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Monitoring and improving reproductive performance of crossbred dairy cattle in Tigray Region, Ethiopia

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Declaration

I declare that the work presented in this thesis is my own and has been generated by me as the result of my own original research.

Where I have quoted the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.

I have clearly stated in the acknowledgement section any assistance received and any aspects of the project that were carried out by others.

Chapters 3 and 6 of the thesis have been published (see page XI). I confirm that as first author on these papers I wrote the manuscripts myself.

The work described in this thesis has not been previously accepted for, nor is it currently being submitted for another degree or qualification.

Finally, I confirm that this work was entirely done while in candidature for this research degree at the University of Edinburgh.

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Date:

Abstract

Ethiopia maintains an extensive livestock population; however, reproductive performance of cattle and their breeding management are unsatisfactory. Currently, the sole diagnostic tool in the country is rectal palpation, which is inaccurate for early pregnancy in cattle. The study assessed reproductive performance and major reproductive problems using questionnaire survey, and evaluated simple, cost-effective alternative monitoring approaches using on-farm diagnostic tools to determine milk and serum progesterone (P4) and evaluate reproductive status. There were 177 dairy farms (range 1-115 cattle per farm) included in the questionnaire survey. Of these, 47 participated in the quantitative determination of P4 and estradiol profiles that used an enzyme-linked immunosorbent assay (ELISA) and in the on-farm diagnostic trial that used qualitative ELISA (Target P4 and Dipstick (P4 Rapid), and the reproductive status of 319 crossbred [Holstein Friesian (HF) X Zebu] dairy cattle was assessed. Questionnaires indicated that heifers in the study area reach puberty at older age and calve late, and cows have long postpartum estrus and calving interval. Anestrus, repeat-breeding, dystocia, retained fetal membranes (RFM), endometritis and abortion as the major reproductive problems in dairy cattle in the study area. Serum, milk, saliva and urine P4 and serum estradiol profiles of cattle at different reproductive status was determined using quantitative laboratory ELISA. High P4 levels was detected in pregnant and diestrus cattle than cattle that were anestrus and in-heat. Estradiol level was higher in cattle that were in-heat than cattle in other reproductive conditions. On-farm P4 ELISA indicated in-heat (estrus) 10 (3.1%), anestrus 77 (24.2%), repeater (follicular cyst) 9 (2.8%), normally cycling 69 (21.6%) and pregnant 154 (48.3%). The field P4 ELISA findings were validated using quantitative laboratory P4 ELISA, and similar results were obtained. The sensitivity and specificity of on-farm and laboratory P4 ELISA tests for diagnosing pregnancy were 88.6 & 99.4% and 98.1 & 100%, respectively. Once reproductive problems were identified using on-farm P4 ELISA and per rectal palpation, along with reproductive history, 122 cattle (75 cows and 47 heifers) were assigned to a 10-day Controlled Internal Drug Release (CIDR) in combination with prostaglandin F2-alpha (PGF2α) and equine chorionic gonadotrophin

(eCG) based estrus synchronisation protocol to study the estrus response and conception rate. The overall estrus response and conception rates were 97.5% and 78.3%, respectively, with no significant differences in parity, pre-treatment reproductive status (anestrus or cycling (repeat-breeding/silent-estrus)) and farming system (smallholder vs organised commercial farms). The study has shown high estrus response and conception rate. Hence, this protocol is highly recommended to enhance fertility of dairy cattle in the study area and other regions. Finally, the study has determined the macronutrient composition of milk and assessed the effect of reproductive status, farm (nutritional) management, stages of lactation, parity and breed on milk composition in 246 dairy cows. This consisted of 184 crossbred cows from smallholder (n= 76: 36 non-pregnant and 40 pregnant) and organised commercial dairy farms (n= 108: 62 non-pregnant and 46 pregnant) in and around Mekelle, Ethiopia and by way of comparison, 62 HF cows (25 non-pregnant and 37 pregnant), either managed indoors or outdoors in a commercial farm in Edinburgh, United Kingdom. The mean milk fat, protein, lactose, total solid (TS) and solid-no-fat (SNF) recorded were 2.36%, 3.46%, 4.37%, 10.39% and 7.82%, in crossbred dairy cows, compared with 5.05%, 3.71%, 4.72%, 13.68% and 8.43%, in HF cows, respectively. Significantly lower ($p<0.05$) level of macronutrient was recorded in milk from crossbred cows than HF cows. Milk fat in both breeds was affected by reproductive status, farm (nutritional) management and stages of lactation, but not by parity. In both cattle breeds, the milk fat content was significantly higher ($p<0.05$) in pregnant than non-pregnant cows. Milk protein content was significantly ($p<0.05$) affected by reproductive status (in crossbreds) and stages of lactation (in both breeds), but not by farm management or parity. Reproductive status (in crossbreds), stages of lactation (in both breeds) and parity (in crossbreds) affected lactose level; however, farm management had no effect on lactose level in both breeds. Milk fat was the most affected macronutrient content in both breeds. Low milk fat level in crossbred cows could be as a result of samples obtained from early milking coupled with nutritional management. In conclusion, the present study has determined the major reproductive problems in crossbred dairy cattle, assessed their actual reproductive status using rapid, cost effective, simple and applicable on-farm P4 tests, and established P4 and estradiol profiles at different reproductive status. The major breeding

problem was poor estrus detection evidenced when animals reported anestrus were confirmed normally cycling using on-farm as well as laboratory P4 assays. These studies offer opportunities for establishing simple field reporting of reproductive status in these crossbred dairy cattle, which can have a major impact on breeding management and productivity.

Key words: Cattle, Cow, Estrus, Nutrient, Estradiol, Macronutrient, Progesterone, Reproductive performance, Reproductive problems, Reproductive status, Synchronisation, Ethiopia, Zebu.

Lay Abstract

Ethiopia has huge a cattle herd of more than 130 million, but due to poor herd management there is room for improvement in their fertility. Currently, the only method of fertility examination in cattle is per rectal palpation, which is inaccurate for early pregnancy, and can only be performed by trained veterinary professionals and artificial inseminators. The present study evaluated reproductive performance and major fertility problems in and around Mekelle, Ethiopia. The study used a questionnaire survey, and evaluated simple, cost-effective alternative monitoring approaches using on-farm testing methods to measure the amounts of progesterone, the hormone of pregnancy in both blood and milk, and evaluate the fertility status in crossbred dairy cattle [Zebu (native breed to Ethiopia) X Holstein Friesian (HF - European breed with high milk yields to improve native breed productivity)]. One-hundred and seventy-seven (177) dairy farms were included in the questionnaire survey. Of these, 47 participated in the on-farm diagnostic trial, and fertility status of 319 female cattle was assessed. Simple and rapid tests were used to measure progesterone and evaluate fertility status on the farm. These were compared for effectiveness with a laboratory test. Questionnaires indicated that fertility in the herd is suboptimal: heifers (cows that have not previously given birth) in the study area reach puberty at older age and calve later, cows return to breeding cycles after a longer interval following calving, and have an extended interval between calves. Non-cycling, repeated cycling, difficult birth, failure of expulsion of placenta, uterine infection and abortion were the major fertility problems in dairy cattle in the study area. On-farm progesterone tests effectively diagnosed breeding cyclicity and pregnancy. Findings from field progesterone tests were compared with laboratory tests, and results were similar, indicating effectiveness for on farm use. One hundred and twenty-two cattle had identifiable fertility problems (75 cows and 47 heifers). These were administered with drugs that can improve fertility, to study their effectiveness in stimulating the fertile time-period and achieving a pregnancy. The overall success of stimulating the fertility period and conception rates were 97.5% and 78.3%, respectively. These findings were similar between cows and heifers, between non-cycling and cycling cattle, between farming systems (smallholders

and organised commercial farms). This high response indicates the potential of this method to enhance fertility of dairy cattle in the study area and other regions. Finally, the study has evaluated composition of milk and assessed the effect of fertility status, farm (nutritional) management, stages of lactation, number of previous calvings and breed on milk composition. Lower levels of nutrients were recorded in milk from crossbred cows than in purebred HF. Reproductive status had significant effects on milk fat level in both breeds, and on milk protein and sugar levels in crossbred cows. In both breeds, the level of milk fat, protein and sugar levels were significantly affected by stages of lactation (the period of time cows yield milk). Farm management had significant effect on milk fat level in both breeds, but not on milk protein and sugar levels. In both cattle breeds, the milk fat content was significantly higher in pregnant than non-pregnant cows. Milk fat was the most affected nutrient content in both breeds. In conclusion, the present study has determined the major fertility problems in crossbred dairy cattle, assessed their actual fertility status using rapid, cost effective, simple and applicable on-farm tests, and established progesterone and estradiol profiles across different reproductive states. The major breeding problem in the study area was poor observation when cattle are in estrus, as shown by the number of animals reported non-cycling that were confirmed normally cycling using on-farm as well as laboratory progesterone tests. These studies provide the rationale for establishing simple field reporting of fertility status in these crossbred (Zebu X HF) dairy cattle, which can have a major impact on breeding management and productivity.

Key words: Cattle, Cow, Estrus, Nutrient, Estradiol, Macronutrient, Progesterone, Reproductive performance, Reproductive problems, Reproductive status, Synchronisation, Ethiopia, Zebu.

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Dedication

To my mum - Mihret Tesfasilassie Berhe who passed away April 2011

To my foster parents - Gebrehiwot Fisseha and Zafu Aregay

To my wife - Nigste Hadera Tesfay

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Abbreviations

µl	Microlitre
AI	Artificial insemination
ANOVA	Analysis of variance
BCS	Body condition score
BSA	Bovine serum albumin
C	Carbon
cAMP	Cyclic adenosine monophosphate
CIDR	Controlled internal drug release
CL	Corpus luteum
CV	Coefficient of variance
CYP19A1	Cytochrome P450 family 19 subfamily A member 1
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMA	Dairy milk analyser
eCG	Equine chorionic gonadotrophin
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle stimulating hormone
g	Gram
g	Gravitational force
GDP	Gross domestic product
GH	Growth hormone
GnRH	Gonadotrophin-releasing hormone
H_2SO_4	Sulphuric acid
hCG	Human chorionic gonadotrophin
HCl	Hydrochloric acid
HF	Holstein Friesian
hr	Hour
HRP	Horse radish peroxidase

IFN-τ	Interferon-tau
IGF-1	Insulin-like growth factor 1
ISO	International Organisation for Standardisation
IU	International unit
kg	Kilogram
km	Kilometre
L	Litre
LH	Luteinising hormone
LH-R	Luteinising hormone-receptor
m	Metre
m.a.s.l.	Meters above sea level
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
MRC	Medical Research Council
mRNA	Messenger-ribonucleic acid
n	Number
^{o}C	Degree Celsius
P4	Progesterone
P450scc	Cholesterol side-chain cleavage enzyme
PBS	Phosphate-buffer saline
PdG	Pregnanediol glucuronide
pg	Picogram
PGF2α	Prostaglandin F2alpha
PKA	Protein kinase A
PMSG	Pregnant mare's serum gonadotrophin
PRID	Progesterone-releasing intravaginal device
QCS	Assay quality control
R	Correlation coefficient

RFM	Retained fetal membranes
RIA	Radioimmunoassay
rpm	Revolution (s) per minute
SD	Standard deviation
SEM	Standard error of the mean
SNF	Solid-no-fat
TAI	Timed AI
TMB	Tetramethylbenzidine
TS	Total solid

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CHAPTER 1: LITERATURE REVIEW

1.1. OVERVIEW OF LIVESTOCK IN ETHIOPIA

Ethiopia maintains an extensive livestock population of more than 130 million (53.4 million cattle (Statistical Bulletin, 2011), 25.02 million sheep, 21.88 million goats and 38.13 million poultry) (NABC, 2010). Livestock are well adapted and distributed across diverse ecological conditions and management systems in Ethiopia (Lobago et al., 2006). About 85% of the Ethiopian population are engaged in the agricultural sector, which is the backbone of the country's economy (IBC, 2004), whereas industry contributes about 5% and services 10%. Livestock contributes about 43.5% of the gross domestic product (GDP) and 61% of total export; by comparison, industry contributes to only 13.4% and services 43.1% of GDP (NABC, 2010). Livestock and its products are important sources of food and income, but dairying has not been fully exploited and promoted in the country.

1.2. DAIRY CATTLE PRODUCTION IN ETHIOPIA

Cattle play the most important role in the farming economy (Ftiwi and Tamir, 2015) followed by sheep and goats in Ethiopia. The livestock population in Ethiopia are primarily indigenous type, mostly Zebu (NABC, 2010). Although the country holds huge livestock population, production and reproductive performance of its livestock are disappointing. Poor genetic performance of the local cattle breeds, poor nutrition, poor management, infertility, reproductive disorders and diseases, and lack of veterinary professionals are some of the major constraints in the dairy industry in the country (Mekonnen et al., 2010; NABC 2010; Bitew and Prasad 2011). Ethiopian livestock are genetically diverse mainly as result of natural selection influenced by environmental factors. Accordingly, indigenous livestock are better conditioned to withstand feed and water shortages, disease challenges and harsh climates (Lobago, 2007). A recent report from Bahir Dar and Gondar, Ethiopia has shown that more (73%) local cattle were reported to be healthier and required less veterinary services compared to crossbreds

(Zebu X Holstein Friesian/HF) (Roschinsky et al., 2014). However, their milk and meat productivity is very low. According to a previous report, the milk and meat production in the country, during 1983-1985, was estimated at 960,000 tonnes and 246,000 tonnes, respectively, with per capita consumption of 17.1L milk and 5.6kg meat (ESAP, 1995). Although the main source of milk in the country is the dairy cow, cows are used mainly for breeding replacement and draught oxen, so milk production is of secondary importance to the livestock farming community.

Dairy cattle production in the country is classified into four major systems: rural smallholder (mixed crop-livestock) production, pastoral and agro-pastoral production, urban and peri-urban smallholder dairy production, and specialised commercial dairy production systems (IBC, 2004; Lobago, 2007). In the rural smallholder production system, smallholder farmers predominantly raise indigenous (Zebu) cattle, and farming is subsistence in nature, while, some farmers located near urban centres who have access to milk markets raise crossbred cattle for selling milk (Lobago, 2007). The pastoral and agro-pastoral production system is practiced in lowland areas of the country where arid and semiarid agro-climates dominate. This system features sparsely populated pastoral rangelands, where subsistence is mainly based on livestock and livestock products except in agro–pastoral areas, where some crops are produced for both subsistence and sale. In this system goats, cattle, sheep, and camels are reared. The main source of food is milk, so pastoralists tend to keep large herds to ensure a sufficient milk supply and income (IBC, 2004). In the urban and peri-urban smallholder dairy production system, farmers are fully involved to the system or use it as their part-time business. In this production system, small numbers of crossbred dairy cattle are kept in a zero-grazing system to produce milk for both home consumption and sale. Animals are fed with natural grass hay and crop residue, supplemented with various amounts of agro-industrial by-products. This system is relatively accessible to a market place for milk and other dairy products, and to government services, like AI services, animal feed and veterinary services (IBC, 2004). The specialised commercial dairy production system consists of specialised dairy farms owning crossbred and/or pure exotic breeds of dairy cattle (Lobago, 2007). Farms in this

system are concentrated in and around major cities and produce milk for sale. Farms can be small, medium or large scale depending on cattle numbers, with large scale farms concentrated in and around the capital city (Lobago, 2007). This PhD study focused on urban and peri-urban smallholder dairy production and specialised (organised) commercial dairy production systems.

1.2.1. Dairy Cattle Breeds in Ethiopia

1.2.1.1. Local Breeds (Zebu)

The word Zebu has been used worldwide for the last 250 years to refer to *Bos indicus* cattle. The external feature which distinctly separates Zebu from European type cattle is the hump present over the shoulders or the posterior part of the neck (Kanwal, 2010). It has been suggested that there are over 25 different breeds of indigenous cattle in Ethiopia (Lobago, 2007). Of 53.4 million cattle population in Ethiopia, 99.3% are pure Zebu breeds (Statistical Bulletin, 2011). Barka, Fogera, Borana, Horro, Sheko, Afar and Arsi are among the Ethiopian local breed cattle having good productive performance. The Fogera and Horro breeds, which are well known for their milk production, are reared around Lake Tana and Eastern Wellega regions, respectively. Horro breeds have a brownish, brick-red skin with almost 90 percent of them of uniform colour (Figure 1.1 G & H) (Tefera, 2013). Foggera are resistant to internal parasites (Tefera, 2013). The Borana, a renowned beef breed/population, found in the southern and eastern parts of the country (NABC, 2010), is also used for milk production (Gojjam et al., 2002; Yilma et al., 2006; Tefera, 2013). The Sheko breeds, which are considered to have tolerance to high tsetse challenge, are found in the southwest part of the country (NABC, 2010).

Tigray is one of the regional states of Ethiopia where livestock production is widely practiced (Genzebu et al., 2012). Raya-Azebo, Irrob, Abergele, Adwa, Arado, Begait (Barka) and Medense are among the local breeds of cattle in the region. Arado is the most populous breed of cattle in the highland part of the region, and they are good for ploughing and are docile (Tefera, 2013). Begait, which are the largest (Zerabruka, et al., 2007) and most excitable breed, require proper restraining during handling, and are not good for

ploughing (Tefera, 2013). Begait have a well-developed udder and are high milk producers (Zerabruka et al., 2007). The physical appearance of some of the Ethiopian cattle breeds is demonstrated in Figure 1.1.

Indigenous cows are the major source of milk in Africa (Yilma et al., 2006), from where 97% of the milk in Ethiopia is obtained (Tedla et al., 1991). However, the milk production of indigenous cattle breeds is very low (ILCA, 1981). Milk yields from unimproved Ethiopian local cows are generally less than 500 litres (L) per lactation (Yilma, 1999); however, when they are crossbred with HF breeds, production increases. The milk yield of Zebu cows, for example Kenana and Butana, which are located in Sudan, is about 1500 L per lactation (Cunningham, 1983).

Reports indicate that the mature body weight of tropical Zebu breeds ranges from 280 to 650 kg, age at first calving ranges from 24 to 62 months and calving interval ranges from 330 to 650 days (reviewed by Mukasa-Mugerwa, 1989; Taneja, 1999). The scale of these variations in productive and reproductive performance suggest that there are large differences between animals in their genotype, nutrition, location, management and climatic factors (reviewed by Mukasa-Mugerwa, 1989; Taneja, 1999). All indigenous cows have very high maternal instinct and do not give milk in the absence of their calves. Production is low if their calves are not around them (Tefera, 2013).

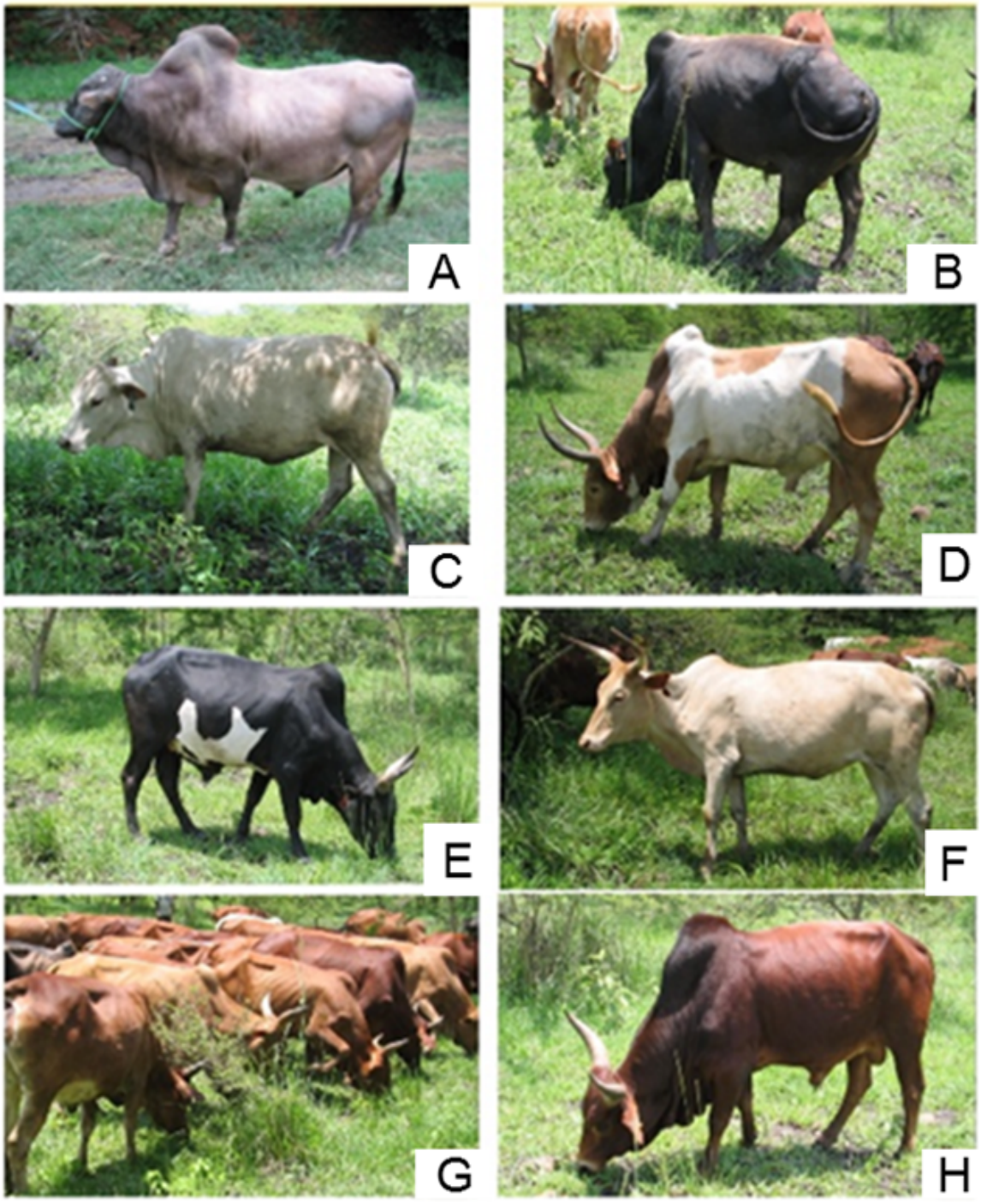

Figure 1.1: Some indigenous cattle breeds of Ethiopia.

Sheko bull (A & B); Sheko heifer (C); Abigar bulls (D & E); Abigar cow (F); a herd of Horro cattle (G); and, Horro bull (H). The Sheko are the only indigenous hump-less cattle of the subspecies *Bos taurus* in Eastern Africa and are trypanosomosis tolerant; Abigar tolerate harsh environment (**Source**: Tefera, 2013).

1.2.1.2. Exotic Breed Cattle

European breeds, especially HF and Jersey, have been imported to Ethiopia for many years and crossed with the indigenous cattle breeds. A previous repot has shown that exotic cattle breeds constitute 0.1% of the Ethiopia cattle population (Statistical Bulletin, 2011). Total milk production from pure HF cows in the country is over 3000 L (Genzebu et al., 2012). It has been reported that HF cattle in the country produce 4.5 times more milk than local breeds and nearly 1000 L more milk than the best lactating crossbred (HF X Zebu) cattle (Demeke et al., 2000). Although HF breed have a better breeding performance, Genzebu et al. (2012) reported a longer calving interval in this cattle breed (460 days) than Barka (local) breed (397 days) under the same good management conditions in Debre Zeit Agricultural Research Centre (DZARC), in the central highlands of Ethiopia. This shows the effect of environment on fertility, as HF breeds are genetically favourable to a temperate environment (El-Wishy, 2013).

