterization of post-thaw viability of Boran embryos.

It is concluded that the Boran should be considered quite different in comparison with *Bos taurus* breeds not only by its morphology, physiological peculiarities and ecological characteristics but also in specific reproductive functions. It is indeed an iconic tropical cattle breed everywhere and of Ethiopia in particular.

And hence, the following points are recommended for similar improvement in Boran:

- Boran is a unique breed with immense potential for economic traits: it handle the harsh conditions well and get into calf sooner. It is well adapted and efficient, has incredible hybrid vigour when crossed with any breed, but alarmingly endangenered. It is iconic indigenous breed deserving conservation and multiplication by application of modern biotechnologies. Thus the present finding warrants the need for fine tuning of advanced protocols for a wider application
- The higher number of ovarian follicles of each estrus cycle in Boran affirms that it is a breed that can be reproductively exploited through ovum pick up and *in vitro* embryo production technologies. Therefore, it would be a wise decision to apply this technology sooner for the multiplication of Boran breed
- Despite the fact that embryo technologies enable the maximization of elite female the major contribution of use of quality semen is undeniable. Therefore, semen to be used to inseminate superovulated cows has to be evaluated for quality before each insemination
- Today, scientific evidences witnessed that most modern breeds have an appreciable zebu ancestry, which attests indigenous cattle breeds represent a unique genetic resource. However, they are at a juncture when there is an urgent need to conserve and/or improve livestock productivity for the benefit of the present and future human generations or let to exteniction. The motivation of the present research finding, has paved a way for the extrapolation of these reproductive technologies for the betterment of other indigenous zebu breeds such as Sheko, Horro, Begayit, Fogera etc.

CHAPTER 7

3 Years old Nellore bull, Brazil, May2016

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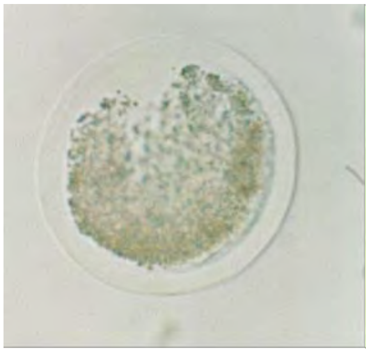
Annex 1. Bovine embryos: examples of developmental stage and quality - **Stages 1 to 9 (Mapletoft, 2013)**



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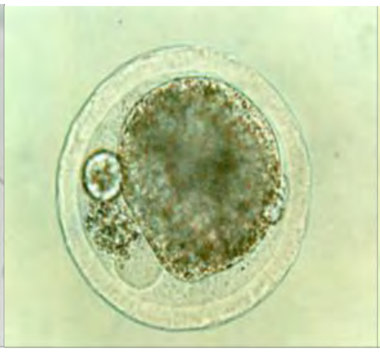

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Stage Code:1
Quality Code:4

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Quality Code: 4

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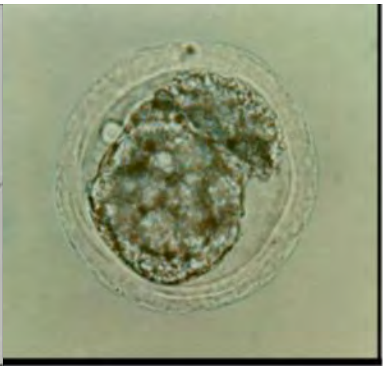

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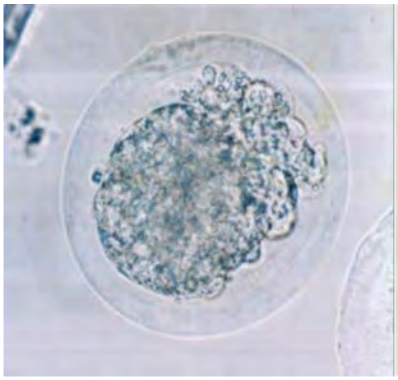

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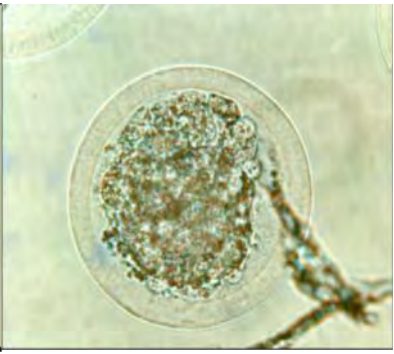

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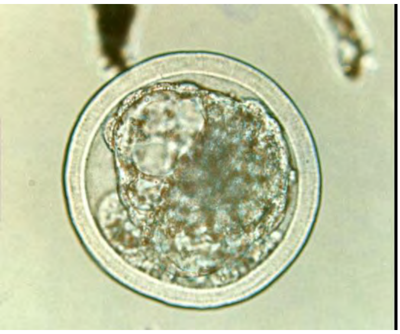