1.2.1.3. Crossbred Cattle

Crossbred cattle constitute 0.64% of the Ethiopian cattle population (Statistical Bulletin, 2011). The available AI options in Ethiopia are 50%, 75% or 100% exotic inheritance, which are provided to upgrade crossbreed levels. Previous studies suggested that crossbred cattle were favourable for milk production under tropical conditions (Madgwick and Goddard, 1989; Ahlborn-Breier and Honenboken, 1991; Touchberry, 1992; Zarnecki et al., 1993), while other studies suggested using pure European cattle breeds in the same tropical regions (Swan and Kinghorn, 1992; Simpson and Conrad, 1993; McDowell et al., 1996). However, the particular environmental conditions may dictate which genetic mix is most appropriate (Barbosa et al., 2008). In the temperate climatic conditions of the USA, productivity of *Bos indicus* x *Bos taurus* F_1 crossbred cows has been reported to be outstanding for reproduction and maternal performance relative to that of *Bos taurus* x *Bos taurus* F_1 cross cows when mated to produce terminal-cross calves by Red Poll or Simmental sires (Crouse et al., 1993). Furthermore, a study in the USA demonstrated that crossbreeding has improved the reproductive performance when comparing pure breed Holsteins to three crossbreeds (Heins et al., 2006). Barka, Fogera, Borana, Horro, Sheko,

Afar and Arsi are among the Ethiopian local breed cattle having good productive performance, and are suitable when crossed with exotic breeds in an intensive dairy production system. The productive capacity of local breed dairy cattle in the country has been improved by crossbreeding them with mainly HF and Jersey cattle breeds (NABC, 2010). A recent report shows that Zebu crossed with HF have yielded more total milk (1740 to 2381 L) than local breeds (869 L of milk), whereas the milk from pure HF breed was the highest (3028 L) (Genzebu et al., 2012), under good management practice.

1.2.2. Reproductive Performance of Dairy Cattle in Ethiopia

Reproductive performance of cattle in Ethiopia is not satisfactory (Negussie et al., 1998). Local cattle breeds reach puberty later than *Bos taurus* x *Bos indicus* crossbreeds or pure exotic (*Bos taurus)* cattle, and age at first calving is longer than for exotic breeds or their crosses (Alberro, 1983). Although efforts have been made to improve local cattle breeds through crossing with exotic breeds, mainly HF, these crosses do not attain satisfactory reproductive performance due to various reasons, such as poor nutrition, poor breeding management and reproductive diseases (Haftu et al., 2014; Dubuc, 2011; Tschopp et al., 2014). Similarly, both local and crossbred cows have longer first postpartum estrus and have long calving interval (Shiferaw et al, 2003; Kumar et al., 2014). We have recently demonstrated that heat detection problems (28%), lack of awareness (18%), lack of infrastructure (16%), management problems, such as provision of unbalanced diet, poor housing condition, (14%), inexperience of AI technicians, diseases and lack of incentive to the technicians and owners are the major factors affecting conception in local/crossbred dairy cattle in Tigray Regional State, Ethiopia (Ashebir et al., 2016).

Anestrus is defined as a condition in which the ovaries are quiescent without signs of cyclicity (Noakes, 1996). In dairy cattle, the anestrus period after calving should not exceed 60 days (VanRaden et al., 2004). Cattle which do not exhibit estrus signs during this period are considered in pathological anestrus (Mwaanga and Jonowski, 2000; Zduńczyk et al., 2002). Anestrus (failure of ovarian cyclicity) could be due to insufficient release or production of gonadotrophins to induce follicular growth and maturation or it may reflect the failure of the ovaries to respond to gonadotrophins (Zulu et al., 2000).

Anestrus is one of the major problems affecting reproductive performance of local and crossbred cattle in Ethiopia (Negussie et al., 1998; Mureda and Mekuriaw, 2007; Nuraddis et al., 2011; Dinka, 2012; Kumar and Tkui, 2014). Benti and Zewdie (2014) reported that abortion (12.2%), repeat-breeding - a condition when cows fail to conceive despite multiple inseminations (10.3%), anestrus (10.3%) and retained fetal membranes (RFM; 7.6%) are the major reproductive health problems in local breed cattle in Southern parts of Ethiopia. Another recent report from Central (Debre Zeit) Ethiopia has shown that anestrus (12.9%) was the major reproductive problem followed by repeat-breeding (11.4%) in HF cows (Hadush et al., 2013). However, another report showed lower prevalence of anestrus along with abortion, and uterine/vaginal prolapses in smallholder dairy cows in and around Nazareth, Ethiopia (Gizaw et al., 2007.).

Repeat-breeding is another major problem (Fischer et al., 1998; Gizaw et al. 2007; Dinka, 2012; Hadush et al. 2013; Mandefro and Negash, 2014; Benti and Zewdie, 2014). Repeat-breeding is generally defined as a cow or heifer repeatedly returning to estrus after 3 or more services, while having normal duration of estrous cycles (17-25 days) associated with true estrus and without anatomical abnormalities or infections (Zemjanis, 1980; Perez-Marin et al., 2012). The cause of repeat-breeding is not clear and could be multifactorial, may result from a problem in the cow, the bull or other factors such as environment or farm management (Perez-Marinet al., 2012). Maternal factors causing repeat-breeding are age, genetic problems, uterine infections, conformational defects, hormonal imbalance, early embryonic mortality and nutritional problems (Perez-Marin et al., 2012). Failure of fertilisation is one of the factors contributing to repeat-breeding, which could be due to failure of ovulation, delayed ovulation, defects in the ovum, poor semen quality, low sperm concentration, poor motility, improper handling of semen and improper AI, inflammatory conditions and anatomical defects of the reproductive tract of the cows (Karunakaran et al., 2012).

Anestrus and repeat-breeding can be controlled and treated by reversing the factors responsible for their occurrence. Proper feeding management, veterinary health care and breeding management can possibly improve fertility and productivity of dairy cattle.

Exogenous hormonal administration has been used to treat cattle with problem of anestrus and repeat-breeding (Rhodes et al., 2003b; Lucy et al., 2004; Honparkhe et al., 2008; Hailu et al., 2015).

1.2.3. Developments in Improving Reproductive Performance of Crossbred Cattle in Ethiopia

There are various developments in the country aimed at improving reproductive performance and productivity. One of the main developments addresses artificial insemination (AI) services in urban and rural areas of the country. AI services have been extensively supported, both at the veterinary units, and/or as a door-to-door service to improve the genetic potential and increase milk production. The Government Agriculture Bureau has established at least one bull rearing and semen storage and processing centre in each region in the country. Currently, the AI service is supported by an estrus synchronisation programme. The programme is carried out extensively throughout the country whereby local/crossbred heifers and cows are administered a single dose of PGF2α which is followed by insemination of animals 48 and 72hr post treatment. Reports from Tigray Regional Government Agriculture Bureau showed a high estrus response of cows following administration of prostaglandin F2α (PGFα), though the conception rate was low (31.5%; Giday, 2014). Further to the government's effort to support the dairy production sector, particularly through improving reproductive potential and productivity, some organised commercial farms have started importing and using semen from genetically improved sires. This includes importation of sex-sorted semen to obtain female calves and enrich availability of adequate replacement heifers in the farms.

1.3. REPRODUCTION IN DAIRY CATTLE

1.3.1. Puberty and Sexual Maturity in Heifers

Generally, puberty is defined as the process whereby animals become capable of reproducing (Robinson and Shelton, 1977). However, in heifers, puberty is defined as the first estrus that is followed by a normal luteal phase (Moran et al., 1989). This involves a

complex series of interactions of genetic and environmental factors that direct endocrine events, which result in puberty (Estill, 2015). The hypothalamic release of pulsatile gonadotrophin-releasing hormone (GnRH) is considered the trigger for mammalian puberty, as it initiates the release of luteinising hormone (LH) and follicle stimulating hormone (FSH) that are required for gonadal activity and gametogenesis (Fortes et al., 2016).

Replacement heifers are fundamental to the dairy industry; hence, herds could be improved by culling less reproductive and productive performing cows and replacing them with well-fed, healthy, genetically superior, and properly managed cows (Heinrichs and Hutchinson, 2015). A number of factors including genetic, nutrition, body weight, diseases and parasites can affect age at puberty (McDonald, 1980; King et al., 1983). These factors cause a delay in first service. Any delay of first service will increase age at first calving and increase associated costs (Heinrichs, 1993). *Bos indicus* heifers are older at puberty compared to *Bos taurus* heifers (reviewed in Yelich and Bridges, 2012). According to Alberro (1983), the Ethiopian Zebu (*Bos indicus*) cattle reach puberty at the age of 22.4 months. Similarly, puberty in crossbred (Zebu x HF) heifers is usually 24 months (Duguma et al., 2012). However, this is not consistent throughout Zebu or crossbred cattle in the country and varies with breed, region/environment and management. Whereas, exotic breeds, for example HF heifers reach puberty earlier than Zebu or Zebu x HF. Furthermore, *Bos taurus* heifers attain puberty at 6-12 months of age, generally at a body weight of 200-250 kg (Forde et al., 2011). Others reported that age at first service in British HF heifers is 16.5 months (Cooke et al., 2013)

Heifers reaching puberty ovulate potentially fertile oocytes, which may be accompanied by visual signs of estrus (reviewed in Cooke, 2009; Wathes et al., 2014). Up to 25% of cattle can show estrus without ovulating (Byerley et al., 1987). Usually the first ovulation is not accompanied by heat and is followed by a short luteal phase, with the subsequent fall in progesterone (P4) helping to promote the first behavioural changes (Gasser, 2013). Morrow et al. (1976) reported that 6% and 25% of estrous cycles in HF heifers resulted in silent and non-standing estrus, respectively. Such problems together with poor estrus

detection affect reproductive performance of dairy cattle in Ethiopia (Alemayehu and Moges, 2014). These reproductive problems can be largely resolved by incorporating estrus synchronisation into the breeding management programme. Studies have shown that treatment with P4 can enhance onset of puberty by stimulating pulsatile LH secretion that accelerates follicle growth to the preovulatory stage (Imwalle et al., 1998; Patterson et al., 1990; Lucy et al., 2001). Heat detection aids can also be applied in breeding management to improve estrus detection efficiency in organised commercial farms with large herds. However, their feasibility and affordability under smallholder farming conditions require further study.

1.3.2. Ovarian Anatomy, Physiology and Endocrine and Paracrine Control of Ovarian Functions in Cattle

1.3.2.1. The Ovaries

The term "ovary" is derived from the Latin word "ovum," meaning egg (Edson et al., 2009). The ovaries are paired glands, and are primary organs of reproduction in the female. They produce oocytes and steroid hormones (including estrogen and P4). During embryonic development, cells from the inner cell mass undergo differentiation to become specialised stem cells that form various tissues of the fetus (Britt, 2008). Primordial germ cells arise from a set of progenitor cells set aside during early embryonic development (Seydoux and Braun, 2006; Britt, 2008). In the female, the primordial germ cells are precursors of oogonia (Britt, 2008). Primordial germ cells migrate from the embryonic yolk sac through the hindgut to the genital ridge and populate in the gonads (ovaries in female and testes in male) (Lawson and Hage 1994; Britt, 2008; Conti and Giudice, 2008). About two-third of the original population of germ cells in the fetus are lost by the time of birth; however hundreds of thousands of oocytes are present in a neonatal calf's ovaries and thousands can remain in the ovaries of mature cows (Erickson, 1966).

In cattle, ovaries are berry-shaped and, in heifers and young cows, they are located at the side and slightly below the uterine horns in the pelvic cavity, whereas in multiparous cows they are situated in the abdominal cavity (Noakes et al., 2009). The ovaries have two

extremities, tubal and uterine extremities. The tubal extremity of the ovary is attached to the fallopian tube by infundibulopelvic ligament (Daftary and Chakravarti, 2011), whereas, the uterine extremity is attached to the uterus by the ovarian ligament. The ovaries have two parts, cortex and medulla. The surface epithelium, tunica albuginea and numerous ovarian follicles (Figure 1.2) and corpus luteum (CL) constitute the cortex, while the medulla is consisted of numerous blood vessels, lymphatics and nerves (Williams and Erickson, 2012; http://www.oecd.org/chemicalsafety/). In the cortex of the ovary, folliculogenesis, a process of attaining successively higher levels of organisation by means of cell proliferation and cytodifferentiation, when small primordial follicles mature to become preovulatory follicle (Williams and Erickson, 2012). The growing follicles, with two or three layers of granulosa cells are situated deeper into the cortex than primordial follicles (Tian and Zhao, 2000).

The weights of both right and left ovaries are almost the same at birth, and they grow rapidly from birth to puberty (Tian and Zhao, 2000). However, ovaries on the right side are bigger and more active than ovaries on the left at sexual maturity (Pierson and Ginter, 1987; Schneebeli and Döbeli, 1991). Similarly, studies in human reported that the right ovary tends to undertake more ovulation than the left ovary (Potashnik et al., 1987; Check et al., 1991).

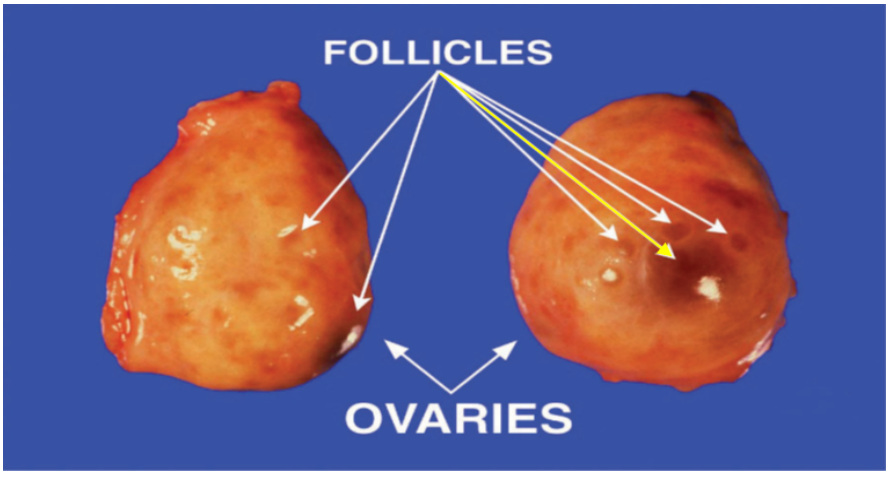

Figure 1.2: Ovaries in cattle with blister like follicles; yellow arrow dominant follicle (**Source**: SRS, 2016).

1.3.2.2. Ovarian Follicles and Folliculogenesis

Follicles are fluid filled, blister like structures (Figure 1.2) containing the developing oocytes or eggs (SRS, 2016). The adult ovary contains a reserve of inactive primordial follicles containing a small non-growing oocyte and a layer of non-dividing pregranulosa cells encapsulated by the follicular basal lamina (Rodgers and Irving-Rodgers, 2010). There are numerous follicles on each ovary varying in size from barely visible to 18-20mm in diameter (SRS, 2016). The largest follicle on one of the ovaries is termed the "dominant follicle" (Figure 1.2, Right - yellow arrow) and is the most likely candidate for ovulation when the animal comes into heat; whereas, more than 95% of the other follicles on the ovaries regress and undergo atresia without ovulating and are replaced by new growing follicles (SRS, 2016).

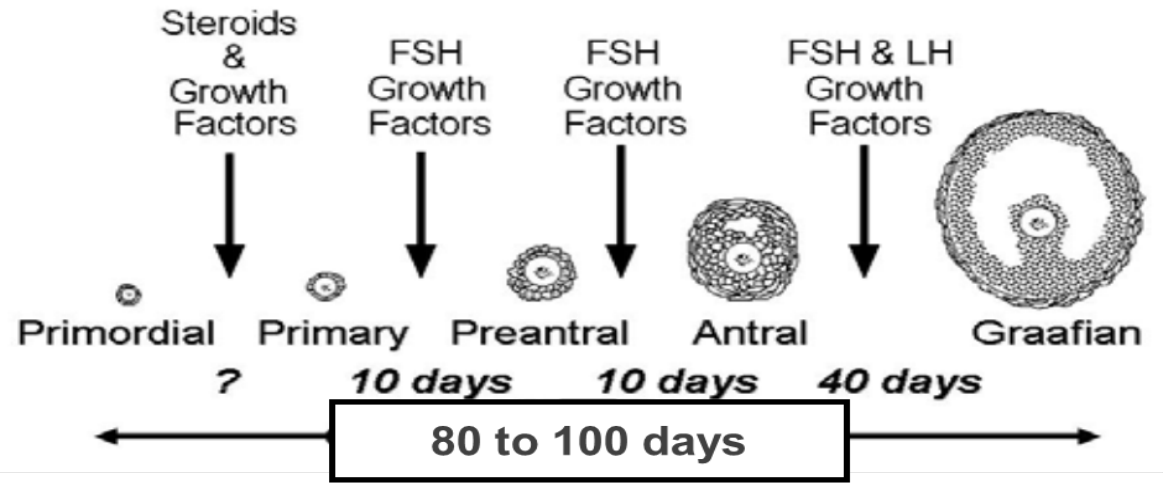

Figure 1.3: Approximate timeline of bovine folliculogenesis for each stage and examples of hormones and factors that regulate transition from one stage to the next (**Source**: Britt, 2008).

Fertility in female mammals depends on the ability of ovaries to have an adequate primordial follicle pool (Nilsson and Skinner, 2009) and their ability to produce Graafian follicles, which can ovulate fertilisable oocytes (Erickson, 2008). There are two different pools of follicles on the ovaries of cattle, the non-growing pool that contains primordial follicles, and the growing pool that contains primary, secondary and tertiary follicles

(Kanitz et al., 2001). The primordial follicles enter into the growth phase by leaving the arrested pool and grow into primary follicles, where by the oocytes increase in size and the surrounding squamous pre-granulosa cells become cuboidal and proliferate to form a layer of cuboidal cells around the growing oocyte (Fortune et al., 2000). The mechanism by which initiation of growth of primordial into primary follicles controlled by steroids (Britt, 2008), growth factors and c-kit (reviewed by Web et al., 1999a). Oocyte growth is a slow process (Hansen, 2013). Once an ovarian follicle emerges from the pool of resting follicles and initiates growth, it takes approximately 100 days (Figure 1.3) to reach the point where ovulation can occur and the oocyte contained within the follicle is released (reviewed by Britt, 2008; Hansen, 2013; Webb et al., 2016). The number of follicles commencing growth in a given time is predictable, as it is based on the size of the primordial store that drops exponentially with time (Webb et al., 1999a).

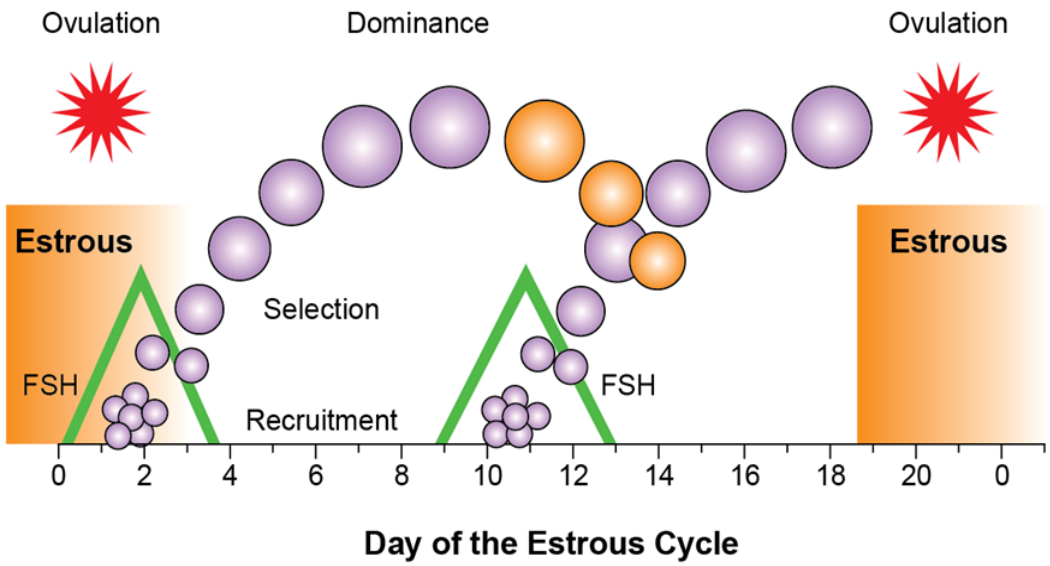

Figure 1.4: Relationship between circulating concentrations of follicle FSH and stages of a bovine follicular wave (recruitment, selection, and dominance).

A transient increase in FSH (solid line) initiates recruitment of a cohort of follicles, from which a single follicle is normally selected to become the dominant follicle. If the CL regresses in the presence of a viable dominant follicle ovulation will occur (second follicular wave). However, in the absence of luteal regression, the dominant follicle becomes atretic (regresses; brown circles) (**Source**: Modified from Kojima and Patterson, 2003; Smith et al., 2012).

During folliculogenesis, various developmental stages, which include primordial follicle recruitment, preantral follicle development, selection and dominance and follicle atresia take place (Richards et al., 1995; Smith et al., 2012). The concept of recruitment is used for the entrance of follicles into the growth pool in a wave-like growth pattern (reviewed by Kanitz, 2003; Kojima and Patterson, 2003; Smith et al., 2012). The key hormone for the endocrine initiation of follicular waves is FSH (Adams et al., 1992; Sunderland et al., 1994; Fricke et al., 1997). Over 95% of estrous cycles are composed either two or three follicular waves (Adams, 1999). *Bos Taurus cattle* usually have two to three follicular waves (Kojima and Patterson, 2003; Smith et al., 2012), and each wave begins with the growth of a cohort of antral follicles from a pool of growing small follicles, which is referred to as recruitment (Smith et al., 2012). Cattle with two follicular waves usually have shorter estrous cycle length of 18 to 20 days compared to those with three follicular waves which are 20-24 days long (Ginther et al., 1989; Kojima and Patterson, 2014).

During the first stage of folliculogenesis, recruitment, a cohort of small follicles usually 4-6 mm in diameter begin their final growth (Atkins et al., 2013). During recruitment, theca cells organise into distinct layers around early developing follicles and establish essential cell-cell interactions with the granulosa cells (reviewed by Kanitz, 2003). The granulosa cell-derived kit-ligand promotes the formation of theca cell layers around the primary follicles (Parrott and Skinner, 2000). Recruitment of a cohort of follicles is associated with initiation of expression of messenger-ribonucleic acid (mRNA) for cholesterol side-chain cleavage enzyme (P450scc) and aromatase (P450arom) in granulosa cells (Kanitz, 2003). This stage is initiated by a transient rise in FSH (Adams et al, 1992) and recruited follicles require GnRH for survival (Atkins et al., 2013).

Normally, three to six follicles with a diameter of 4 to 5 mm occur after recruitment of follicles into a follicular wave (Savio et al., 1988; Sirois and Fortune, 1988; Sunderland et al., 1994). However, others reported recruitment of higher number of follicles in a wave (Adams, 1999; Bellmann, 2001). The recruited follicles produce estradiol and inhibin that cause a decrease in circulating concentration of FSH that leads to the second stage of

folliculogenesis, selection, which occurs approximately 36 to 48 hr after the initiation of follicle recruitment (Atkins et al., 2013).

The third stage of the follicular wave is dominance. At this stage, one of the recruited follicles is subsequently selected from the cohort for continued growth (reviewed by Kanitz, 2003; Smith et al., 2012; Atkins et al., 2013), whereas the subordinate follicles becomes atretic (Atkins et al., 2013). The selected follicle acquires LH receptors in the granulosa cells and also more free Insulin-like growth factor 1 (IGF-1) compared with the subordinate follicles, both of which make the selected follicle capable of producing estradiol and inhibin in a low-FSH environment (Atkins et al., 2013). It has been reported that the temporal expression of LH-R is different between follicular cells, as the theca cells produce LH-R early in the follicular growth phase, while the granulosa cells acquire LH-R near the preovulatory LH surge (Peng et al., 1991). In agreement to this, others reported that LH-R is expressed only in the granulosa cells of one follicle of ≥8 mm in diameter per cow (Bao et al., 1997; Manikkam et al., 2001). This suggests a relationship between follicular dominance and LH-R production in granulosa cells (Robert et al., 2003). The selected follicle becomes larger than other follicles and becomes capable of surviving in a low FSH environment, unlike the subordinate follicles that are not (Atkins et al., 2013), and becomes dominant in size and influence over smaller follicles (Smith et al., 2012; Atkins et al., 2013).

The presence of a dominant follicle causes suppression of circulating FSH levels, and the subordinate follicles, which are gonadotrophin-dependent (Figure 1.3), become atretic (Webb et al., 2003; Britt, 2008; Smith et al., 2012; Atkins et al., 2013; Webb et al., 2016). The dominant follicle grows approximately 1 to 2 mm per day (Sirois and Fortune, 1988; Knopf et al., 1989) and reaches its maximum size of 10 to 20 mm (Fortune et al., 1988; Savio et al., 1988; Ginther et al., 1989; Knopf et al., 1989). A viable dominant follicle, which is present at CL regression, will generally become the ovulatory follicle (e.g. second follicular wave in Figure 3) (Smith et al., 2012). If luteolysis occurs during the growth phase of the dominant follicle, final maturation and ovulation occurs (reviewed by Kanitz, 2003), whereas, the remaining follicles become atretic. Different stages of follicular

growth and atresia have been associated with changes in mRNA expression for the gonadotrophin receptors, key steroidogenic enzymes and growth factors (IGF-I and -II) and their binding proteins (reviewed by Kanitz, 2003). During a nonovulatory follicular wave (e.g. first follicular wave in Figure 1.4), the dominant follicle eventually becomes atretic and a new follicular wave is initiated (Smith et al., 2012).

The pre-ovulatory follicles contain two distinct sublineages of granulosa cells, the mural granulosa cells and cumulus cells, arising during folliculogenesis as the cell population segregate upon formation of the fluid-filled follicular antrum (reviewed by Russell and Robker, 2007). The mural granulosa cells, which line the follicle wall, are separated from the theca cell layer by a basement membrane, and the cumulus cells surround the oocyte (reviewed by Russell and Robker, 2007). The LH-R appear on the surface of granulosa cells only in the late stages of folliculogenesis, and probably only in the dominant follicle that is destined (Robert et al., 2003). Luteinising hormone plays an important role in the regulation of ovarian function, such as ovulation and transformation of follicular cells into luteal cells (Smith et al., 1994).

1.3.2.3. The Corpus Luteum (CL)

The CL is a small, dynamic endocrine gland formed following ovulation from the secretory cells (granulosa and theca cells) of the ovarian follicles and plays an essential role in regulation of reproductive cycle and pregnancy (Baerwald et al., 2005; Tomac et al., 2011). The primary function of the CL is production of P4 during the luteal phase of the estrous cycle in non-pregnant female cattle and during pregnancy (reviewed by Schams and Berisha, 2004). It consists of endothelial cells, steroidogenic luteal cells, fibroblasts, smooth muscle cells and immune cells (O'Shea et al., 1989). The formation of the CL, referred to as luteinisation, is initiated by a series of morphological and biochemical changes in theca interna and granulosa of the preovulatory follicle (reviewed by Schams and Berisha, 2004). Both folliculogenesis and luteinisation are characterised by irreversible and profound physiological and morphological transformation processes,

which eventually result in the ovulation of a fertilisable egg and the conversion of the estrogen-producing follicle into a P4 producing CL (Vanselow and Fürbass, 2010).

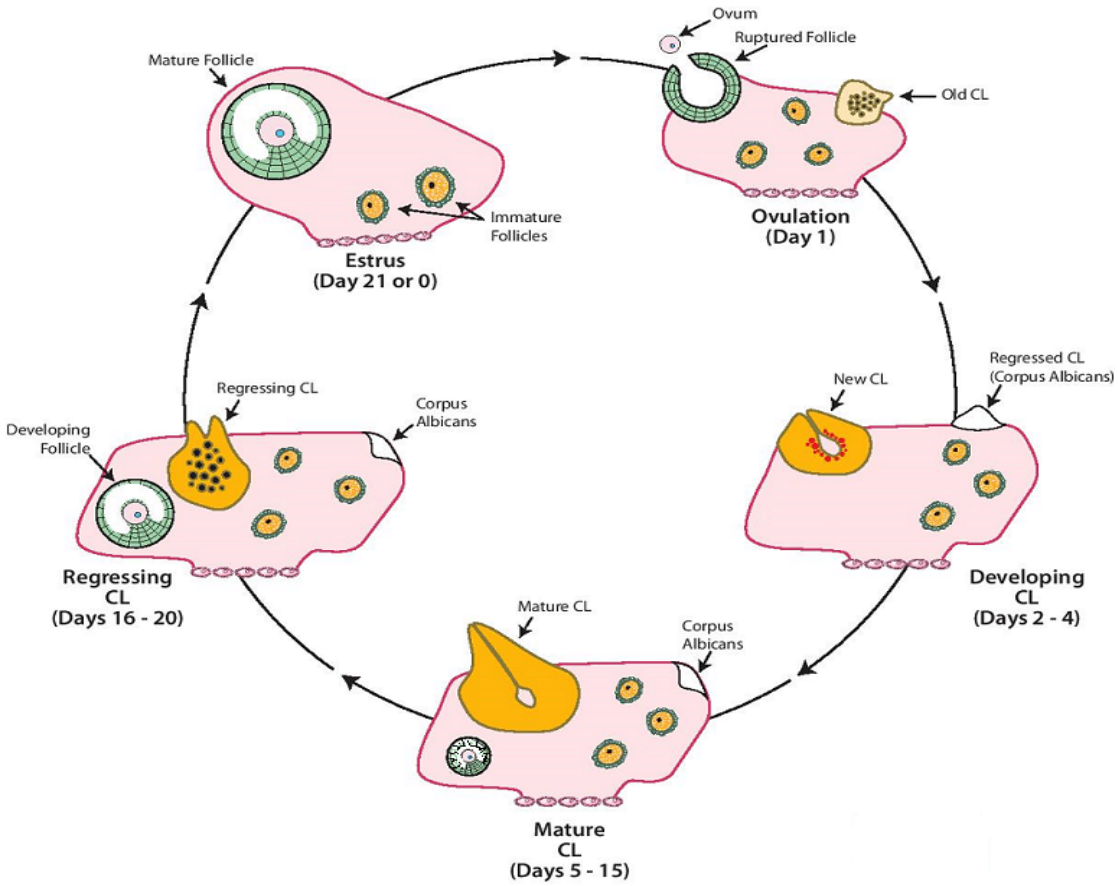

Figure 1.5: The ovarian changes during a typical 21-deay estrous cycle in non-pregnant cow (**Source**: Whittier, 1993).

In cattle, the CL of pregnancy remains at its maximum size throughout the gestation and a significant decrease could be seen only after calving (Ginther, 1998). The CL in diestrus cattle is slightly smaller than in pregnant cattle, and contains a central lacuna unlike in pregnant cattle where it disappears (Noakes et al., 2009). The colour of the CL depends on the pregnancy status of the cow. In pregnant cattle, the colour of the CL is yellow. The development and regression of the CL, and of the follicles, are continuous processes in normally cycling non-pregnant cattle (Whittier, 1993), see Figure 1.5. For successful

establishment of pregnancy, maintenance of a functional CL beyond its normal cyclic lifespan is required, to sustain production of P4 (Ziecik et al., 2011).

In normally cycling, non-pregnant cows, the CL regresses due to the effects of PGF2α from the uterus, leading to basal P4 level (Purohit, 2010), whereas, in pregnant cattle, the CL persists throughout pregnancy and continues producing P4. In the absence of implantation, or at the end of pregnancy, the CL ceases production of P4, and its tissue mass decreases in size accompanied by loss of cellular integrity, defined as luteal regression or luteolysis (Niswender and Nett, 2005), which allows the start of a new ovarian cycle (Whittier, 1993) (Figure 1.5).

The ovarian cycle is characterised by repeated patterns of cellular proliferation, differentiation and transformation that accompany follicular development and formation and regression of the CL (reviewed by Schams and Berisha, 2004). The differentiation of follicular cells into luteal cells is caused by the preovulatory LH surge (reviewed by Schams and Berisha, 2004). The LH surge is characterised by increased steroid production and a switch from producing estradiol to P4 and of enzymes responsible for these changes (Juengel and Niswender, 1999). LH and growth hormone (GH) are the primary luteotropic hormones that support the development and function of the CL in domestic animals (reviewed by Schams and Berisha, 2004). Previous classical binding studies have shown that an increase in the number of LH receptors in the bovine CL (Garverick et al., 1985; Okuda et al., 1999) was preceded by an increase in mRNA encoding this receptor (Kobayashi et al., 2001).

1.3.2.4. Estradiol and P4: Synthesis and Roles

The major site of estrogen and P4 synthesis is the ovary. The ovarian follicle and CL are the major estradiol and P4 production sites, respectively. Theca interna cells produce androstenedione and testosterone, which diffuses in to the granulosa cells. The granulosa cells synthesise estradiol and estrone from androgenic precursors produced by the theca interna cells (Bullock et al., 2001). Detailed steroid hormone synthesis pathway is illustrated in Figure 1.6.

Estrogen is a primary female sex hormone and is primarily synthesised by the ovarian follicle (Harris, 1972). Estrogen is also produced in small amounts in the adrenal glands, brain, and fat of both sexes (Roselli et al., 1997; Oettel, 1999). Furthermore, in some species like humans, sheep and horses, the placenta produces estrogen derived from either fetal androgens, placental progestins, or other steroid precursors (Bowen, 2000). There are three forms of estrogen, all of which have 18 carbon atoms (C18). Estrone, which has a single hydroxyl group, is a weak estrogen produced by the ovaries and fat tissue (Bullock et al., 2001). It is also converted from steroid precursors and external environment precursors. Estradiol is the most active form of estrogen (Viganó et al., 1990; Bullock et al., 2001; Guyton and Hall, 2011) having 2 hydroxyl groups, and is mainly produced by the ovaries. Estriol is the weakest of the three forms of estrogen and has 3 hydroxyl groups. This hormone is produced by the placenta and the liver, but not by the ovary (Bullock et al., 2001).

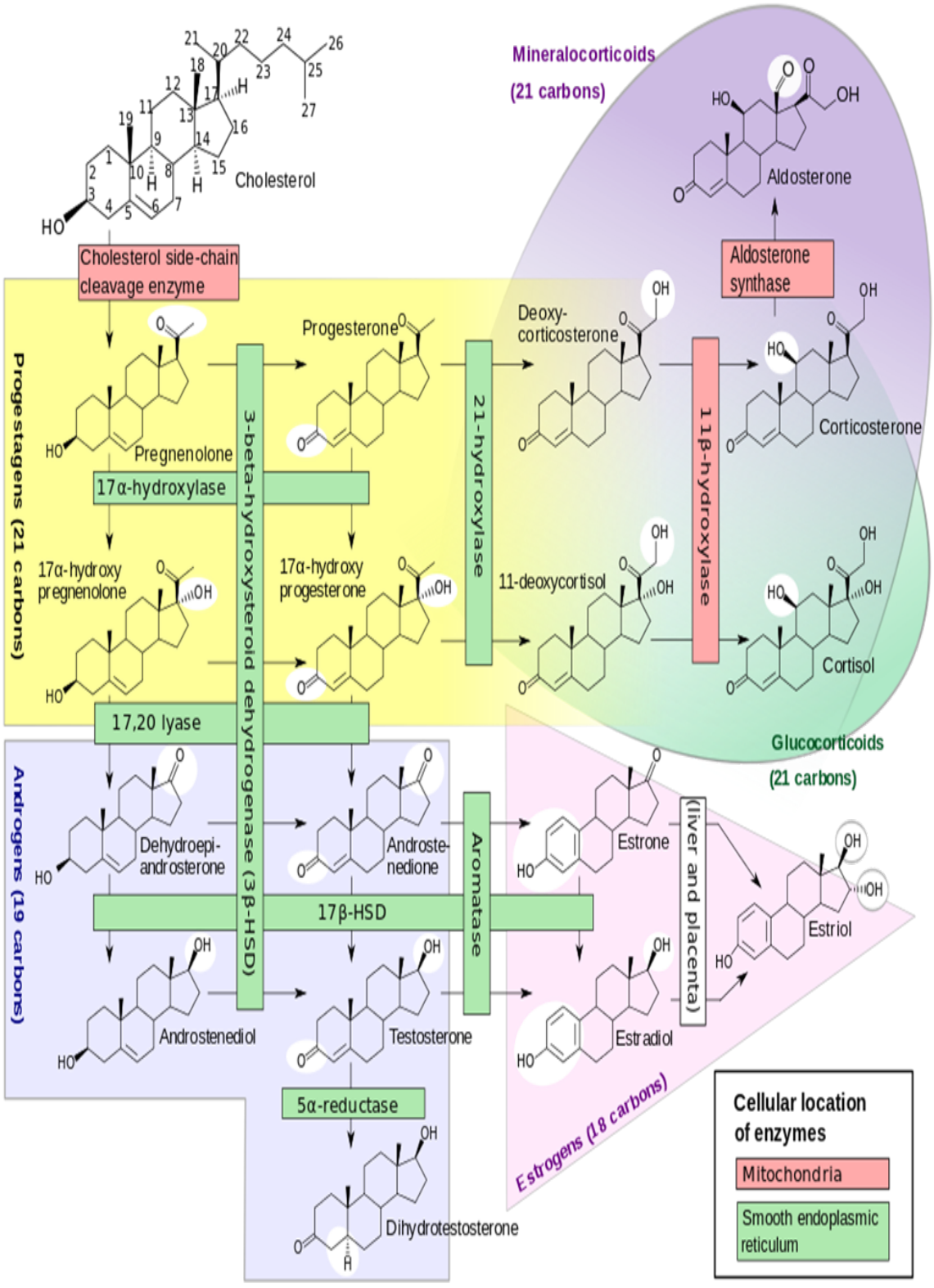

Figure 1.6: Steroidogenesis pathways (**Source**: Häggström and Richfield, 2014).

The actions of estrogens were first described by Charles R. Stockard and George N. Papanicolaou in guinea pigs (Stockard and Papanicolaou, 1917), which was then followed with similar findings in rats in 1922 by Joseph A. Long and Herbert M. Evans (Long and Evans, 1922). Production of estrogens from the ovaries fluctuates during the reproductive cycle. The level of estrogens is high when cattle are in heat and around the end of pregnancy. Estrogen, specifically, estradiol, is the primary signal to the brain that induces expression of estrus, but only in the absence of P4 (Vailes et al., 1992). Estrogens also play important roles in growth and development, the regulation of reproductive cycles (Bowen, 2000), and affect many other body systems. In pregnant cows, estrogens stimulate growth of the myometrium and antagonise the myometrial-suppressing activity of P4. In many species, the high levels of estrogen in late gestation induces myometrial oxytocin receptors, thereby preparing the uterus for parturition. Estrogens also stimulate mammary gland development, both ductal and alveolar growth (Bowen, 2000). Furthermore, in species like humans and horses, where placental estrogens are synthesised from androgens produced by the fetus, maternal estrogen levels are usually an important indicator of fetal wellbeing (Bowen, 2000).

Immediately after the Graffian follicle ruptures, a CL develops from the collapsed follicle. The CL is the principal site of P4 production (Bullock et al., 2001). The primary hormone that stimulates the production of P4 by luteal cells is LH (Niswender and Nett, 1988). In cows treated with an antiserum against LH, it has been reported that the CL regressed, causing a reduction in P4 concentration to basal levels within a short period of time (Hoffmann et al., 1974). Most of the LH receptors are located on the small luteal cells and are stimulated by LH to secrete P4 through a mechanism involving formation of cyclic adenosine monophosphate (cAMP), activation of protein kinase A (PKA), and subsequently increased P4 production (Schams and Berisha, 2004). IGF-1 mRNA is expressed in theca interstitial cells and granulosa cells of the ovarian follicles (Schams et al., 1999). Large luteal cell, where GH receptors (protein and mRNA) are located (Lucy et al., 1993; Kirby et al., 1996; Koelle et al., 1998), are responsible for 80% of total P4 production by the CL (Niswender et al., 1985). It has been reported that GH stimulates P4

and oxytocin secretion by bovine CL (Liebermann and Schams, 1994) and supports the development of the CL in vivo (Lucy et al., 1994; Juengel et al., 1997). Furthermore, in studies of bovine CL in vitro, GH has been suggested to be a more powerful stimulator of PGF2α and P4 production in the early bovine CL than LH (Kobayashi et al., 2001). Previous studies in cows have shown that as follicles grow, the expression of granulosa cell FSH receptor declines, while LH receptor, cholesterol side-chain cleavage enzyme, 3β-hydroxysteroid dehydrogenase and cytochrome P450 family 19 subfamily A member 1 (CYP19A1/aromatase) increase, as do the concentrations of estradiol and P4 in the follicular fluids (reviewed in Bao and Garverick, 1998; Webb et al., 1999b; Fortune et al., 2001; Knight and Glister, 2003; Beg and Ginther, 2006).

Around estrus, P4 level is low in cattle both in blood and milk. Between 6th to 17th days of the estrous cycle the P4 level in serum or milk remains high, which subsequently declines from day 17 onwards if the animal is not pregnant (Figure 1.7). However, if the cow is pregnant, P4 concentrations in the body will remain at a higher level until around the end of pregnancy (Rhodes, 2015).

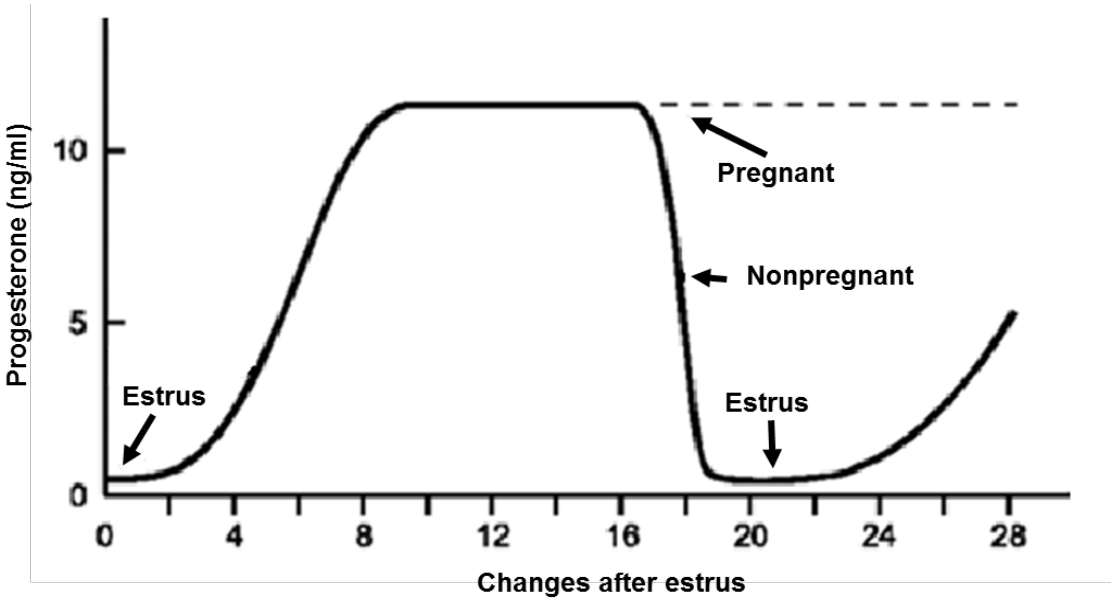

Figure 1.7: Changes in P4 levels throughout the estrous cycle (**Source**: Silvia and Heersche, 1986).

In addition to the CL, the placenta of all mammals produce progestins. In some species, for example women, horses, sheep and cats, the placenta secrets sufficient P4 to maintain the pregnancy (Senger, 2005; Ziecik et al., 2011; VDIB, 2015). In these species, ovariectomy after establishment of the placenta will not affect P4 secretion and pregnancy outcome. However, in species such as cattle, pigs, goats and dogs, luteal P4 is necessary throughout gestation, because placenta production is insufficient (Bowen, 2000; Ziecik et al., 2011).

1.3.3. Estrous Cycle in Dairy Cattle

Estrus is defined as the state at which female animals exhibit sexual receptivity and accept the male (Roelofs et al., 2010). The duration of estrus is 10-18 hr, however this can vary between individual cattle (range <8 to >30 hr) (O'Connor and Senger, 1997). The average duration of standing estrus in cows is 18 hr during which they stand to be mounted by a bull or fellow cows (Roberts, 1986). However, others reported an average standing estrus of 7.1 hr in lactating HF cows housed on an outdoor wood-chip pad in Ireland (Sveberg et al., 2011). Estrous cycle is defined as the length of time from one estrus period to the next involving the whole sequence of hormonal, reproductive as well as behavioural changes (Goehring, 2015). The average length of the estrous cycle in cattle is 21-days, ranging from 17 to 24 days (Wishart, 1972; Salisbury et al., 1978).