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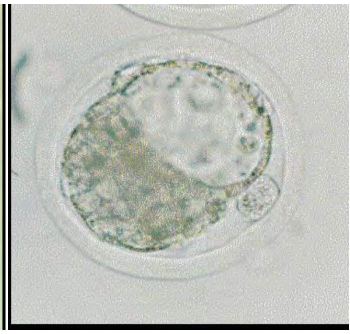

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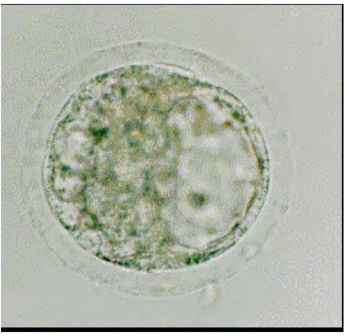

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Quality Code: 1

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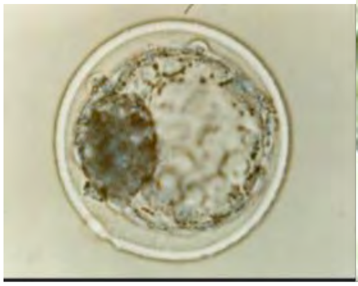

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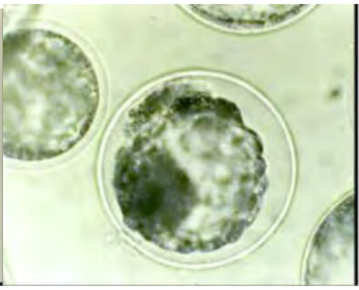

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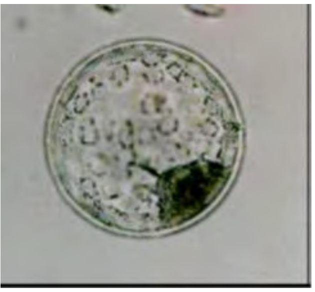

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Quality Code: 2

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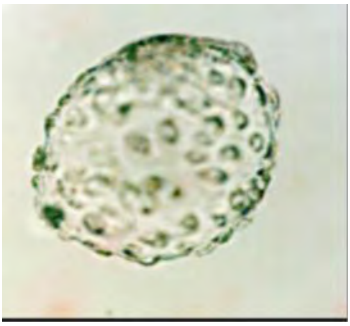
Stage Code: 8
Quality Code: 1



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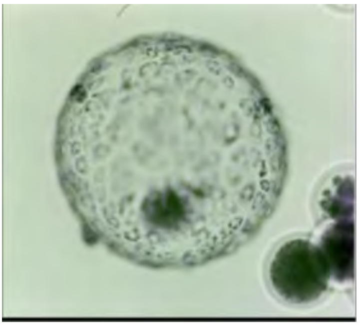

Cycle Day: 9
Stage Code: 9
Quality Code: 1

Annex 2. Lists of embryo donors and recipients of Boran and Boran*Holstein crossbred cows

No.	Cow ID	Breed	Body weight	BCS	Exotic blood level	Flushing
1	B 01	Boran	355	6	0%	4 x
2	B 06	Boran	410	8	0%	2 x
3	B 23	Boran	385	7	0%	3 x
4	B 30	Boran	340	6	0%	1 x
5	B 36	Boran	360	7	0%	2 x
6	B 40	Boran	375	7	0%	2 x
7	B 41	Boran	380	7	0%	3 x
8	B 44	Boran	475	8	0%	3 x
9	B 53	Boran	370	7	0%	5 x
10	5HB54272.3	Boran*Holstein	540	7	96.87%	1 x
11	6HB 2181163.9	Boran*Holstein	365	8	98.43%	1 x
12	6HB 2181165.3	Boran*Holstein	495	7	98.43%	2 x
13	6HB 2181165.4	Boran*Holstein	520	8	98.43%	1 x
14	6HB 444628.2	Boran*Holstein	437	6	98.43%	1 x
15	6HB 444664.2	Boran*Holstein	490	7	98.43%	1 x
16	6HB2181153.et	Boran*Holstein	375	6	98.43%	2 x
17	7HB 44462333.2	Boran*Holstein	525	7	99.22%	2 x
Recipient						PD test
18	B 36	Boran	360		0%	Negative
19	B 46	Boran	350		0%	Negative
20	HB 42.2	Boran*Holstein	400		50%	Positive
21	5HC 342952.4	Barka*Holstein	476		96.87%	Positive
22	6HC 2152312.4	Barka*Holstein	421		98.43	Negative
23	7HB 912412.5	Boran*Holstein	398		99.22%	Positive
24	6HC 3429524.1	Barka*Holstein	480		98.43%	Negative
25	3HC 33610.7	Barka*Holstein	432		87.5%	Negative
26	6HB 2181165.3	Boran*Holstein	490		98.43%	Positive
27	6HC 3429525.1	Barka*Holstein	389		98.43%	Negative