The estrous cycle has four different stages: pro-estrus (18^{th} to 20^{th} day), estrus (0 day), met-estrus (1^{st} to 5^{th} day) and di-estrus (6^{th} to 17^{th} day) (reviewed by Rao et al., 2013). The estrous cycle is controlled by many hormones, such as GnRH, FSH, LH, P4, estradiol, inhibin and PGF2α (Roche, 1996; WAAESD, 2005). These hormones collectively provide control and feedback mechanisms between the hypothalamus, pituitary gland and ovaries, which regulate ovarian follicular dynamics, CL function and ovulation (Pring et al., 2012). The exposure to P4 or inhibition of P4 action by a P4 antagonist in early to mid-diestrus regulates the onset of uterine release of PGF2α from endometrium, causing shortening or extension of the inter-estrus interval in sheep and cows (Schams et al., 1998; Schams and Berisha, 2001; Schams and Berisha, 2002). This indicates regulatory activity of steroids in ovarian function. Concentrations of P4 are low when cattle are in-heat (reviewed by

O'Connor 1993; Ashwood, 2012). The basal (low) concentration of P4 due to luteolysis induces frequent increases in GnRH pulses (Chabbert-Buffeta et al., 2000). These increases in GnRH pulses stimulate expression of GnRH receptors on the pituitary gonadotrophes (Nett et al., 2002). An increase in pituitary sensitivity to GnRH, due to low P4 level, stimulates the dominant follicles to produce sufficient estradiol to induce estrus (Chabbert-Buffeta et al., 2000). When cattle are in-heat, the level of estradiol increases leading to an increase in LH, and P4 becomes basal. A surge in LH and FSH concentrations occurs at the onset of estrus and induces ovulation. Immediately after ovulation, the level of P4 increases due to the presence and development of the CL (Figure 1.8). The CL will be persistent and P4 level remain elevated if the animal is pregnant. The CL regresses in cows that fail to become pregnant, due to release of PGF2α by the endometrium after day 12 of the estrous cycle and P4 levels decline around day 17 of the cycle, and the animal returns to estrus (briefly reviewed by O'Connor 1993; Kojima 2009; Forde et al., 2011).

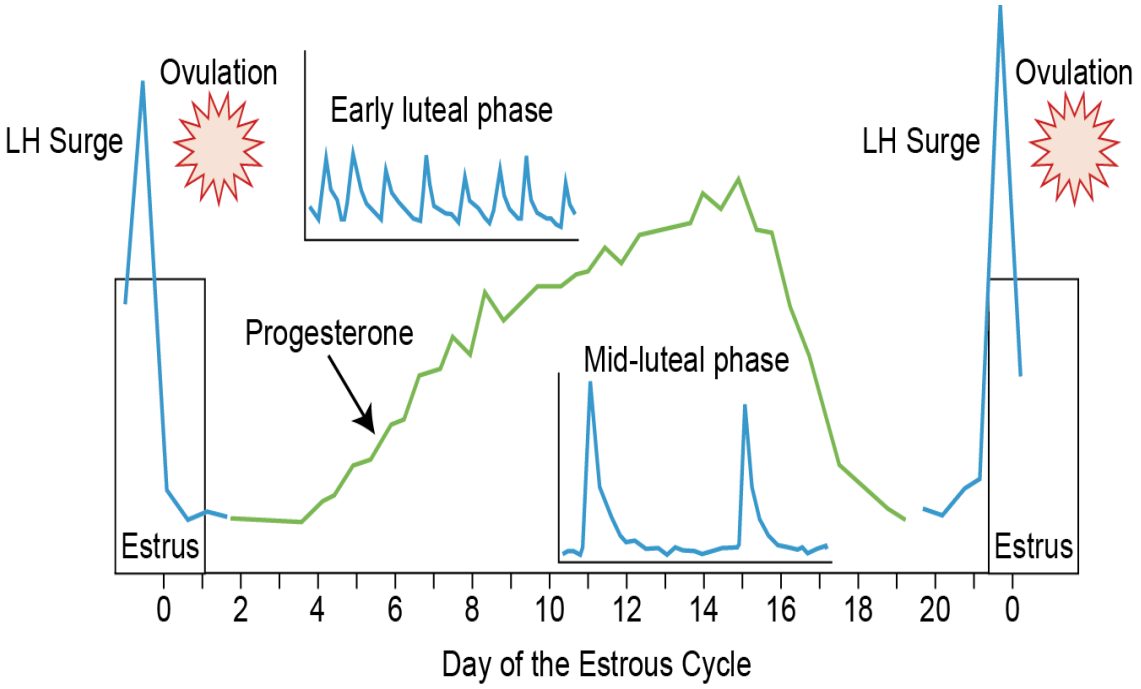

Figure 1.8: The estrous cycle in cattle; a relationship between pulsatile patterns of LH (blue line) and the typical profile of P4 (green line) concentrations during the estrous cycle of cattle. Inserts show change in frequency of LH pulses over the cycle (**Source**: Kojima, 2009).

1.3.4. Estrous Behaviour and Detection in Cattle

High circulating estradiol is a key factor leading cattle to exhibit estrus, this is in the presence of a low level of P4 (Vailes et al., 1992). When cattle are in estrus the level of estradiol is not critical but should be above a threshold (approximately 10pg/ml), however the P4 level is critical and needs to be below a relatively low threshold (about 0.6 ng/ml) (Britt, 1995). Standing to be mounted by a bull or herd mate is the primary and most definitive outward sign of estrus in cattle (Forde et al., 2011; Graves, 2012) and is the best indicator of the fertile period (Fricke, 2015). Secondary signs of estrus in cattle include roughened tail-head; dirty streaks and marks on lower hips, sides, or shoulders (in wet weather); nervousness and restlessness; riding or mounting other animals; grouping together; clear mucus discharge; swollen and red vulva; chin resting and rubbing; frequent urination; bawling; sniffing behaviour; decreased milk production and off feed (Duponte, 2007; Graves, 2012).

Good reproduction management is key for successful dairy farming and detection of estrus is the first step in achieving pregnancy; the second step is timely insemination (Roelofs and van der Kooij, 2015). One of the most important factors causing reduced fertility is the inability to detect cows in estrus (Law et al., 2009). Inadequate and inaccurate estrus detection causes cows to become repeat-breeders (Bilby, 2010) and to be incorrectly considered anestrus. In many farms, estrus detection accuracy is less than 50% (Bilby, 2010), which suggests utilisation of estrus detection aids may be required (Rorie et al., 2002). It is important to monitor estrus in cattle at least twice a day. Animals in heat in the morning should be inseminated that afternoon, and animals in heat in the afternoon should be bred the next morning (Graves, 2012).

There are various heat detection aids that can help to identify cattle in estrus when no one is around them. Estrus detection aids include tail paint (whereby painted tailheads can be monitored for rubbing activity), pedometers, HeatWatch (helps for 24 hr surveillance effective for detecting standing estrus whereby activation of a sensor sends a radio telemetric signal containing a code for transmitter identification, date, time and duration of standing event), detector animal (teaser bull) with chinball marker, radiotelemetric

pressure transducers, measurement of milk temperature (Maatje and Rossing, 1976; Kyle et al., 1997; Rorie et al., 2002; Bilby, 2010; Graves, 2012; Michaelis et al., 2013; Rao et al., 2013; AHDB, 2014). Determination of P4 level in milk or serum/plasma is another widely used method of estrus detection in cattle, and the method is effective and reliable (Booth et al., 1979; Eddy and Clark, 1987; Friggens and Chagunda, 2005; Simersky et al., 2007). Ultrasonography has also been used to confirm animals in estrus (Hansar et al., 2014). Application of estrus synchronisation can also help to improve estrus detection and inseminate cattle sooner (Graves, 2012; Britt, 1995) or to enable to the insemination of animals without the need for estrus detection (Patterson et al., 2011). In Ethiopia, the usual means of estrus detection is visual inspection (observation) (Mukasa-Mugerwa et al., 1991; Lobago, 2007). Estrus detection in most farms in Ethiopia is not satisfactory, which could be due to a lack of application of heat detection aids. One of the research questions in this study was to try simple and affordable means of detecting estrus in dairy cattle, and also determining the status of animals in other reproductive stages (determining whether animals are cycling or noncycling, or pregnant), with the aim of evaluating breeding management in the study area. The second question focuses on quantifying P4 and estradiol levels in cattle at different reproductive stages, and the third question examines bringing cycling and noncycling cattle into estrus following estrus synchronisation and determining the conception rate. The aims of these research questions were to address the gap in identifying reproductive status of cattle and aid estrus detection.

Inaccurate estrus detection is one of the main factors affecting reproduction in cattle. In addition to human error in detecting cattle in estrus, there are several other factors that can obscure estrous behaviour in cattle. Type of housing is one of the causes of failure of estrus detection. Cattle housed in tie-stalls express lower estrous behaviour than those in free-stalls (Felton et al., 2012). Environmental temperature can affect the duration of estrus. Cattle that come to estrus during cooler parts of the day tend to have a shorter duration of estrus than those coming into estrus during the warmer part of the day (Drost and Thatcher, 1987). Floor surface is an aspect of farm environment that may affect expression of estrus and mounting behaviour in dairy cattle. Cows kept on dirt floors show

more consistent estrous behaviour (standing and mounting activities and longer duration of estrus) than cows on concrete floors (Britt et al., 1986). Foot and leg problems, cow density, nutrition, lactation number, days postpartum, and milk production are some of the other factors affecting estrous behaviour in cattle (O'connor, 1993). However, Britt et al. (1986) reported no effect of postpartum days, milk yield and season of the year on sexual behaviour.

1.3.5. Comparison of Reproductive Systems and Their Control between *Bos taurus* and *Bos indicus*

The reproductive characteristics of *Bos indicus* cattle has been little studied, and there is limited scientific information available compared with *Bos taurus* cattle. Previous reports have shown the existence of differences between Zebu and *Bos taurus* cattle in hypothalamic, pituitary and ovarian activities and relationships leading to the existence of fertility difference between the two species, even under similar feeding regimes and other farm management (Griffin and Randel, 1978; Rhodes et al., 1982; D'occhio et al., 1990; Britt, 2008).

The reproductive performance of *Bos indicus* is comparatively lower than *Bos taurus* breeds (Reynolds, 1967; Temple, 1967; Plasse, 1973) or *Bos indicus* and *Bos taurus* crossbreeds (Mukasa-Mugerwa, 1989). *Bos indicus* cattle generally reach puberty at an older age compared with *Bos taurus* (Syrstad, 1987; Galina and Arthur, 1989; Mukasa-Mugerwa, 1989; Abeygunawardena and Dematawewa, 2004; Brito et al., 2004; Johnston et al., 2009; Yelich and Bridges, 2012), on average, 6-12 months later than *Bos taurus cattle* (Warnick, 1965; Wiltbank et al., 1969). Ethiopian Zebu heifers reach puberty at 22.4 months (Alberro, 1983). Duguma et al. (2012) reported that crossbred (Zebu x HF) heifers attain puberty at 24 months. In contrast, HF heifers attain puberty much earlier, at 6-12 months (Forde et al., 2011). In heifers, age at puberty can be affected by several factors, such as genetics, management, environment/climate, nutrition and the purpose for which they are being reared (Warnick, 1965; Wiltbank et al., 1969; Werre, 1980; Mukasa-Mugerwa, 1989; Abeygunawardena et al., 1994; Abeygunawardena and Dematawewa, 2004). *Bos indicus* cattle attain puberty when they reach 60% of adult body weight

(Abeygunawardena and Dematawewa, 2004); whereas, *Bos taurus* breeds reach puberty at 30-55% of adult body weight (Mukasa-Mugerwa, 1989; Abeygunawardena et al., 1994). Nutritional status and body energy reserves are important to the hypothalamic-hypophyseal-gonadal axis integrity in cattle (Schilloz et al., 1992). Proper provision of nutrition has a positive impact on body weight gain, sexual maturity and onset of ovulation. A previous study from Brazil has shown that heifers fed with protein supplements gained more live weight (378 g), reached sexual maturity at a higher rate (95.7%) and shown earlier ovulation than the control (non-supplemented) group (Meirelles et al., 1994).

Ultrasonographic examination of bovine ovaries has revealed differences between breeds of cattle in the number of ovarian follicles (Alvarez et al., 2000). The development of ovarian follicles can be monitored using ultrasonography once follicles reach approximately 1 to 3 mm (Britt, 2008). *Bos taurus* cows have two or three follicular waves per cycle, whereas *Bos indicus* cows usually have 4 waves (Bo et al., 2003; Kojima and Patterson, 2003; Smith et al., 2012). Brahman and Senepol cows, which are *Bos indicus* cattle, have been reported to have more small antral follicles than Angus (*Bos indicus*) cows on all days of the estrous cycle (Segerson et al., 1984; Britt, 2008; Carvalho et al., 2008). Zebu breeds are less fertile despite having large numbers of ovarian follicles, which could be due to breed specific optimal number of follicles (Britt, 2008). Carvalho et al. (2008) studied the effect of treatment with an intravaginal progesterone-releasing device (CIDR) and estradiol benzoate between different breeds of cattle, and reported that the maximum diameter of dominant follicles in Zebu (Nelore) heifers (9.5 mm) was significantly smaller than in crossbred (Zebu x *Bos indicus*/Aberdeen Angus) heifers (12.3 mm). Others reported a maximum diameter of the ovulatory follicle ranging from 14 to 17 mm in HF (Ginther et al., 1989; Sartori et al., 2004) and 11.3 to 12.3 mm in Zebu cattle (Figueiredo et al., 1997; Sartorelli et al., 2005). Furthermore, it has been reported that *Bos taurus* female cattle have larger maximum CL diameter (between 20 and 30 mm) (Ginther et al., 1989; Sartori et al., 2004) than *Bos indicus* females (between 17 to 21 mm) (Rhodes et al., 1995; Figueiredo et al., 1997; Segerson et al., 1984).

1.3.6. Pregnancy and Parturition in Dairy Cattle

Pregnancy is the period from conception to parturition. The average length of gestation in cattle is 285 days (range 276-295 days) (Bazer and First, 1983; Rodriguez et al, 1983). If pregnancy is not successful following insemination, the P4 level falls after mid-cycle following degeneration of the CL by maternal PGF2α produced from the endometrium. However, in pregnant animals, the release of PGF2α is blocked by the presence of the conceptus, allowing the CL to continue secretion of P4. The conceptus produces an anti-luteolytic factor for the maternal recognition of pregnancy. In ruminants, this factor is interferon-tau (IFN-τ). It is secreted by the trophoblast cells of sheep and cattle from around day 13 and acts on the endometrium in a paracrine manner to prevent luteolysis, thereby maintaining the CL and production of P4 (Roberts, 1989). The mechanism of action of IFN-τ is through blocking expression of oxytocin receptors. Oxytocin is responsible for episodic release of the luteolysin PGF2α from the endometrium and this requires oxytocin coupling with its receptor. IFN-τ inhibits estrogen receptor expression, which in turn, blocks oxytocin receptors expression on the endometrial luminal epithelia (Spencer et al., 1995). In pregnant cows, the level of P4 in milk or serum remains at a higher level until the end of pregnancy. P4 is a key hormone of pregnancy and is essential for establishment and maintenance of pregnancy in all mammals. It is important for promoting uterine secretion for conceptus growth and development, initiating the window of uterine receptivity, then during pregnancy, inducing quiescence and non-contractility of myometrium to avoid abortion and protection of the embryo against the maternal immune system (Lewis, 2003; Soloff et al., 2011).

The success of pregnancy starts prior to insemination. Pregnancy can be affected by maternal problems and problems from the sire or semen quality if performing AI, which may be influenced by poor management practice. Some of the factors from the dam side are poor body condition, disease, abortion, problem of reproductive tract, anovulation, delayed ovulation and embryonic mortality. The problems in the sire/AI include poor semen quality, improper time of insemination, inappropriate insemination. To achieve a

successful pregnancy, factors affecting fertility should carefully be evaluated and appropriate measures taken.

1.3.7. Common Methods of Diagnosing Pregnancy in Dairy Cattle

Early diagnosis of pregnancy is an essential management tool to shorten the calving interval in dairy cows (Karen et al., 2015). Furthermore, detection of non-pregnant cows as early as possible after insemination reduces inter-insemination interval for herds performing synchronisation and resynchronisation without estrus detection (Lucy et al., 2011). There are various ways of monitoring pregnancy in dairy cattle.

Farmers consider return to estrus 18-24 days post AI as the easiest and least costly method of determining non-pregnancy (Fricke, 2010). Animals that do not come to estrus after 24 days post service are usually considered by the farmers as pregnant. However, this time interval can be affected by factors such as prolonged luteal phase, short duration of estrous behaviour (Dransfield et al., 1998) and silent estrus, whereby animals may return to estrus after the expected 17-24 days post service, and in case of silent estrus, even if animals which were inseminated return to estrus, they do not show any external signs. Furthermore, heat detection efficiency can also affect monitoring animals for their return to estrus to predict pregnancy or non-pregnancy.

One of the methods regularly used to monitor fertility of dairy cattle is per rectal palpation (Hansar et al., 2014). It has advantages over other methods being cheap and providing immediate results. However, the time the examination is carried out 45-60 days postpartum, combined with inadequate experience and skills of AI technicians to detect the structures on the ovaries are some of the drawbacks of applying this method in Ethiopia (Belihu, 2002; Lobago et al., 2007). An alternative method to monitor fertility is the use of ultrasonography to determine the structures (CL/follicle) on the ovaries and identify the reproductive stage of the animal (briefly reviewed by Royal et al., 2000a; Fricke, 2010; Hansar et al., 2014), including fetal sex and twin fetuses (Fricke, 2002). Ultrasonography is an accurate and instant method to diagnose fertility of cattle; however, it is expensive and requires management skills (Lucy et al., 2011).

Hormonal analysis is one of the methods applied to evaluate fertility of beef and dairy cattle. P4 is one of the steroid hormones commonly measured to diagnose pregnancy as well as cyclicity of cattle. Serum/plasma, milk and saliva P4 levels can be detected using laboratory ELISA or RIA (Laing and Heap, 1971; Lamming and Bulman, 1976; Romagnolo and Nebel, 1993; Muhammd et al., 2000; Lobago et al., 2007; Domènech et al., 2011). Furthermore, the P4 level in urine can also be determined using ELISA and used to diagnose fertility (Prabu and Rameshkumar, 2012). Laboratory ELISA/RIA procedures require a comparatively longer time than the ultrasound technique or rectal palpation. However, they accurately predict ovarian cyclicity or pregnancy status of animals. Rapid ELISA techniques that enable fertility evaluation of cattle at field/farm level have been widely used (Nebel et al., 1987; Nebel, 1988; Rajamahendran et al., 1990; Osman et al., 2012; Järvinen et al., 2014). The kits for rapid ELISA tests have been commercially available to dairy producers and veterinarians since the mid-1980's. The procedure was considered a cow side test since it could be performed on the farm or in a veterinary clinic (Friggens, et al., 2008). Rapid ELISA kits are available in two forms. The first form is a solution kit whereby P4 antibodies are precoated in small cups, accompanied by wash buffers, enzyme conjugates and substrates and results are recorded based on colour formation. The other form is as kits utilising a Dipstick. In the latter case, P4 level is determined based on the number of lines produced following immersion of a P4 antibody-coated Dipstick into a glass tube containing 0.5ml of milk. Both kits are qualitative and designed to determine relative rather than absolute concentrations of P4, and results are classified as either low or high (Peters and Ball, 1995).

The most commonly used method of pregnancy diagnosis in Ethiopian dairy farms is rectal palpation, performed by AI technicians or veterinarians. However, follow up of cows for return to estrus 21-days post AI is also the traditional way of detecting nonpregnancy in most farms. Rectal palpation is usually carried out two months or later post service. Farmers' accuracy of estrus detection determines the success of observing non-return to estrus, as a method of pregnancy check. However, silent estrus animals and animals coming to estrus in the evening would likely be undetected. This leads to false

diagnosis of pregnancy and keeping non-pregnant animals for prolonged periods without rebreeding them. For successful integration into a reproductive management system, an ideal early pregnancy test for dairy cattle would be sensitive, specific, inexpensive, simple to conduct under field conditions, and able to determine pregnancy status at the time the test is performed (Fricke, 2010).

1.4. SYNCHRONISATION OF ESTRUS IN DAIRY CATTLE

Estrus synchronisation is a farm management technique in which the estrous cycle of animals is controlled by the use of exogenous hormones so that they come into estrus and ovulate at a desired period (Fike et al., 1999; Kojima, 2004). Estrus synchronisation can avoid or reduce the need for estrus detection and increases fertility and productivity of cattle (Larson et al., 2006). Furthermore, estrus synchronisation facilitates planning of AI, shortens first day service in postpartum cows, reduces the calving interval and helps to maintain uniform calf crops and replacement heifers (Larson et al., 2006; reviewed by Lamb et al., 2010). The first successful synchronisation of estrus in cattle was reported in 1948 (Christian and Casida, 1948). It is considered uneconomic within the dairy industry when animals are not pregnant beyond the optimal voluntary waiting period (Groenendaal et al., 2004; Meadows et al., 2005), usually 60 days (DeJarnette, 2015). A deviation from this period in cows is called postpartum anestrus. Pre-pubertal heifers which did not attain puberty and are not bred during the optimal age at first service need proper management attention, as dairy farms need to replace cows that have poor reproductive and productive performance due to age or other factors. Hence, postpartum anestrous cows and pre-pubertal heifers can be included in estrus synchronisation programmes (Larson and Voelz, 2014). Cycling cows can also be synchronised for estrus to enable farmers to manage when their herd calves. By way of an example from Ethiopian culture, the majority of Orthodox Christian people fast for a prolonged period around Easter. This has a negative impact on the dairy industry, as milk consumption falls during these fasting days (Ayenew et al., 2009; Yilma et al., 2011). Application of estrus synchronisation in such cases can help the farmers to schedule and monitor the breeding and calving season so that losses during this religious fasting period can be reduced.

Table 1.1: Sources and functions of the major reproductive hormones and commercial products (**Source:** Fike et al., 1997; Lammoglia et al, 1998; Michael and Thomas, 2005).

Hormone	Source	Function	Commercial Products
GnRH	Hypothalamus	Stimulates the release of FSH and LH	Cystorelin®, Factrel®, Fertagyl®, OvaCyst®, Receptal
FSH	Anterior pituitary	Stimulates development of ovarian follicles	Folltropin®
Estrogen	Ovarian follicle	Stimulates behavioural estrus and the LH surge	Synthetic estradiol salts (valerate, cypionate, benzoate)
LH	Anterior pituitary	Stimulates final maturation of a follicle and ovulation	Not used in current systems
eCG	Placenta	FSH- and LH-like activity	Syncrostim®, Folligon, Pregnecol
hCG	Placenta	LH-like activity	Chorulon®, Vecetor
P4	CL	Maintains pregnancy	Melengestrol acetate, intravaginal P4 inserts (CIDR/PRID)
PGF2α	Uterus	Regress the CL	Lutalyse, Estrumate, ProstaMate, In-Sync

*eCG: equine chorionic gonadotrophin; hCG: human chorionic gonadotrophin; PRID: progesterone-releasing intravaginal device.

Synchronising estrous cycles depends on controlling the functional life span of the CL (Hansel and Convey, 1983). This can be achieved either by long-term administration of a progestin/ progesterone with subsequent regression of CL during the time the P4 is administered (Britt, 1987) or by administration of a luteolytic agent that shortens the normal life span of the CL (Knickerbocker et al., 1988). PGF2α results in regression of the CL leading to a decrease in blood P4 concentration, which causes increased GnRH. This increase in GnRH stimulates secretion of FSH and LH. FSH stimulates follicular development and follicular waves, while LH stimulates final maturation of the dominant follicle (Foster and Ryan, 1981; Timothy, 2003), estradiol secretion (Conn and Melmed, 1997) and ovulation (Timothy, 2003). Estrus can be synchronised by individual or combinations of hormones. Various hormones, their sources and available products are

indicated in Table 1.1. Mechanisms of estrus synchronisation are indicated on Figure 1.9. Estrus and ovulation occur within 2 to 8 days after P4 withdrawal or within 48 to 120 hr after injection of PGF2α/ estradiol valerate or benzoate (Murugavel, 2003).

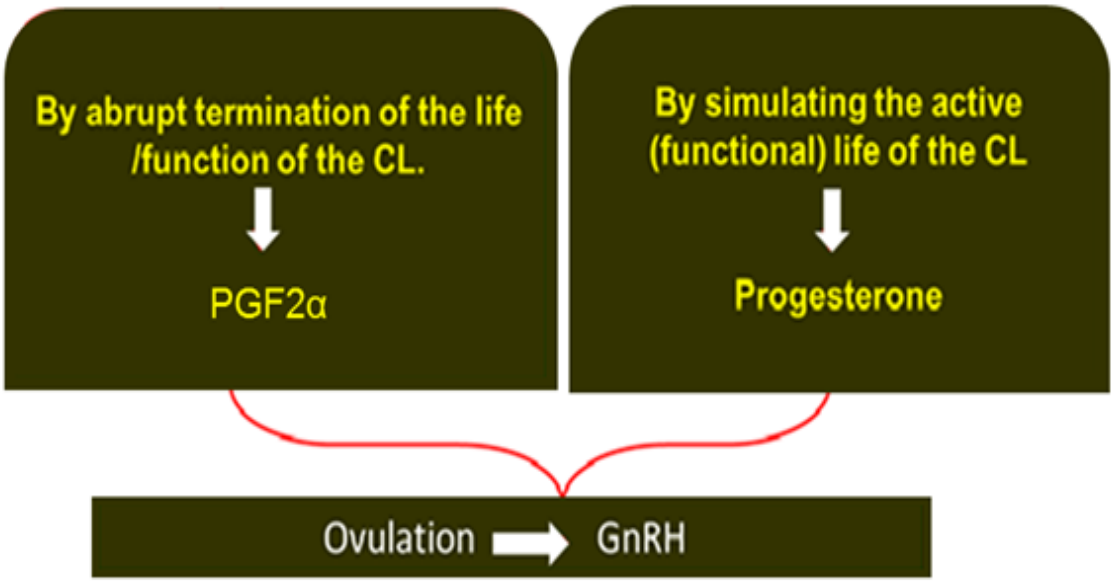

Figure 1.9: Mechanisms of estrus synchronisation in cattle.

The use of AI in Ethiopia is rapidly growing, and imported genetically improved semen for high milk production and sexed semen are being introduced and used in some organised commercial dairy farms. However, the AI programme and the use of imported semen is challenging and becoming ineffective due to poor estrus detection. Further to this farm management problem, poor expression of estrus and short duration with low intensity of estrus signs in Ethiopian Zebu cattle causing failure in estrus detection have been reported (Mukassa-Mugerwa, et al., 1989; Tegegne, et al., 1989; Bekele et al., 1991). Problems with estrus detection coupled with other reproductive problems such as anestrus and repeat-breeding in local and crossbred cattle affects profitability of the emerging dairy industry in the country. Application of an estrus synchronisation programme as a management scheme, in this regard, is urgently required. Commonly used estrus synchronisation protocols are discussed below.

1.4.1. GnRH or Combination with PGF2α

The GnRH is a peptide hormone, which stimulates the release of FSH and LH from the anterior pituitary gland. Usually, GnRH is administered in combination with PGF2α when synchronisation of estrus and ovulation is planned (Pursley et al., 1995; Geary and Whittier, 1999). However, GnRH can also be administered alone for inducing cyclicity or synchronisation of ovulation (Stevenson et al., 2000; Hailu et al., 2015). Although GnRH can be used in cycling cattle and can stimulate cyclicity in anestrous cows (Stevenson et al., 2000), it has been reported that it is less effective in initiating a new follicular wave or inducing ovulation in non-ovulatory or anestrus rather than in cycling cows (Chebel et al., 2006; Macmillan, 2010; Dickson et al., 2012).

Ovsynch, Cosynch and Selectsynch are examples of GnRH and PGF2α combined protocols, with each involving an injection of GnRH on day-0 and an injection of PGF2α on day-7 (briefly reviewed in DeJarnette, 2015). Ovsynch and Cosynch involve administration of a second GnRH on day-9. Administration of the first GnRH injection stimulates endogenous release of LH to stimulate final maturation of the dominant follicle and ovulation resulting in CL formation, and beginning of maturation of a dominant follicle. Administration of PGF2α on day-7 regresses the CL. Around 48 hr post PGF2α administration (Ahmed et al., 2016), the dominant follicle matures, and a second GnRH is administered to stimulate ovulation. Ovsynch is a fixed-time AI synchronisation protocol, with AI given 16 hr after the second GnRH, and extensively used in lactating dairy cattle (Stevenson et al., 1990; Pursley et al., 1995; Pursley et al., 1997; Pursley et al., 1998). Cosynch is an estrus synchronisation protocol used as an alternative to Ovsynch whereby cattle are inseminated immediately following the second GnRH administration. Selectsynch is a protocol that involves insemination of animals following estrus detection usually 24 hr to 5 days after PGF2α administration. In this protocol, a second GnRH is given if animals are not seen in estrus following PGF2α administration. GnRH - PGF2α combined protocols can also be used in combination with CIDR insertion along with first administration of GnRH.

Report have shown a pregnancy rate of 50% using an Ovsynch protocol in crossbred cows (Carabă and Velicevici, 2013; Ahmed et al., 2016). Others reported pregnancy rates of 55% and 52% when using Cosynch and Ovsynch protocols, respectively using timed breeding; however, the pregnancy rate at standing estrus (following estrus detection) was 61% (Geary and Whittier, 1999). Recent estrus synchronisation protocols for timed AI (TAI) such as the 5-d Cosynch + CIDR have reported success in improving pregnancy rates up to 70% in beef cows when compared to other protocols (Bridges et al., 2008; Gunn et al., 2009). A study at Mekelle University, Ethiopia has modified the available GnRH-PGF2α-GnRH protocol (Ovsynch or Cosynch) by either simultaneous administration of GnRH and PGF2α, or by administration of PGF2α at 11-day intervals, and GnRH administered simultaneously with the second PGF2α, to reduce the number of farm visits. In both cases, cattle were inseminated/mated twice at 48 & 72 hr, and a high overall conception rate (72.7%) was obtained (Mekonnin et al., 2016). The protocol was validated by a subsequent study in 10 crossbred dairy cattle by colleagues at Mekelle University and a lower conception rate (37.5%) recorded (Weldeselassie et al., 2015), which may be due to the small number of animals, animal condition, or insemination technique used. Sample size dictates the amount of information in the study and determines the precision or the level of confidence that study sample estimates; and more data provides more information, hence our estimate will be more precise (Marley, 2017). Furthermore, pregnancy rates following estrus synchronisation in Ethiopia may depend on how AI technicians are paid; payments per success of pregnancy may bring improvement to proper use of AI technologies and reduce prolonged postpartum estrus.

1.4.2. PGF2α

The PGF2α based protocol is one of the most commonly used estrus synchronisation protocols. It initiates degeneration of the CL and causes the animals to come into estrus and subsequently ovulate. Therefore, PGF2α is only effective in animals with a well-developed CL. It has no effect in animals that do not have CL, including anestrous cows, pre-pubertal heifers, cycling cattle in their first five to six days of the estrous cycle. Nor does it have an effect on the follicular wave (DeJarnette, 2015). The effect of exogenous

PGF2α in luteinising CL was first reported in 1972 (Seguin, 1980). It can be administered once or twice at different intervals (MacMillan, 1978; Seguin et al., 1978; Plunkett et al., 1984). Single administration of PGF2α is commonly carried out in cycling animals bearing well-developed CL, and animals are bred according to estrus. In contrast, double administration is provided at an interval of 10 to 14 days in animals with unknown stage of the estrus cycle or doubtful/undetected CL, and is used to ensure that a high proportion of animals have a responsive CL at the time of the second PGF2α treatment (Colazo, 2005; reviewed by Islam, 2011). The pregnancy success following administration of PGF2α depends on the cyclicity of the animal. Cattle need to be in diestrus; however, animals between day one to five of the cycle are not responsive to PGF2α. Hence, the second administration of PGF2α covers the animals that were not responsive when administered between day one to five of the cycle (Selk et al., 1988; Rosenberg et al., 1990; Kristula et al., 1992; Mekonnin et al., 2016).

1.4.3. Progesterone Based Protocols

Progesterone was originally used to synchronise the bovine estrous cycle (Lamond, 1964; Gordon, 1976). P4 is effective in initiating a new follicular wave or inducing ovulation in anovulatory or anestrous cows (Macmillan, 2010; Dickson et al., 2012). It has been shown that P4 can be used to treat cows with a problem of repeat-breeding/ ovarian follicular cysts, in which case their fertility improved (Kim et al., 2006; Todoroki and Kaneko, 2006; Amer and Badr, 2007). P4 is available in the natural (progesterone) or synthetic (progestin) state. P4 products are available in various forms for estrus synchronisation in cattle. It is available as a powder (melengestrol acetate), injectable solution, ear implant and vaginal inserts. The commonly used vaginal inserts/ devices in cattle and buffaloes are PRID and CIDR.

CIDR is an intravaginal P4 insert used in combination with other hormones to synchronise/induce estrus in dairy/beef cattle (Lamb, 2010), sheep and goats (Ainsworth and Downey, 1986; Wheaton et al., 1993). It was developed in New Zealand and has been used for several years to advance the first pubertal estrus in heifers and the first postpartum

estrus in cows (Lamb, 2010). The device is T-shaped with flexible wings that collapse to form a rod for insertion into the vagina with an applicator (Grant, 2006; Lamb, 2010). A tail is attached to the other end of CIDR, which is opposite to the wings to facilitate its removal (Grant, 2006; Lamb, 2010). Plasma P4 concentrations are able to be maintained above 2 ng/ml during periods of CIDR insertion of up to 4 weeks, while low levels of P4 (< 0.4 ng/ml) are reached within 6 hr of device removal (Jubb et al., 1989). CIDR has advantages over other P4 devices, including PRID because it can more easily be applied and has higher retention rate with lower degrees of trauma (Broadbent et al., 1993). Vaginal devices release P4 at a constant rate (Dyer and Graves, 2014), which simulates the function of the CL. The estrus response and conception rate of cattle following CIDR treatment varies with breed, management, breeding method (natural or AI), breeding season, geographical location.

Previous study has shown that administration of CIDR devices for 7 days during a TAI protocol increased the proportion of functional CL in anestrous cows after AI and pregnancy per AI compared to protocols without CIDR (Chebel et al., 2010). Some previous reports on estrus response and conception rate of cattle following CIDR treatment are detailed in Table 1.2.

Table 1.2: Estrus response and conception rate of cows/heifers to CIDR treatment based on previous reports.

Protocol	Breeding time post treatment	Number of animals	Estrus response (%)	Conception rate (%)	Authors
7 day CIDR + PMSG (eCG) on day 7	At induced estrus	30 cows/ heifers	100	70	Singh et al., 2006
7 days CIDR-B + PGF2α on day 6	Within 5 days	133 HF heifers	74.0	46.6	Broadbent et al., 1993
12 day CIDR-B + PGF2α on day 10	Within 5 days	188 HF cows/ heifers	76.2	37.9	Broadbent et al., 1993
7 day CIDR-B + PGF2α on day 7	12 hr after estrus	5 Aceh cattle	80	100	Siregar et al., 2015
7 day CIDR + PGF2α on day 7	6 - 12 h after estrus	127 HF / Crossbred cows	79.5	51.5	Romano and Fahning, 2013
9 day CIDR + PGF2α on day 7	6 - 12 hr after estrus	128 HF / Crossbred cows	87.5	45.5	Romano and Fahning, 2013
7 day CIDR + PGF2α on day 7 + GnRH 48 hr later	60-72 hr	73 Crossbred cows	100	40.9	Dickson et al., 2012
Select Synch + CIDR + TAI	At 84 hr	278 beef cows	59	58.0	Larson et al., 2006
CO-Synch + CIDR	84 hr after PGF2α	288 beef cows	59	53.8	Larson et al., 2006
Ovsynch + CIDR	Twice by bull	20 buffalo cows	75	35	Azawi et al., 2012
14-day CO-Synch + CIDR	TAI from 14-24 hr after estrus	33 beef heifers	51.5	41.2	Powell et al., 2012
5 day Co-Synch + CIDR	TAI from 14-24 hr after estrus	33 beef heifers	57.6	52.6	Powell et al., 2012
7 day Ovsynch + CIDR	TAI at 17 hr	28 cows	86.7	32.1	Kim et al., 2007
7 day Ovsynch + CIDR	TAI 72 hr	23	?	43.5	Kawate et al., 2011

PMSG: pregnant mare's serum gonadotrophin; %: the mean percentage of estrus response and conception rate.

1.5. IMPACT OF NUTRITION AND BODY CONDITION SCORE ON CATTLE FERTILITY

Fertility can be affected by several factors, such as environment, age, parity, genetic and congenital factors, diseases, hormonal disturbances and farm management (such as poor nutrition, suckling/cow-calf interaction and improper breeding practices) (Osmanu, 1979; Mukasa-Mugerwa et al., 1991; Peters, 1991; Webb et al., 2004; Garnsworthy, 2011). Regardless of the factor, poor fertility reduces both annual and lifetime milk yield per cow, and increases the number of replacements needed (Garnsworthy, 2011).

There is a strong association between nutrition and reproduction (reviewed by Garcia-Garcia, 2012), and it has been suggested that reproductive efficiency of cows is largely dependent on their nutrition (Carroll et al., 1988; Butler and Smith, 1989; Robinson, 1990; O'Callaghan and Boland, 1999; Robinson et al., 1999; Boland et al., 2001). Nutrition has a significant impact on numerous reproductive functions such as hormone production, folliculogenesis, fertilisation, and early embryonic development (Armstrong et al., 2003; Boland and Lonergan, 2005). Improper nutritional management has a negative effect on ovarian activity (Webb et al., 2004). Suboptimal provision of nutrition delays puberty, reduces conception rate and increases pregnancy losses in heifers (Milagres et al, 1979; Fleck et al, 1980; Lemenager et al, 1980). Zebu and crossbred cows tend to have good reproductive activity/performance during the rainy season when grazing conditions and nutrient availability are optimal (reviewed by Mukasa-Mugerwa, 1989). As most crossbred dairy cattle are reared indoors and have very limited access to grazing conditions, feed supplementation is required for good milk production and fertility response. A previous report in Ethiopia (ILCA, unpublished data) has shown that Borana heifers supplemented with 1.5 kg of concentrate (1/3 oilseed cake, 2/3 wheat bran) for 90 days during the dry period reached puberty earlier (596.4 vs 633.5 days), were heavier at puberty (230.7 vs 202.4 kg) and had larger ovaries than the control heifers (reviewed by Mukasa-Mugerwa, 1989). Moreover, in superovulation and embryo-transfer programmes, the nutritional condition of the donor cow has a significant impact on the response of the

gonads to gonadotrophin treatment and production of quality embryos (Nolan et al., 1998; Yaakub et al., 1999).

The reduction of food consumption pattern and nutritional status of cows in estrus synchronisation programs, affects the expression of estrus, due to the lack of growth and maturation of ovarian follicles caused by a negative energy balance (Centurion-Castro et al., 2013). A previous study has shown the effect of feed supplementation on weight changes, milk yield and postpartum estrus in cows included in three treatment groups: groups B cows were supplemented with dhaincha, ipil-ipil, treated straw, fish meal and common salt; group C cows with UMMB and green grass; and, group A with treated straw, fish meal and common salt; whereas, group D were control cows (non-supplemented). Supplemented cows gained body weight over a period of 32 weeks, showed an increase in milk yield and expressed behavioural estrus 84-218 days postpartum, whereas control cows neither gained weight nor manifested estrus during the study period, suggesting that feed supplementation during lactation in cows improves body condition and stimulates ovarian cyclicity earlier (Alam et al., 2009).

Nutrients that act independently of energy balance have been reported to directly or indirectly alter reproductive performance; these include protein (Armstrong et al., 2001), starch (Armstrong et al., 2001; Burke et al., 2006; Roche et al., 2006), monopropylene glycol (Chagas et al., 2007), minerals and trace elements (Underwood and Suttle, 2001), and fats (Staples et al., 1998; Boken et al., 2005) including specific lipids such as n-3 fatty acids (Ambrose et al., 2006), rumen inert fats (Staples et al., 1998), and possibly conjugated linoleic acid (Baumgard et al., 2005).

Body condition score (BCS) is used to evaluate the amount of body fat and is usually carried out visually and/or by touch, on the tail head and/or the loin area (Royal et al., 2002b). It is recommended that animals be scored for condition in the morning before they have had any access to food or water, as these alter the assessment of BCS (Nicholson and Butterworth, 1986). In a scale of 1-5, cow with BCS of 1 are considered as emaciated, whereas with 5 are considered as obese (Wildman et al., 1982). The BCS and its

description based on the animal's condition is indicated in Table 1.3. The postpartum delay in hyperphagia is associated with body tissue reserves being mobilised to support milk production (Bauman and Currie, 1980). It appears that cows have a physiological target level for body reserves in early lactation (Garnsworthy and Topps, 1982). Therefore, fatter cows at calving will tend to lose more body fat than thinner cows (Garnsworthy, 2007). Cows that are producing a high volume of milk tend to have lower BCS indicating that nutrients are partitioned toward milk production rather than increasing body reserves (Dechow et al., 2003). Britt (2008) suggested that dairy cows that lose more body condition during the first 5 weeks postpartum exhibit a more negative balance than those that lose less body condition.

Table 1.3: Assessment of body condition by description, BCS, backfat thickness, and total body fat content (**Source:** Schröder and Staufenbiel, 2006).

Description	BCS	Backfat Thickness (mm)	Total body fat content (kg)
Emaciated	1.0	<5	<50
Very poor	1.5	5	50
Poor	2.0	10	76
Moderate	2.5	15	98
Good	3.0	20	122
Very good	3.5	25	146
Fat	4.0	30	170
Adipose	4.5	35	194
Obese	5.0	>35	>194

It has been reported that up to one-third of the total milk solids produced in early lactation is produced from the body tissue reserves (Bauman and Currie, 1980). The relationship between BCS at calving and loss of BCS in early lactation is reported to be very strong and is influenced by genetics (Garnsworthy, 2007). A previous report from Zimbabwe has shown that cows supplemented with sweet sorghum-lablab silage (6kg/cow/day) in late pregnancy had better (higher) BCS at calving than the unsupplemented cows (Nyoni et al., 2001). Poor BCS at calving and negative energy balance postpartum affect reproduction negatively (Buckley et al., 2003; Rhodes et al., 2003a; Roche et al., 2007).

The BCS is used as a management tool in deducing how well the current feeding needs are being met relative to stage of lactation in dairy cows (Royal et al., 2002b). Hence, management of body reserves is critical for reproductive success and requires an accurate assessment of a cow's "body condition" (Chagas et al., 2007).

1.6. MILK AND ANALYSIS OF MILK COMPOSITION

1.6.1. Milk

Milk or raw milk is defined as the normal clean and fresh secretion obtained by emptying the udder of a healthy animal that is properly fed and kept, excluding that obtained during the four days immediately following parturition (NZFR, 1984). It is secreted by the mammary gland of mammals and is the main source of nutrition for new-borns (Hettinga, 2009).

The milk production potential of *Bos indicus* (Zebu) cattle is lower than *Bos taurus*. In tropical countries, milk production of Zebu cattle is improved by crossbreeding with *Bos taurus* (Tadesse and Dessie, 2003). Crossbred dairy cattle produce three times more milk, with longer lactation period and shorter calving interval than Zebu breeds (Kiwuwa et al., 1983). A previous study in Ethiopia has shown that 50% Holstein crosses had a four-fold increase in terms of lactation milk yield, 305-day milk yield, milk yield per day of lactation and lifetime yield with higher lactation period than the Ethiopian Borana breed (Haile et al., 2009).

Natural milk for human consumption is obtained from different species of domestic animals such as cattle, goats, sheep, horses, donkeys and camels (Barłowska et al., 2011). The main sources of milk in Ethiopia are the indigenous cattle, accounting for 97% of the country's annual milk production (Tedla et al, 1991), with an average 1.85 L/head/day (Tegegne et al., 2013). Non-dairy alternatives that have a similar taste to dairy milk are also available in the market. Plants, such as soybeans, rice and almonds are sources of non-dairy milk. Non-dairy milk is an alternative to natural milk particularly to people with

lactose intolerance, vegan and during religious fasting for people who should not take animal products during these fasting periods.

Milk production increases rapidly from early lactation to peak at around 40 to 70 days after calving (Jaynes, 2014), which then decreases until the cow dries up (Foley et al., 1972; Mech et al., 2008). Milk yield is higher in early than other stages of lactation, which is due to the presence of higher secretory activity per cell (Capuco et al., 2001; reviewed in Capuco et al., 2003), which drops in the latter stages of lactation due to a decrease in secretory cells number (Capuco et al., 2001). Milk yield is largely determined by genetic and nutritional factors (Millogo, 2010). Milk production can also be affected by milking frequency and efficiency of milk removal (Depeters et al., 1985; Gisi et al., 1986) and milking techniques, with milking in combination with suckling increasing milk yield (Bar-Peled et al., 1995).

1.6.2. Nutritional Composition of Milk

The composition of milk varies depending on feed intake (Haug et al., 2007), species, and breed, but it always contains significant amounts of carbohydrate, fat, and protein, and is an important source of calcium (Hettinga, 2009). Bovine milk contains carbohydrates (approximately 4.6%), fat (approximately 4.3%) (Walstra et al., 2008), and proteins (approximately 3.5%) (Fox et al., 2000). Three-quarters of the protein in bovine milk is casein; the remainder consists of whey proteins, especially β-lactoglobulin and α-lactalbumin (Fox et al., 2000). Milk fat is a complex mix of tri- and diglycerides, complex lipids, and liposoluble substances (Debry, 2001; Walstra et al., 2008).

1.6.3. Methods of Evaluating Milk Composition

In Ethiopia despite a large cattle population and an emerging commercial dairy industry, particularly in urban and periurban areas of the country, there is very little awareness of milk composition by the consumers. Furthermore, very few scientific reports are available on milk composition of crossbred or other dairy cattle breeds in the country. Milk production, processing and marketing chain is the main challenge in the study area (according to farmers' comments during the study) and other parts of the country (Ayenew

et al., 2009). About 95% of the marketed milk at national level is channeled through an informal system (Yilma et al., 2011). In western countries, milk composition is clearly labelled on the milk container, with a recognised colour coding of labels for full fat, skimmed or/and semi-skimmed milk. The majority of the farms in Ethiopia are smallholders, and the experience with milk supply, as in many other parts of Africa, is that most consumers go with their milk containers/bottles (or milk containers are left in the farms by the consumers prior milking) to dairy farms to collect milk. Alternatively, there may be direct delivery of fresh raw milk and milk products by producers to consumers in the immediate neighbourhood and sales to itinerant traders, milk cooperatives or individuals in nearby areas (Ayenew et al., 2009). In almost all these supplies, there is no concept or no question arises from consumers (or farmers) on milk composition as long as they get fresh whole milk. This is due to a lack of awareness about milk composition and its nutritional value among consumers. Milk composition is very rarely analysed at farm level due to a lack of milk composition analysing equipment. Furthermore, dairy cattle farmers have limited knowledge of dairy product handling, coupled with lack of dairy infrastructure such as cooling facilities and unavailability of clean water in the production areas (Yilma et al., 2011).

The composition of milk could have great value for the dairy farmer. It determines the economic value of the milk and provides valuable information about the metabolism of the corresponding cow (Aernouts et al., 2011). Furthermore, analysis of milk composition provides information on milk quality alterations (Forsbäck et al., 2010). There are various methods used to analyse the composition of milk. Infrared (IR) spectroscopy has been widely used to analyse the macronutrient content of cow (Rutten et al., 2009; Soyeurt et al., 2011; Arnould et al., 2015) and human (Corvaglia et al., 2008; Menjo et al., 2009) milk. Gas chromatography with mass spectrometer is used to quantify components of bovine milk (Hettinga, 2009; Tunick et al., 2013). The quality of raw milk can also be analysed for changes due to cow diet, chemical contamination or enzymatic deterioration using chemical tests (Azzara and Campbell, 1992). Aldehydes, ketones, and alcohols which are volatile components in milk may also be useful indicators of milk quality

(Marsili, 2000; Valero et al., 2001), and can be analysed using headspace sampling techniques (Hettinga, 2009). Miris milk analyser is an example of a recently developed technology used for analysing macro components of milk, such as lactose, fat and protein (Fusch et al., 2015).

AIMS OF THIS THESIS

General Aims:

- To assess reproductive performance and status, prevalence of reproductive problems and methods to improve fertility of crossbred dairy cattle in and around Mekelle, Tigray, Ethiopia.
- To trial simple, cost effective and reliable on-farm P4 tests for monitoring reproductive status in crossbred dairy cattle.
- To determine steroid hormone (P4 and estradiol) and macronutrient components of milk in crossbred dairy cattle

Specific Aims:

- ✓ To study the prevalence of reproductive problems and assess performance in crossbred dairy cattle (Zebu x HF) in and around Mekelle, Tigray, Ethiopia using a questionnaire survey.

- ✓ To study the reproductive status of crossbred dairy cattle with on-farm assays (Target P4 and Rapid P4/ Rapid P4 Estrus Detection test (RPEDT/Dipstick)) in the study area, and to compare the findings with laboratory ELISA measuring salivary (cows and heifers), milk (lactating cows) and serum (heifers and non-lactating cows) levels of P4, conducted in The University of Edinburgh, UK.

- ✓ To trial and introduce simple, appropriate, cost effective, and rapid on-farm P4 assays (Target P4 and Dipstick) and to assess the reproductive status of crossbred dairy cattle as a means to alleviate the problem/doubt of accuracy of monitoring, when rectal palpation, which is the only routine method of diagnosis, is used in Tigray region as well as other parts of Ethiopia.

- ✓ Based on the above results, to establish a hormonal (P4 in serum, milk, saliva and urine, and estradiol in serum) profile of crossbred dairy cattle in different reproductive conditions (anestrus, estrus, diestrus, pregnancy or cystic ovarian condition) in the

region, as there are no reports of clinical ranges to date, and to compare field findings from on-farm P4 assays with laboratory P4 ELISA measuring the level in serum/ milk in the immunoassay laboratory, MRC Centre for Reproductive Health, The University of Edinburgh, UK.

- ✓ Based on on-farm P4 test results, to evaluate estrus response and conception rate of crossbred dairy cattle following synchronisation/induction of estrus and ovulation by treatment with CIDR in combination with PGF2α and eCG.
- ✓ To determine macronutritional composition of milk in crossbred dairy cows and evaluate the effect of reproductive status, farming system, stages of lactation, parity and breed on milk composition.
- ✓ To recommend the best reproductive management approaches based on the findings and the available scientific evidence.

CHAPTER 2: MATERIALS AND METHODS

2.1. STUDY AREA

The studies reported upon in Chapter 3, 5 and 6 were undertaken in and around Mekelle, Tigray, Ethiopia. Mekelle is the largest city in northern Ethiopia, and is the capital city of Tigray Region. It is one of the sixth largest cities in Ethiopia located at 39° 29`E and 13° 30` N, covers 28km square that is 783km^2 and is at an altitude of 2000m above sea level. The climate of the study area is representative of Ethiopian highlands. The mean annual rainfall of the study area is 628mm and is associated with the north and south oscillation of inter-tropical conversion zone. The rainfall is bimodal with short rainy season occurring from March to May and from the middle of September to February. The annual minimum and maximum temperatures are 12°C and 30°C, respectively (BoPED, 2011). A map of the study area in Ethiopia is indicated in Appendix 1. On-farm P4 findings (Chapter 5) were compared with and validated against a laboratory procedure in The MRC Centre for Reproductive Health, The University of Edinburgh, United Kingdom. Studies covering Chapter 4 and 7 were conducted in The MRC Centre for Reproductive Health, The University of Edinburgh, United Kingdom.

2.2. STUDY FARMS AND ANIMALS

2.2.1. Dairy Farms and Management Practices

Dairy farms in Ethiopia included in the study (Chapter 3-7) were either smallholder farms or organised commercial farms rearing crossbred (Zebu x HF) dairy cattle. Smallholder farms, as the name suggests, have small cattle numbers with the majority located in or around residential areas, and established with small capital; whereas, organised commercial farms are established with more investment capital and most are located away from residential areas. Both smallholder and organised farms use similar feed, such as grass hay, straw, concentrate composed of wheat by-products, crop residues and local beer residue (Attela), however cattle in organised farms tend to receive more green fodder and regulated ration, plus regular watering, and most have proper breeding records. All

crossbred cattle that were included in the study were under indoor management. Crossbred dairy cattle had body condition score (BCS) of 2.5-4 (on a scale of 1-5) and an average milk yield of 15L per day in lactating dairy cows.

A commercial dairy farm (Langhill Dairy Farm) rearing pure HF cattle in Edinburgh, United Kingdom was also included in the study for comparison, as well as for validation of on-farm P4 and milk composition findings (Chapter 5 and 7). Cows in Langhill Dairy Farm were managed either intensively (indoor) or extensively (outdoor). Cows in intensive management (indoor) were provided with ration of grass silage, whole crop, Langhill total mixed ration meal and molasses, and had BCS of 2.8 and produced an average milk yield of 38L/day. Cows under outdoor husbandry were grazed on the grassland, except when they come for milking at which time they received rations. They had BCS of 2.5 producing an average milk yield of 25L/day.

2.2.2. Study Farms and Animals

One hundred and seventy-eight (178) dairy farms were included in the entire study (Chapter 3-7). Of these 177 were dairy farms in Ethiopia (range 1-115 cattle per farm) that housed 736 crossbred dairy cows and 277 crossbred dairy heifers aged two years or above, also housed 55 crossbred dairy bulls and 398 crossbred dairy calves. These crossbred cattle were crosses of pure Zebu and HF cattle, with a higher proportion of Holstein Friesian pedigree (≥75% HF). In Ethiopia, semen production is exclusively operated by the National Artificial Insemination Center and regional artificial insemination centers, though a small amount of exotic semen is imported by a private breeding service in Addis Ababa (Gizawa et al., 2016), including sex-selected semen by a few organised farms in Mekelle, Ethiopia. Crossbred dairy cattle produce more milk and have longer lactation period compared with pure Zebu breed. Moreover, they are more docile and easily managed compared with the local breed of cattle (Tefera, 2013). Since the human population in Ethiopia in general and in Tigray Region in particular is increasing, there is more demand on milk, meat and their products. Furthermore, the emerging dairy farms in urban and periurban areas of the country and the increasing effort and support provided by the Ethiopian government in improving the productive and

reproductive performance of local breed cattle through crossbreeding by a door to door provision of AI services, has raised our attention to inclusion of crossbred dairy cattle in the present study (see also Section 1.2.1 for reasons why crossbred cattle are used instead of pure Zebu breeds).

The first study (Chapter 3), for surveying common reproductive problems and reproductive performance of their cows and heifers, included 177 dairy farms (that housed 736 crossbred dairy cows and 277 crossbred dairy heifers aged two years or above). Chapter 4, hormonal (P4 and estradiol) profile study, incorporated a total of 336 crossbred dairy cattle (232 cows and 104 heifers) from 47 dairy farms owned by smallholder/ organised commercial dairy farmers. The on-farm assessment of reproductive status (Chapter 5) included 325 dairy cattle: 6 HF cows from one British farm and 319 crossbred dairy cattle (224 cows and 95 heifers) from 47 Ethiopian farms. The 6 HF cows were included for preliminary study (Chapter 5) to trial the rapid on-farm P4 (Dipstick/P4 Rapid and Target P4) kits in assessing reproductive status prior to the actual field work in Ethiopia, and to be familiar with the test procedures. Both cattle breeds were at different reproductive states, either in-heat, anestrus, repeat-breeder (repeater) or pregnant based on ultrasonography findings (HF cows) or visual inspection (follow up)/ per rectal palpation records. One hundred and twenty-two (122) crossbred dairy cattle (75 cows and 47 heifers) from 28 farms (19 smallholder farms and 9 organised commercial farms) were included in estrus synchronisation study (Chapter 6). A total of 246 lactating dairy cows (184 crossbred cows from 47 farms in and around Mekelle, Ethiopia and 62 HF cows in a commercial farm, UK) were included in analysis of nutritional composition of milk in relation to reproductive status, stages of lactation, management, parity and breed (Chapter 7).

2.3. ASSESSMENT OF REPRODUCTIVE PERFORMANCE AND PROBLEMS

2.3.1. Questionnaire Preparation

The questionnaire (Appendix 2) was translated into the local language (Tigrigna) (Appendix 3) so that questions were properly understood and appropriately answered, and piloted beforehand.

2.3.2. Questionnaire Data Collection

A cross-sectional survey was used to collect information on reproductive performance and assess the common reproductive problems of crossbred dairy cows and heifers. Farm owners, attendants, managers, and veterinary professionals were given a short briefing about the overall aims of the study. They were asked to complete the questionnaire survey information regarding age at first service, age at first calving, calving interval, first postpartum estrus, reproductive health problems, breeding history (AI, natural mating, parity) and other related questions including the year of farm establishment.

2.4. SAMPLE COLLECTION FOR ON-FARM AND LABORATORY ELISA

2.4.1. Milk Collection

Udder and teats were properly washed and dried prior to milking. Evening (PM) milk (10-20ml per cow) was collected manually into plastic centrifuge tubes (Alpha Laboratories, Hampshire, UK) twice at eleven (11) day intervals from 6 HF cows (British Farm) for preliminary study to trial rapid on-farm P4 tests for assessing reproductive status (Chapter 5), before the main field study in Ethiopian farms. Whole milk (2.27L) was also bought from a supermarket in Edinburgh, UK for preparation of P4 standards and to evaluate the effectiveness of the on-farm kits prior to using them in assessing reproductive status of crossbred dairy cattle in Ethiopia. Milk was also collected from 198 lactating crossbred dairy cows in and around Mekelle, Ethiopia (Chapter 4-7). Furthermore, milk from 62 HF

cows was collected (early milking) on one occasion, utilising a milking machine to study nutritional composition of milk (Chapter 7). Of these 62 HF cows, milk was also collected manually (same as from crossbred cows) from 21 cows to evaluate the effect of milking method on milk composition (Chapter 7). Samples from crossbred cows were taken manually from all four quarters after discarding the first four milk drops. Milk was collected only from cows with clinically healthy udder and teats into collecting tubes labelled with the relevant cow identification number. In most smallholder farms and a few organised commercial dairy farms where cattle did not have an identification number, a laboratory code/identification for labelling collected samples was allocated. Samples were processed immediately at the farms (Chapter 5 & 6), except in the case of inadequate working conditions, such as excessive wind, and dust which could affect the results. On those occasions, samples were transported using an icebox to the local laboratory for analysis. However, for hormonal profile study (Chapter 4) and analysis of milk composition (Chapter 7), samples from crossbred dairy cows were frozen at -20^{o}C and transported with ice pack to The MRC Centre for Reproductive Health, College of Medicine and Veterinary Medicine, The University of Edinburgh, Edinburgh, United Kingdom. Samples were frozen at -80^{o}C until processed.

2.4.2. Blood Collection and Serum Separation

Blood Collection*:* Cattle were restrained by farm personnel (farmers/farm attendants and/or our study team members) with blood drawn into 10ml plain vacutainer tubes (BD Vacutainer®, BD, Plymouth, UK) from the jugular vein of dry and lactating cows and heifers (Chapter 4-6), before and after blood collection, the injection site was disinfected with 70% alcohol.

Serum Separation: Blood was allowed to clot at room temperature. Serum was separated and stored in 5ml capacity plastic transport tubes (Alpha Laboratories, Hampshire, UK) 24 to 48 hr after blood was collected. For hormonal (P4 and estradiol) profile assessment (Chapter 4), serum samples were labelled appropriately, frozen at -20^{o}C and transported with ice packs to The MRC Centre for Reproductive Health, College of Medicine and

Veterinary Medicine, The University of Edinburgh, Edinburgh, United Kingdom. Samples were then frozen at -80^{o}C until processed. In contrast, serum samples were immediately processed to study reproductive status at the farm level (Chapter 5) and the estrus response and conception rate of cattle following estrus synchronisation/induction (Chapter 6).

2.4.3. Saliva Collection

Animals were properly restrained by farm personnel (farmers/farm attendants and/or our study team members) during sample collection. To study hormonal (P4) profiles of crossbred dairy cattle (cows and heifers) in Tigray, Ethiopia (Chapter 4), saliva (5ml from each animal) was collected twice at 11-day intervals using a 7ml capacity plastic transfer pipette directly from the buccal commissures by aspiration. In cases where enough saliva was not present, animals were provided with some green feed or hay/straw to stimulate salivation, and in some cases saliva dropping out of the mouth was collected directly to the sterile, plastic transport tubes (Alpha Laboratories, Hampshire, UK). Samples were transported from the farms to the Immunology and Biochemistry Laboratory, College of Veterinary Medicine, Mekelle, Ethiopia in an insulated box containing ice packs and stored at -20^{o}C until transported to the UK for analysis. Samples were centrifuged at 4000*g* for 10 min at 20^{o}C to remove any contaminating food particles and stored at -80^{o}C in The MRC Centre for Reproductive Health, College of Medicine and Veterinary Medicine, The University of Edinburgh, Edinburgh, United Kingdom. In some cases, samples were frozen and centrifuged multiple times, to allow food particles to be removed. Sample collection and separation of food particles were, with minor modifications, based on previous reports (Gao et al., 1988; Kanchev et al., 1988).

2.4.4. Urine Collection

Urine (5ml/cow) was collected by waiting until cows urinated naturally or by stimulating through massaging the ventral commissure of the vulva. Samples were frozen at -20^{o}C prior to transporting to the UK for hormonal profile (P4) study (Chapter 4). Urine samples

were stored at -80^{o}C in The MRC Centre for Reproductive Health, The University of Edinburgh, Edinburgh, United Kingdom until assayed.

2.5. QUALITATIVE (ON-FARM) P4 ELISA PROTOCOLS

2.5.1. Preliminary Assessment of Reproductive Status of HF Cows in Edinburgh, UK Using On-farm P4 Tests

The objectives of this experiment were to trial on-farm P4 kits in assessing reproductive status of HF cows before the actual field study in Ethiopian crossbred dairy cattle, and to be familiar with procedures of the tests. The reproductive status of six HF cows was determined using ultrasonography. Milk was collected from each cow immediately after scanning, and reproductive status determined using qualitative on-farm P4 kits (Target P4 and Dipstick/P4 Rapid). A second milk sample collection was performed 11 days later and P4 levels determined immediately. The study compared ultrasonography findings with rapid on-farm P4 findings, and the results of the two on-farm P4 kits.

Target P4

Two drops of milk samples were added to the centre of the cup and left for 2 min. Then, two drops of sample wash was added and left for liquid to drain into the cup. Washing was repeated once. After the cup was washed, one drop of enzyme congugate (Horse Radish Peroxidase/HRP) was added and incubated for 1 minute. The cup was filled with wash solution to the top of the inner line and this liquid was then allowed to drain completely. Two drops of freshly prepared substrate was added to the centre of the cup. Results were recorded within 9 min, as either bright blue, light blue, or white with the bright blue test result indicating the presence of low P4 (confirming the animal is not pregnant, in-heat or anestrus), while white indicated high P4 (confirming the presence of CL: pregnancy or luteal phase). All procedures were performed according to the manufacturer's instructions (Target, Biometallics Inc., New Jersey, USA).

Dipstick (P4 Rapid)

Reproductive status of HF cows in a commercial farm (Edinburgh, UK) was assessed using a qualitative on-farm P4 kit, Dipstick according to the manufacturer's instructions (Ridgeway Science Ltd, Gloucestershire, UK). Fresh whole milk (0.5ml) was added to tubes using a plastic transfer pipette after the milk was shaken to mix the fat. Dipsticks were labeled with the cow's identity number, placed into test tubes containing sample and left for 5 min. Results were recorded after 5 min. Dipstick test procedures are schematically illustrated in Figure 2.1. One line (only reference line) represents high P4, the cow may be pregnant or in the luteal phase; two lines (reference and test lines) represent low P4, the animal could be in-heat or without a functional ovary.

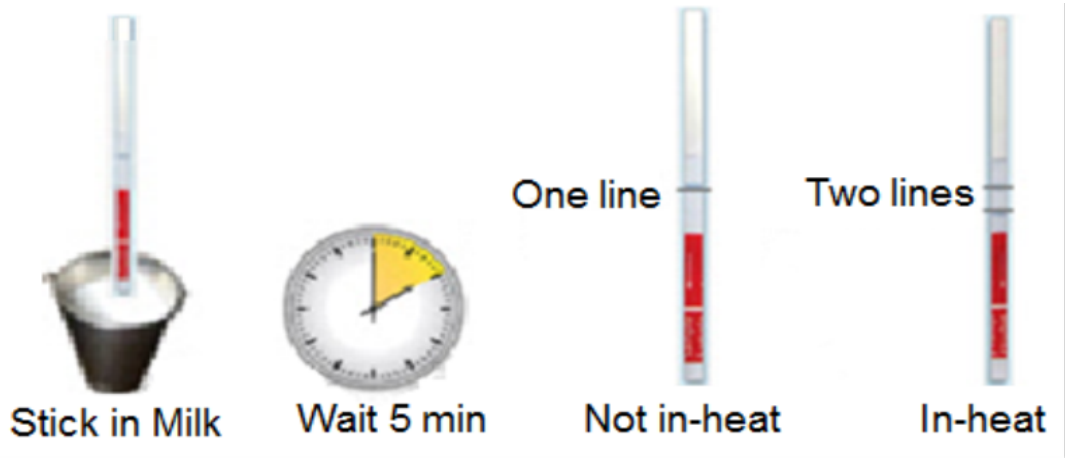

Figure 2.1: Schematic illustration of Dipstick procedures and result interpretation (**Source:** http://www.agriprom.nl/engels/p4_heat_detection.html).

2.5.2. Rapid On-farm Assessment of Reproductive Status in Crossbred Dairy Cattle in Tigray, Ethiopia

Target P4

Qualitative serum/ milk P4 determination was performed using the Target P4 kit according to the manufacturer's instructions (Target, Biometallics Inc., New Jersey, USA). Two drops of fresh whole milk/serum samples were added to each antibody-coated cup and

incubated for 2 min at room temperature. Cups were washed twice with wash buffer and this step was repeated before allowing all wash buffer to drain. One drop of HRP was added to each cup and incubated for 1 min at room temperature. Cups were washed again with buffer and allowed to drain completely. Two drops of substrate (mixture of buffered hydrogen peroxide solution and buffered tetramethyl benzidine solution/aqueous methanol) were then added to each cup. Results were taken according to a standard colour grade chart. White colour indicates high P4 level and the cows/heifers diagnosed as either pregnant or in its mid-cycle (diestrus), whereas, dark blue colour indicates low P4 level and non-pregnancy. Light/faint blue indicates no definite diagnosis, so, a second test would be required.

Dipstick (P4 Rapid)

Quantitative serum/milk P4 determination was also performed to assess reproductive status of cattle using the Dipstick (P4 Rapid) kit (Ridgeway Science Ltd, Gloucestershire, UK), according to manufacturer's instructions. Findings with Dipstick were compared with Target P4 test (Chapter 5). The same milk samples used above were also used for the Dipstick to evaluate reproductive status of crossbred dairy cows. P4 was determined by adding 0.5ml of raw whole milk sample (without additive) to a test tube (supplied by manufacturers) using a fresh plastic pipette for each cow. The Dipstick provided with the kit was placed into a test tube containing sample and left for 5 min. Results were recorded based on the appearance of a line/lines. The appearance of two dark lines indicates low P4, hence the animal is either in-heat, anestrus or suffering from follicular cyst/s whereas, a single line indicates high P4, hence the animal is either pregnant, diestrus or suffering from luteal cyst/s.

2.6. PER RECTAL PALPATION

Immediately after the P4 levels were determined in the second sample, in crossbred dairy cattle, P4 test results were combined with rectal palpation findings. The reproductive tract and ovaries were examined using per rectal palpation to determine pregnancy status (presence of fetus, uterine asymmetry, membrane slip, uterine fluid, placentome, CL) size

of reproductive tract, size of ovaries, presence of any abnormalities or pathological condition such as ovarian cysts, non-functional ovaries, pyometra or other problems.

2.7. SAMPLE TRANSPORT FROM MEKELLE, ETHIOPIA TO EDINBURGH, UK

The appropriate import license was granted from the UK to import samples from Mekelle, Ethiopia to Edinburgh, UK. All biological samples were heat treated at 56^{o}C for 30 min in a water bath in the Immunology and Biochemistry Laboratory, College of Veterinary Medicine, Mekelle University, Ethiopia prior to transporting them to The MRC Centre for Reproductive Health, The University of Edinburgh, Edinburgh, United Kingdom. This heat treatment was a requirement stated on the Import Authorisation of Animal Products/Biological samples for Study or Analysis [TARP(S)2014/23], according to the Trade in Animals and Related Products (Scotland) Regulations 2012, issued by the Agriculture, Food and Rural Communities Directorate of the Scottish Government.

2.8. LABORATORY P4 ELISA

Hormonal profiles of crossbred dairy cattle (cows and heifers) at different reproductive status was assessed using a quantitative laboratory ELISA in The MRC Centre for Reproductive Health, The University of Edinburgh, Edinburgh, UK.

2.9. PREPARATION OF BUFFER SOLUTIONS

All chemicals were of 'Analar' Laboratory Grade from recognised suppliers.

2.9.1. Preservative for ELISA Buffer

Stock Sol.

A) 2-Methyl-4-isothiazoline-3-one Sigma 725765 20% 2g

B) 5-Bromo-5-nitro-1,3-dioxane Sigma B8791-5G 20% 2g

A and B were dissolved in a 10 ml solution of Dimethylsulfoxide/Dimethylforamide (1:1 V:V) (i.e. 5ml Dimethylsulfoxide : 5 ml Dimethylforamide).

Stored at room temperature in a dark bottle.

Working Concentration

1/2000 dilution of stock

2.9.2. Wash Buffer

5x Tris Buffer Solution (TBS): 250mM Tris 750 mM NaCl

Composition: 30.29g Tris (Trizma base: [2-amino-2 (hydroxymethyl)-1,3 propanediol]

43.83g NaCl

400ml deionised H_2O

19ml hydrochloric acid (HCl) and pH adjusted to 7.4 by adding concentrated HCl make up to 1L with deionised H_2O

Working ELISA wash Buffer:

50 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween 20

Composition: 400ml of 5x TBS in a 2 L glass Dewar

1600ml H_2O

1ml Tween 20 (Fisher Scientific, UK)

Mixture stirred with magnetic stirrer.

2.9.3. Assay Buffer PBS 0.1% BSA plus ELISA Preservative

PBS: Phosphate-buffer saline

The following chemicals were dissolved in 800ml distilled H_2O

8g NaCl

0.2g KCl

1.44g Na_2HPO_4

0.24g KH_2PO_4

pH was adjusted to 7.4 with concentrated HCl.

1g of bovine serum albumin (BSA) and 0.5ml preservative were added and the solution was sterilise filtered through a 0.22-micron filter.

2.9.4. Coating Buffer

Composition: 2.12g Na_2CO_3 2.02g $NaHCO_3$ 400ml deionised H_2O

Preparation: the solution was adjusted to pH 9.6 and made up to 500ml before being stored at 4^{o}C.

2.10. CHARCOAL STRIPPING

Standards for determination of the level of P4 in serum and milk were prepared from charcoal-stripped serum and whole milk, respectively. Whereas standards for P4 assay in saliva and urine were prepared in assay buffer (0.1% BSA in PBS). Bovine whole milk and calf serum were charcoal-stripped for preparation of P4 standards for P4 profile studies (Chapter 4) and validation of on-farm P4 (field) findings (Chapter 5).

Activated charcoal (10mg per 1ml of serum or 20mg per 1ml of milk) was added to a glass flask containing intact serum/ intact whole milk or a milk diluted 1:5 in assay buffer and gently stirred with a magnetic stirrer (IKA® RCT Basic, GMBH & CO, Staufen, Germany) for approximately 4 hr at room temperature. Serum samples were spun in a centrifuge at 3000g for 30 min at 4^{o}C. Milk was gently stirred by magnetic stirrer overnight in a cold room and spun using a centrifuge (Beckman Instrument INC, California, USA) the following day, after being transferred into 50ml test tubes. Supernatant of charcoal-stripped serum and milk was transferred into clean test tubes and procedures were repeated until supernatant appeared charcoal free. Milk needed further centrifuging at 10000rpm for 25 min at 7^{o}C using Sigma laboratory centrifuge (Shrewsbury, UK), and the procedure was repeated until supernatant was confirmed charcoal free. Both charcoal-stripped serum and milk were stored at -20^{o}C until assayed.

2.11. EXTRACTION OF STEROIDS

Whole milk, saliva and urine were assayed without steroid extraction (intact) for P4 measurement, whereas, steroids were extracted from serum for both P4 and estradiol measurements. Extraction procedures were as follows: 120 microlitre (µl) (for P4 measurement) or 250µl (for estradiol) of serum, standards or controls were added into 16 x 100mm glass tubes. Then, 2ml (for P4 measurement) or 3ml (for estradiol) freshly opened diethyl ether (Sigma-Aldrich, St. Louis, MO, USA) was added to each glass tube and steroids were extracted on a mulivortexer (IKA® Vibrax VXR basic, Staufen,

Germany) for 5 min. The tubes were centrifuged at 1000g for 5 min and the aqueous lower level frozen in an ethanol absolute/dry ice bath, and the ether layer decanted into a fresh glass tube. Extracted samples were dried overnight in a fume cupboard, or dried down quickly under a stream of nitrogen on a hot block (Dri-Block® DB-3, Techne Ltd, Cambridge, UK) at 40^{o}C for a maximum of 1 hr. Then, extracted samples were reconstituted by vortexing in an equal amount (same as initial volume) of charcoal-stripped serum and assayed immediately or stored short term at 4^{o}C until assayed.

2.12. PREPARATION OF P4 STANDARDS

Estradiol standards came with the manufacturer's kit. Charcoal-stripped serum and milk (whole or diluted milk 1:5 in assay buffer) were used to prepare P4 standards for determination of P4 level in serum and milk, respectively, whereas, P4 standards used for determining P4 level in saliva and urine were prepared (diluted) with assay buffer. A total of 8 standards with different concentrations of 40.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.613, 0.0 ng/ml, were prepared to measure P4 concentration in serum and whole milk, while another 8 standards of different concentrations of 8.0, 4.0, 2.0, 1.0, 0.5, 0.25, 0.125, 0.0 ng/ml, to measure P4 in saliva and urine. In all cases, the highest P4 standard was serially diluted. Standards were aliquoted and stored at -20^{o}C until assayed.

2.13. PREPARATION OF QUALITY CONTROLS

Assay quality control (QCS) (Lyphocheck fertility control, Bio-RAD, California, USA) were used during the entire assay procedures for both P4 and estradiol measurements. Controls used for serum, saliva and urine P4 assays were diluted (prepared) in assay buffer (0.1% BSA in PBS); whereas, for milk P4 assay, QCS were diluted (prepared) in charcoal-stripped milk. Two controls of low and high P4 were prepared for determination of P4 in serum, milk and saliva.

2.14. LABORATORY ELISA PROTOCOLS

2.14.1. P4 ELISA

A quantitative in-house laboratory ELISA developed by Dr. Forbes Howie (The MRC Centre for Reproductive Health, The University of Edinburgh, Edinburgh, UK) was adapted to quantify the level of P4 in serum, whole milk, saliva and urine (Chapter 4) and validate findings of on-farm P4 tests (field study) (Chapter 5).

Before running the ELISA, all samples, standards and controls were thawed at 4^{o}C, and then equilibrated at room temperature. Whole milk and saliva samples were warmed in a Dri-Block heater (DB-2D, Bibbi Scientific Ltd., Stone, Staffordshire, UK) at 40-50^{o}C for 40 min to homogenise the milk or break the mucin present in saliva.

The ELISA was performed by coating 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) with 100µl of primary antibody (rabbit anti-progesterone, AbD Serotec, Kidlington, UK) per well at a dilution of 1:1000 in ELISA coating buffer (100mM Na Bicarbonate, pH 9.6); plates were covered with a plate sealer (parafilm) and incubated overnight at 4^{o}C. Plates were then washed 3 times with wash solution (see Section 2.9.2 for wash solution), standards, samples and controls (50µl per well) were added to each well, which was immediately followed by adding 50µl of Progesterone 3 - HRP conjugate (Meridian Life Sciences, Inc., Memphis, USA) 1:500 in assay buffer. Plates were incubated at room temperature for 2 hr on a microtitre plate shaker (IKA®, Schuttler MTS4, IKA Labortechnik, Staufen, Germany). Plates were washed 5 times, and 100µl of substrate solution (3,3,5,5-Tetramethylbenzidine/ TMB; EMD Millipore Corporation, Temecula, CA, USA) was added to each well. Plates were incubated at room temperature without shaking for serum and milk samples, or at 30^{o}C on an Orbital Incubator (Stuart®, Stone, Staffordshire, UK) for saliva samples, all in the dark. After 20 min, once the blue colour was developed, the reaction was stopped by adding 100µl of sulphuric acid ($2NH_2SO_4$) solution. Finally, plates were read on a plate reader at 450nm.

Standard curves were drawn with a total of 8 different concentrations 40.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.613, 0.0 ng/ml, to measure P4 concentration in serum (Figure 2A) and whole milk, while 8.0, 4.0, 2.0, 1.0, 0.5, 0.25, 0.125, 0.0 ng/ml, to measure P4 in saliva (Figure 2B). Samples, standards and controls were made in duplicate.

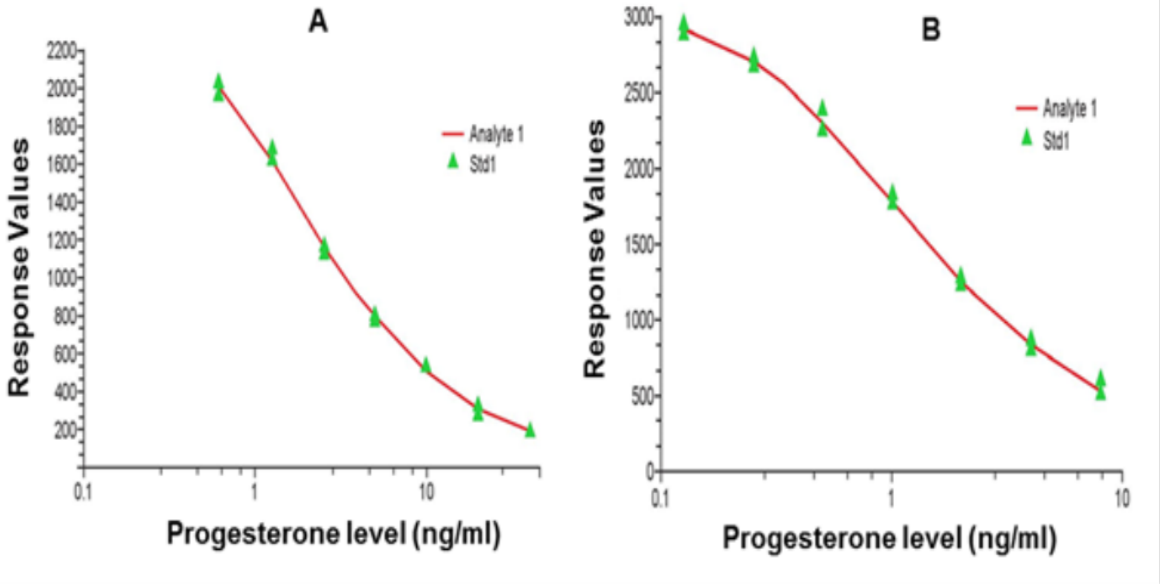

Figure 2.2: Representative examples of standard curves for serum (A) and saliva (B) P4 (ng/ml) determination using the laboratory ELISA test.

Urine P4 ELISA: The same ELISA protocol as saliva was used to determine P4 levels in urine, without extraction. The P4 level in each urine sample was normalised to the sample creatinine level. Creatinine was determined using the creatininase/creatinase specific enzymatic method described by Bömer et al. (1979) utilising a commercial kit (Alpha Laboratories Ltd., Eastleigh, UK) adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd., Welwyn Garden City, UK). Within run precision was CV < 3% while intra-batch precision was CV < 5%.

2.14.2. Estradiol ELISA

Estradiol was measured using an Estradiol Sensitive ELISA kit according to the manufacturer's instructions (Demeditec Diagnostics GmbH, Kiel, Germany). The

analytical sensitivity was calculated by the manufacturer from the mean minus two standard deviations of twenty (20) replicate analyses of Standard 0 and was found to be < 1.399 pg/ml. Steroid hormones were extracted prior to assaying as described in Section 2.11. Inter- and intra-assay CV were determined as for P4, and were 15.2 & 18.1% and 13.6 & 13.5%, for high and low pools of controls, respectively.

2.15. ANTIBODY CROSS-REACTIVITY TEST

Cross-reactivity tests were carried out for 12 related steroid hormones once the quantitative laboratory (in-house) P4 ELISA was optimised (Chapters 4 & 5). Cross-reactivity of the estradiol assay for other steroids was validated by the manufacturer. For the P4 assay, a cross-reactivity test was carried out for estrone, estradiol, estriol, dehydroepiandrosterone (DHEA), testosterone, dihydrotestosterone (DHT), 17α-hydroxyprogesterone, androstenedione, 11-deoxycortisol, corticosterone, cortisone and cortisol. Steroids were prepared in assay buffer at dilutions of 80ng/ml. Cross-reactivities were determined as: calculated mean divided by actual concentration and multiplied by 100. Accordingly, cross-reactivity of P4 assay was, estrone: 0.17%, estradiol: 0.28%, estriol: 0.18%, DHEA: 0.02%, testosterone: 0.36%, DHT: 0.15%, 17α-hydroxyprogesterone: 2.9%, androstenedione: 0.14%, 11-deoxycortisol: 0.46%, corticosterone: 0.18%, cortisone: 0.04% and cortisol: 0.04%.

2.16. INTER-ASSAY AND INTRA-ASSAY COEFFICIENT OF VARIATION

Inter- and intra-assay coefficients of variance (CV) were calculated from two controls (described in Section 2.13) of low and high P4 in duplicate in each of eight assays. Accordingly, the inter-assay CV for low and high pools were 17.4 and 13.1% for serum, 2.6 and 7.1% for milk, and 8.6 and 12.7% for saliva P4, and the intra-assay CV were 8.9 and 11.6% for serum, 2.0 and 11.2% for milk, and 11.7 and 4.7% for saliva P4.

2.17. ESTRUS SYNCHRONISATION USING CIDR-PGF2α-eCG COMBINATIONS

2.17.1. Synchronisation Protocol

The perineal region of each animal was thoroughly washed with water. An Eazi-Breed CIDR™ (CIDR®, Pfizer Ltd., Kent, UK) device, containing 1.38g of P4, was inserted into the vagina and allowed to remain in-situ for 10 days. On the 8th day after device insertion, a dose of 500µg of PGF2α (Estrumate, Schering-Plough Animal Health Corp., Summit, Germany) was administered intramuscularly (IM). On the day of device removal (Day 10), 500 IU of eCG (Intervet UK Ltd, Walton, UK) was administered. The estrus response was compared according to parity (cows vs heifers), pre-treatment group (anestrus, repeaters and silent estrus) and farming systems (smallholder vs organised commercial farms). Animals were inseminated using frozen semen or naturally mated at 48 and 72 hr post device removal.

2.17.2. Early Pregnancy Diagnosis

Animals were examined for presence of pregnancy 20-24 days (average of 21 days) post insemination by on-farm milk (lactating cows) P4 test using Dipstick (P4 Rapid, Ridgeway Science Ltd, Gloucestershire, UK) or on-farm serum (dry cows and heifers) P4 test using Target P4 kits (Target P4, Biometallics Inc., New Jersey, USA).

2.17.3. Confirmation of Pregnancy

Pregnancy was confirmed by rectal palpation/ ultrasonography (KX5200V, Kaixin®, Xuzhou Kaixin Electronic Instrument Co., Ltd, Xuzhou, Jiangsu, China) 44-90 days post insemination/mating. Conception rate was compared according to the variables above and between breeding methods (AI vs natural mating/bull).

2.18. MILK COMPOSITION

2.18.1. Milk Collection

Milk samples were collected either manually (crossbred /HF cows) or milked by machine (HF cows) to analyse milk composition (Chapter 7). Collection techniques are discussed in Section 2.4.1.

2.18.2. Milk Analysis

Macronutritional composition of milk was analysed using Miris Dairy Milk Analyser (DMA). Procedures were according to manufacturer's instructions (MIRIS AB, Uppsala, Sweden). Frozen milk samples were thawed overnight at 4^{o}C and 1-4 hr at room temperature. Samples were then warmed at 40^{o}C for 5-10 min. Wash solution and deionised water were also simultaneously thawed at 40^{o}C in a water bath. Macronutrient content of milk, such as fat, carbohydrate/lactose, protein, total solid (TS: which includes proteins, lactose, minerals, acids, enzymes, vitamins, including fat) and solid-no-fat (SNF: TS minus fat) were determined using the analyser. Prior to milk composition analysis milk samples were homogenised using Miris Sonicator (Miris). This homogenisation was used to break large fat globules into smaller, more equally distributed globules in an emulsion. Milk (2.5ml per sample) was injected into the analyser and macronutrient components in milk were analysed within one minute, and repeated twice per sample. The mean value was taken for further analysis. The levels of macronutrient components of milk were also evaluated in relation to reproductive condition (non-pregnant vs pregnant), farming system, stage of lactation, parity and breed (crossbred cows in Ethiopia vs pure HF cows in the United Kingdom). Furthermore, in pregnant cows, milk composition was compared between stages of gestation: 1st trimester, 2nd trimester and 3rd trimester.

2.19. DATA ANALYSIS

Data in the entire study were analysed using GraphPad Prism (GraphPad Software, Inc., CA, USA). Throughout, statistical significance was reached with $p<0.05$. Questionnaire survey data (Chapter 3) were analysed using the two-tailed Fisher's exact test. Hormonal

profile data (Chapter 4) were checked for normal distribution using the D'Agostino-Pearson test, and analysed using one-way analysis of variance (ANOVA). Post hoc Dunn's multiple comparisons test was used to compare P4 level between pregnant cows and pregnant heifers, between 1st, 2nd and 3rd trimester pregnancies within cows and heifers, between in-heat and diestrus cows and heifers and between diestrus and pregnant animals. Data from on-farm assessment of reproductive status (Chapter 5) were analysed with the two-tailed Fisher's exact test. Sensitivity, specificity, and predictive values were analysed using MediCalc® statistical software (MediCalc statistical software, 2015). Findings from estrus synchronisation (Chapter 6) were analysed using Fisher's exact test and Chi-square test. Results from analysis of milk composition (Chapter 7) were analysed using an unpaired t- test, or one-way ANOVA.

CHAPTER 3: ASSESSMENT OF REPRODUCTIVE PERFORMANCE AND PROBLEMS IN CROSSBRED (HOLSTEIN FRIESIAN X ZEBU) DAIRY CATTLE

Abstract:

A cross sectional study was conducted in and around Mekelle, Tigray, Ethiopia from November 2013 to May 2014 with the aims to assess reproductive performance and prevalence of reproductive problems in crossbred (HF x Zebu) dairy cattle. A questionnaire survey methodology was used. The study sampled 177 randomly selected dairy farms comprising 1013 crossbred dairy cattle (736 cows and 277 heifers). The mean age at first service, age at first calving, first postpartum estrus and calving interval were 24.8 months, 35.3 months, 114.5 days and 401.5 days, respectively. Prevalence rates of reproductive problems recorded in the study area were anestrus (37.8%), repeat-breeding (21.0%), dystocia (11.6%), RFM (11.5%), endometritis (6.6%), abortion (6.4%), prolapsed uterus/vagina (2.9%), stillbirth (2.0%) and freemartin (0.2%). Incidence of abortion did not vary with parity and stages of gestation. Breeding and the possible causes of missing insemination/mating while animals were in estrus are problematic. In conclusion, this study has revealed that the reproductive performance of crossbred dairy cattle was not optimal, and anestrus, repeat-breeding, dystocia, RFM, endometritis and abortion were the major reproductive problems. More effective breeding and reproductive health management should be considered to improve the fertility of this crossbred cattle herd. Awareness and training should be provided to the farming community to improve the productivity in the region.

Key words: Crossbred Dairy Cattle, Reproductive Performance, Reproductive Problems.

3.1. INTRODUCTION

Ethiopia owns the largest livestock population in Africa and one of the largest in the world, having 52 million cattle, 33 million sheep, 30 million goats, 2.5 million camels and 38 million poultry (CSA, 2012). About 85% of the Ethiopian population are engaged in the agricultural sector, which is the backbone of the country's economy, with livestock and its products being important sources of food and income (IBC, 2004). Cattle are well adapted and distributed among diverse ecological conditions and management systems (Lobago et al., 2006). However, dairying has not been fully exploited and promoted. Cattle play the most important role in the farming economy followed by sheep and goats. This cattle population is primarily of indigenous Zebu type (NABC, 2010). Barka, Fogera, Borana, Horro, Sheko, Afar and Arsi are also among the Ethiopian local breed cattle that are crossbred with exotic breeds (HF and Jersey).

Dairy cattle production in the country is classified into four major livestock production systems: rural smallholder (mixed crop-livestock) production, pastoral and agro-pastoral production, urban and peri-urban smallholder dairy production, and specialised commercial dairy production systems (IBC, 2004; Lobago, 2007). Infertility is the main problem that affects production in both local and crossbred cows and heifers in Ethiopia (Shiferaw et al., 2003, Duguma et al., 2012; Dinka, 2012). Consequently, the economic gain of calf crops, milk and milk products is not satisfactory. Puberty in heifers is usually at 24 months in Zebu X HF crossbred dairy cattle (Duguma et al., 2012) and, heifers are reported to have an extended later age at first calving. Most cows in smallholder farms do not optimally calve every 12 to 13 months after the first calving (Shiferaw et al., 2003).

Tigray regional state (northern part of Ethiopia) contributes a significant proportion (3.5 million) to the Ethiopian cattle population (CSA, 2012). There is also an increased demand for milk and milk products in and around Mekelle city (Fisseha, 2010), due to increased population growth and urbanisation. Consequently, the number of people involved in small-scale intensive, large-scale intensive and smallholder dairy farming has increased dramatically in the vicinity of the city. This growth effort is supported by government

extension programme through provision of door-to-door AI service to improve the genetic potential and increase milk production. Despite the effort by government to enhance the dairy sector, there is limited ongoing livestock research, particularly on assessment of reproductive and productive performance and reproductive problems of dairy cattle.

3.2. SPECIFIC AIMS

- ✓ To assess prevalence of reproductive problems.
- ✓ To evaluate reproductive performance of crossbred (Zebu x HF) dairy cattle in and around Mekelle, Tigray, Ethiopia using questionnaire survey.

3.3. MATERIALS AND METHODS

3.3.1. Study Area

The study was conducted from November 2013 to May 2014 in and around Mekelle city, which is the largest city in northern Ethiopia, and the capital city of Tigray. It is located at 39° 29`E and 13° 30` N at an altitude of 2000 m.a.s.l. The climate of the study area conforms to that of Ethiopia Highlands. The mean annual rainfall is 619 mm, which is bimodal with short rainy seasons occurring from March to May and from mid-September to February. The annual minimum and maximum temperature is 11.8°C and 29.9°C, respectively (BoPED, 2011).

3.3.2. Study Farms and Animals

The number of farms sampled in the study was determined by $N=0.25/SE^2$, where N= number of sampled farms, SE=Standard error (Arsham, 2005). A list of 210 farms housing crossbred dairy animals were considered as the sampling frame. From this, 173 farms were selected using systematic random sampling. Considering standard error of 0.038 with 95% confidence interval as follows, $N=0.25/ (0.038)^2 = 173$. In total, 177 dairy farms were included in the survey to enhance precision. These farms housed 736 crossbred (Zebu x HF) dairy cows and 277 crossbred dairy heifers aged two years or above. They also housed 55 crossbred dairy bulls and 398 crossbred dairy calves during the study period.

3.3.3. Questionnaire Data Collection

The questionnaires were piloted beforehand. A cross-sectional survey was used to collect information on reproductive performance and assess the common reproductive problems of crossbred dairy cows and heifers. Farm owners, attendants, managers, and veterinary professionals were given a short briefing about the overall aims of the study. They were asked to complete the questionnaire survey information regarding age at first service, age at first calving, calving interval, first postpartum estrus, reproductive health problems, breeding history (AI, natural mating, parity) and other related questions including the year

of farm establishment. Definitions of traits used to study reproductive performance and problems are indicated in Table 3.1.

3.3.4. Statistical Analysis

Data was analysed using two-tailed Fisher's exact test where a p value of < 0.05 was considered significant.

Table 3.1: Definition of traits used to study reproductive performance and problems.

Trait	Definition
Reproductive performance	(**Sources**: Flores, 1971; Shiferaw et al., 2003; Swai et al. 2007; Cooke et al., 2013).
Age at first service	The number of days from birth to the day of first AI or naturally mated by bull for the first time, or first embryo transfer.
Age at first calving	The number of days from birth to day of first calving.
First postpartum estrus (Day open)	The number of days from last calving to resumption of first estrus immediately after last calving.
Calving interval	The number of days between two consecutive calvings.
Reproductive problems	(**Sources**: Mwaanga and Janowski, 2000; Swai et al., 2007; Chakurkar et al., 2008; Hovingh, 2009; Dubuc, 2011; Esteves et al., 2012; Perez-Marin et al., 2012)
Anestrus	Absence of periodic manifestation of estrus without pregnancy.
Repeat-breeding	A cow or heifer repeatedly returning to estrus after breeding, usually 3 or more times, while having normal duration of the estrous cycles (17-25 days) and without anatomical abnormalities or infections.
Dystocia	Difficult birth, when parturition was assisted either by the farmer or by a field officer.
RFM	Failure of expulsion of fetal membranes 12 hr after calving or abortion.
Endometritis	Inflammation of the endometrium due to infection causes purulent vaginal discharge.
Abortion	Loss of the fetus between the age of 42 days and approximately 260 days.
Stillbirth	Expulsion of a dead fetus 260 days of pregnancy to term.
Prolapsed uterus/vagina	Protrusion out of uterus or vagina and hanging over vulva.
Freemartin	Genetically female fetus masculinised in the presence of a male co-twin, giving rise to a sterile heifer.

3.4. RESULTS

3.4.1. Reproductive Performance

The average ages at first service and at first calving were 24.8 months (range 10 to 48 months) and 35.3 months (range of 21 to 57 months), respectively. First postpartum estrus and calving interval are indicated in Table 3.2.

Table 3.2: Reproductive performance of crossbred dairy cattle in the study area.

Variable	Range	Mean ± SD
Age at first service (months)	10 to 48	24.8±6.6
Age at first calving (months)	21 to 57	35.3±6.3
First postpartum estrus (days)	9 to 480	114.5±73.7
Calving interval (days)	302.9 to 759.2	401.5±73.0

3.4.2. Common Reproductive Problems

Anestrus, repeat-breeding, dystocia, RFM, endometritis and abortion were the major reproductive problems cited by farmers/attendants or veterinarians in crossbred dairy cattle, with prevalence indicated in Table 3.3. Among 177 farms, 163 (92.1%) were affected by one or multiple reproductive problems. There were similar incidences of abortion in cows and heifers based on stages of pregnancy (Table 3.4). Overall incidence of abortion in crossbred cattle in first, second and third trimesters were, 17.5%, 42.1% and 40.4%, respectively. There was a significantly higher ($p<0.05$) incidence of anestrus in heifers than in cows. Conversely, a higher incidence ($P<0.05$) of dystocia and retained placenta was recorded in multiparous than in primiparous cattle (Table 3.3). Animals were reported to have exposure to one or more reproductive problems one or more times.

Table 3.3: The relative prevalence of major reproductive problems of crossbred dairy cows and heifers in and around Mekelle.

Reproductive problems	Total Incidence*	Incidence in multiparous cows*	Incidence in heifers/ primiparous heifers*	P-value (Fisher's exact test)
Anestrus¶	336 (37.8)	225 (31.5)	111 (63.1)	< 0.0001
Repeat-breeding¶	187 (21.0)	153 (21.4)	34 (19.3)	0.6058
Dystocia	103 (11.6)	92 (12.9)	11 (6.2)	0.0122
RFM	102 (11.5)	99 (13.9)	3 (1.7)	< 0.0001
Endometritis	59 (6.6)	52 (7.3)	7 (4.0)	0.1293
Abortion	57 (6.4)	50 (7.0)	7 (4.0)	0.1698
Stillbirth	18 (2.0)	17 (2.4)	1 (0.6)	0.2259
Prolapsed uterus/ vagina	26 (2.9)	26 (3.6)	0	-
Freemartin	2 (2.0)	-	2 (1.1)	-
Total	890	714	176	

Anestrus¶ and repeat-breeding¶ are compared between multiparous cows and heifers; whereas, dystocia, RFM, endometritis, abortion, stillbirth and prolapsed uterus/vagina are compared between multiparous cows and primiparous heifers; freemartin associated with heifers. Animals were reported to have one or multiple exposures to a single or to multiple problems. *Values in parentheses are percentages. P-value of < 0.05 considered significant.

Table 3.4: Prevalence of abortion based on stages of pregnancy versus parity in crossbred dairy cattle.

Stage of gestation	**Total Aborted***	**Abortion based on parity**		**P-value (Fisher's exact test)**
		Multiparous Cows*	Primiparous Heifers*	
1st trimester	10 (17.5)	7 (14.0)	3 (42.9)	0.0949
2nd trimester	24 (42.1)	23 (46.0)	1 (14.2)	0.2197
3rd trimester	23 (40.4)	20 (40.0)	3 (42.9)	1.0000
Total	57 (100)	50 (100)	7 (100)	0.1698

*Values in parentheses are percentages. P value of < 0.05 considered significant.

The common breeding method in the study area is mixed AI and natural mating by bull (Table 3.5). Among 177 farms, 68 (38.4%) had shown missing insemination/mating due to various factors. Although animals showed typical signs of estrus (including clear vaginal discharge, restlessness, mounting other cows, mounted by other cows), owners failed to get their animals mated or inseminated. Major causes of missed insemination recorded are absence of AI technicians when called to inseminate, or arriving very late (33.8%), poor animal condition (19.1%), carelessness/ignorance or had some other priority to breeding their animals (11.8%) and reproductive/other health problems (8.8%). Other causes of missed insemination while animals are in estrus are indicated in Table 3.6.

Table 3.5: Breeding practises with crossbred cattle in the study area.

Breeding methods	**Number of Farms**	**Percentage (%)**
AI	61	34.5
Natural mating (bull)	46	26
Mixed AI and bull	70	39.5
Total	177	100

Table 3.6: Common causes of missed insemination/mating while animals are in estrus.

Causes/reasons of missed insemination/mating	**Number of farms**	**Percentage (%)**
Lack of AI technician/ bull	3	4.4
Lack of means of communication to AI technician	3	4.4
Absence of AI technician when called to inseminate or coming very late	23	33.8
Reproductive/other health problems	6	8.8
Small animal size/ or emaciation	13	19.1
Avoiding early reduction of milk production that could happen if animals get pregnant immediately post calving	1	1.5
Carelessness/ ignorance or had some other priority to breeding their animals	8	11.8
Poor milk producer (culling of poor milking cows)	1	1.5
Affected by most or all of the above reasons	6	8.8
Voluntary waiting period (cows coming to estrus within 40 days postpartum)	4	5.9
Total	68	100

3.5. DISCUSSION

Reproductive Performance

Age at first service and age at first calving

In the current study, the age at first service and calving were in accordance with previous studies in smallholder dairy farms in Oromia region, Ethiopia (Dinka, 2012). Similar ages were also recorded in crossbred cattle at first service in Gondar Town (Nuraddis et al., 2011) and in Dire Dawa, Ethiopia (Mureda and Mekuriaw, 2007). Ages at first service and first calving were similar to recent studies in small-scale farms in and around Mekelle (Kumar and Tkui, 2014). Further, comparable ages at first calving were recorded in the previous studies in urban and in peri-urban crossbred dairy cattle farms, in central Tigray (Weldeslasse et al., 2012), and in crossbred cattle in Tanzania (Haile-mariam et al., 1993; Asimwe and Kifaro, 2007). However, later age at first calving was recorded in the present study than in crossbred (Arsi breed X Jersey, and Arsi breed X HF) cattle in Asella, Ethiopia (Negussie et al., 1998; Yifat et al., 2009). The current study showed comparatively earlier age in crossbred dairy heifers at first calving than in Zimbabwe (Masama et al., 2003), other parts of Ethiopia (Shiferaw et al., 2003) and in Malawi (Agyemang and Nkhonjera, 1990). Heifers can be bred when they attain 60% of their adult body weight (Hammond, 1960). For a dairy farm to be profitable there should be a focus on heifer's timely growth and conception. Age at puberty and calving are highly related to body weight of the heifers (Moore et al., 1990).

First postpartum estrus and calving interval

Comparatively longer postpartum estrus was recorded in the present study than in Oromia region, Ethiopia (Dinka, 2012). In contrast, the present study showed shorter postpartum estrus period compared to studies in Abernosa Ranch (Bekele et al., 1991) and in small-scale farms in and around Mekelle, Ethiopia (Kumar and Tkui, 2014). In the present study, the calving interval was similar to previous reports (Dinka, 2012; Negussie et al., 1998). In contrast, the present findings revealed shorter calving intervals compared to previous studies in central part of Ethiopia (Shiferaw et al., 2003), Abernisa Ranch, Ethiopia (Haile-

mariam et al., 1993), small scale dairy farms in and around Mekelle (Kumar and Tkui, 2014), Malawi (Agyemang and Nkhonjera, 1990) and Tanzania (Asimwe and Kifaro, 2007). Unlike the present study and other reports on crossbred (Zebu x HF) dairy cattle, pure Zebu (*Bos indicus*) cattle have a much longer calving interval (reviewed by Mukasa-Mugerwa, 1989). Farmers or farm managers in the present study were asked if they had observed overdue pregnancy. They responded indicating animals bearing male calves tend to have 1 to 2 weeks longer gestation periods compared to female calves, which is in accordance with previous reports (Dinka, 2012). However, the underlying cause is not known. Further to bearing male fetus, gestation length could be prolonged due to increasing cow's age and growing fetus weight (Nogalski and Piwczyński, 2012).

Prevalence of Reproductive Problems

Anestrus was found to be the most prevalent reproductive problem followed by repeat-breeding. This is in contrast to a previous questionnaire survey (Bitew and Prasad, 2011). The present study also demonstrated high incidence of anestrus in comparison with a study in crossbred cattle in Bangladesh (Al-Maruf et al., 2014). Our study indicates that repeat-breeding is a major problem affecting fertility of animals in the study area. High incidence of repeat-breeding could be due to a lack of nutrition, improper insemination and timing of AI and poor semen quality (Esheti and Moges, 2014). Our incidence of repeat-breeding is comparatively higher than previous reports from Ethiopia in crossbred cows around Bedelle (Bitew and Prasad, 2011), East Showa (Esheti and Moges, 2014), in indigenous Borana breed cows in Borena Zone (Benti and Zewdie, 2014), in crossbred cows/heifers in Mekelle city, Ethiopia (Mandefro and Negash, 2014) and in crossbred cows in Bangladesh (Al-Maruf et al., 2014). Conversely, the present study showed lower incidence of repeat-breeding than incidences in Goa, India (Chakurkar et al., 2008).

Dystocia is one of the major reproductive problems in crossbred dairy cattle in and around Mekelle, and is comparatively higher than in previous reports from southwestern (Bitew and Prasad, 2011) and other parts of Ethiopia (Esheti and Moges, 2014). Our findings are in contrast to previous studies where incidence of dystocia was higher in multiparous cows

compared to primiparous ones (Johanson and Berger, 2003). Incidence of dystocia in primiparous heifers was less than in multiparous cows. The possible causes of dystocia reported by the farmers and veterinarians were failure of cervical dilatation, fetal oversize, twinning, abnormal presentation, position and posture of the fetus, uterine inertia, hypocalcaemia and obesity of the dam.

Prevalence of RFM was in agreement with previous studies conducted in the southwestern part of Ethiopia (Bitew and Prasad, 2011). Contrary to our finding, lower incidence was reported in crossbred dairy cows in other part of Ethiopia (Esheti and Moges 2014) and crossbred cows in Bangladesh (Al-Maruf et al., 2014). The higher incidence of RFM in multiparous cows than primiparous heifers was consistent with previous studies in Israel (Eger et al., 1985). The reason that RFM was higher in multiparous cows could be due to previous exposure to reproductive problems or other health problems and nutritional deficiencies.

The prevalence of endometritis in the present study was comparatively lower than previous reports in crossbred cows in other part of Ethiopia (Bitew and Prasad, 2011). However, it was higher than reports in other part of Ethiopia (Esheti and Moges 2014) and Bangladesh (Al-Maruf et al., 2014).

Abortion is one of the major causes of economic losses in the cattle industry. Overall incidence of abortion in the current survey was comparatively higher than in reports by Esheti and Moges (2014), although lower than in other studies of crossbred cows elsewhere in Ethiopia (Bitew and Prasad, 2011). Abortion could be due to metabolic or hormonal abnormalities, nutritional deficiencies, trauma, toxicities, or infectious agents (Ortega-Mora et al., 2007; Givens, 2006). In the present study, the majority of abortions occurred in 2^{nd} and 3^{rd} trimester. Reducing this high abortion rate would have a huge economic impact; notably because the investment of time, since cows will not produce milk and calves after abortion. In the present study, although results were not significant, there was comparatively higher prevalence of stillbirth in calves born from multiparous cows to calves born from primiparous heifers. Contrary to our survey, it is speculated that

the occurrence of assisted calving is 2.5 times higher in primiparous heifers than multiparous cows and that there is a high incidence of stillbirth (Olson et al., 2009). This represents an area for future study.

The exact cause of prolapsed uterus is not clear (Noakes et al., 2001) although high estrogen has been proposed to cause prolapsed vagina (Roberts, 1998). Delayed intervention in cases of uterine prolapse may cause fatal septicaemia (Bhattacharyya et al., 2007). Freemartin is not a major reproductive problem in crossbred cattle in and around Mekelle, although it is one of the causes of anestrus and sterility in heifers. The occurrence of freemartin reaches 90-97% in twin pregnancies in cattle (briefly reviewed by Esteves et al., 2012; SDDP, 1999).

This study has noted a high incidence of missed insemination or mating. The use of AI is encouraged by government, with a bull rearing and semen collection centre being established in Mekelle, and few farmers have their own crossbred dairy bulls for breeding purposes. Cows and heifers are bred with a type of semen based on their size. Animals of small size (mostly heifers) are inseminated with a bull semen of 50% HF pedigree. Sometimes, when semen from bulls with 50% HF pedigree is not suitable for the female, semen from genetically selected local bulls, such as Borana breed, is used instead. When animals are not bred when they come to estrus, this will inevitably result in heifers with a long age at first service and first calving, and cows with a longer calving interval, hence a decreased calf crop or replacement heifers. Strategies to improve breeding management should be developed to achieve one calf per year and therefore improve economic development in dairy production.

Although the present study reports valuable information based on questionnaire survey and the available breeding records, we are more confident with the findings from organised farms than smallholder farms. In most smallholder farms, there were no proper breeding records. Most questionnaire data including age of heifers/cows, date of insemination, calving date or other related traits were based on (farmers') memory rather than recorded information. This might have influenced the reliability of some of the data.

In conclusion, this study has noted that anestrus, repeat-breeding, dystocia, RFM endometritis and abortion are the major problems affecting reproductive performance of crossbred dairy cattle in and around Mekelle, Tigray, Ethiopia. Hence, effective breeding, in addition to reproductive and other health management should be considered as a means to improve fertility of crossbred cattle. Furthermore, awareness and training should be given to the farming community to improve productivity for the dairy industry in the region.

CHAPTER 4: SERUM, MILK, SALIVA AND URINE PROGESTERONE AND ESTRADIOL PROFILES IN CROSSBRED (ZEBU X HOLSTEIN FRIESIAN) DAIRY CATTLE

Abstract:

Effective breeding management is a crucial tool to enhance the reproductive and productive performance of dairy cattle. To achieve this, regular monitoring of their reproductive status is important. The aims of this study were to monitor the reproductive status of crossbred dairy cattle based on progesterone (P4) concentration in serum, whole milk, saliva and urine, and estradiol in serum using quantitative laboratory ELISA tests, and to document hormonal profiles at different reproductive stages. Three hundred and thirty-six (336) crossbred (HF x Zebu) dairy cattle (232 cows and 104 heifers) from 47 dairy farms owned by smallholder or organised commercial dairy farmers were included in the study. Matched blood, milk and saliva samples were collected twice at 11-day intervals at afternoon milking, while urine was collected just once. Serum was separated within 48 hr of blood collection. All samples were stored at -80^{o}C until assayed. In both cows and heifers, P4 was higher during pregnancy than in other reproductive conditions. Estradiol was higher during estrus than at other reproductive stages. Estradiol levels were significantly higher ($p<0.05$) in estrus cows compared with heifers. Lower P4 was recorded in saliva compared to other sample types. The mean level of P4 in serum strongly correlated with the value in milk ($r = 0.6368$, $P< 0.0001$), however there was no correlation in P4 concentration between serum and saliva or between P4 in serum and in urine. Hence, determination of P4 in serum and milk using an ELISA test is a reliable and precise method to monitor reproductive status in crossbred dairy cattle. However, P4 concentrations in saliva and urine require further validation to evaluate their prognostic potential. P4 is more reliable than estradiol for determining reproductive status due to variations in estradiol levels recorded.

Key words: Cattle, Estradiol, ELISA, P4, Reproductive Status.

4.1. INTRODUCTION

Effective breeding management is a crucial tool to enhance the reproductive and productive performance of dairy cattle. Failure to detect and correctly interpret signs of estrus can contribute to significant loss in the dairy industry (Barr, 1974; Britt, 1985). Although, visual observation of animals for signs of estrus is a common method of estrus detection across the globe, in developed countries objective tests are used to confirm estrus (reviewed by Rao et al., 2013), including measuring serum/plasma or milk progesterone (P4) levels, and to successfully breed cattle.

Progesterone plays an essential role in various reproductive functions, including regulating the length of the estrous cycle, maintaining pregnancy (McDonald et al., 1952; reviewed by Rekawiecki et al., 2008) and regulating embryonic growth and development (Garrett et al., 1988; Mann et al., 1996). P4 is high during the luteal phase and in pregnancy, however the levels decline if the animal fails to conceive (Forde et al., 2011; Ashwood, 2012). Monitoring P4 in plasma/serum (Muhammd et al., 2000; Lobago et al., 2007) or milk (Romagnolo and Nebel, 1993; Lobago et al., 2007; Domènech et al., 2011) using an Enzyme Linked Immunosorbent Assay (ELISA) or Radioimmunoassay (RIA) has been used to assess the reproductive status of cattle and ensure successful breeding, or inform decisions to cull non-productive cows/heifers. Additionally, it has been used to detect pathology of the reproductive system such as cystic ovaries (Bajema et al., 1994).

Determination of P4 in saliva using ELISA has also been used to determine the reproductive status in farm animals (Kanchev et al., 1988; Gao et al., 1988; Swanand, 2012) as well as in women (Wong et al., 1990). However, determination of P4 concentration alone is not sufficient to predict ovulation because there is a large variation in the timing of P4 decrease relative to ovulation (Roelofs et al., 2006). Ovarian estradiol plays an important role in establishing the timing of uterine receptivity to the developing embryo (Ozturk and Demir, 2010) and embryo survival (Miller and Moore, 1976). In the bovine, estrus is initiated after a rise in circulating estradiol level (Allrich, 1994).

Identifying the estradiol peak can precisely indicate pre-ovulation (Domènech et al., 2011), which may assist prediction of insemination timing yielding high conception rates.

In Ethiopia, pregnancy diagnosis in cattle is only carried out using per rectal palpation by AI technicians or veterinarians (Lobago et al., 2007). Recently, we showed that qualitative on-farm milk/serum P4 ELISA tests effectively diagnosed pregnancy as early as 18-days post service and confirmed cyclicity in cattle (Mekonnin et al., 2015a). However, the on-farm qualitative testing does not quantify P4 levels in cattle, and is designed only to determine the relative P4 concentration (high or low) (Nebel, 1988). As dry cows and heifers are not lactating, it is not feasible to use milk to determine P4 levels, so urine or saliva samples are attractive alternatives for monitoring reproductive status (Gao et al., 1988).

The metabolism of P4 is rapid and occurs mainly in the liver (Cupps, 1991; Stanczyk, 2003; Falcone and Hurd, 2007). The P4 metabolites are released from the liver into the blood circulation, and excreted by the kidneys into the urine (Cupps, 1991; Falcone and Hurd, 2007). Pregnanediol glucuronide (PdG) is a common urinary metabolite of P4 (Klyne and Wright, 1959; Denari et al., 1981; Baulieu and Kelly, 1990; O'Malley and Strott, 1999; Josimovich, 2013). Volkery et al. (2012) reported that plasma concentration of P4 and estrone sulphate correlated with PdG and estrone sulphate levels in matched urine in female alpacas. Similarly, estrone conjugates and PdG in urine were measured using RIA and/or EIA, and their value paralleled the profile of the parent steroid in serum/plasma in women (Denari et al., 1981; Munro et al., 1991).

Changes in total P4 circulation in blood (protein - bound plus free, non-protein bound fractions) have been widely used for accurate monitoring of reproductive status in cows (Robertson and Sarda, 1971; Robertson, 1972; Shemesh et al., 1973; Kanchev et al., 1988; Gao et al., 1988). Over 80% of circulating P4 is bound to plasma proteins (Tallon et al., 1984; Kanchev, 1976) and only the free fraction is biologically active (Riad-Fahmy et al., 1982; McGarrigle and Lachelin, 1984). Sex steroids in saliva can be used as an index of their unbound plasma concentration (Riad-Fahmy et al., 1982; Sorgo et al., 1982; Vining

and McGinley, 1982). Hence, the quantitative correlation between the two fractions could be used to determine P4 concentration in matched blood and saliva samples (Kanchev et al., 1988; Qureshi et al., 1999; Sathe, 2012).

Saliva and urine have the advantage of being non-invasive and relatively stress-free methods and can easily be collected by farmers (Yang et al., 2004; Chen et al., 2006; Sathe, 2012). However, P4 profiles at different reproductive stages of crossbred cattle in Tigray region and other parts of Ethiopia are not well established. The aims of this study were to monitor the reproductive status of crossbred dairy cattle based on P4 concentrations in serum, whole milk, saliva and urine, and estradiol in serum using quantitative laboratory ELISA tests, and to document P4 and estradiol profiles at different reproductive stages.

4.2. SPECIFIC AIMS

- ✓ To monitor the reproductive status of crossbred dairy cattle based on P4 concentration in serum.
- ✓ To establish P4 profile in serum, whole milk, saliva and urine.
- ✓ To establish serum estradiol profiles in crossbred dairy cattle using ELISA kit.

4.3. MATERIALS AND METHODS

4.3.1. Study Animals

The study was approved by The Government of National State of Tigray Bureau of Agriculture and Rural Development, Tigray, Ethiopia, and by The University of Edinburgh Committee on Ethics of Animal Research. Samples were imported to the UK for laboratory analysis by fulfilling all the necessary requirements stated on the Import Authorisation of Animal Products/Biological samples for Study or Analysis [TARP(S)2014/23], according to the Trade in Animals and Related Products (Scotland) Regulations 2012, issued by the Agriculture, Food and Rural Communities Directorate of the Scottish Government. A total of 336 crossbred (HF x Zebu) dairy cattle (232 cows and 104 heifers) from 47 dairy farms owned by smallholder/ organised commercial dairy farmers were included in the study. Their husbandry practice was as described by Hailu et al. (2015).

Selection of Study Animals

Overtly healthy animals, having a mean BCS of 3.1, range 2.5-4 (using a scale of 1-5) were included in the study (DEFRA, 2001). According to breeding history (farmer's observation), animals that had never come into estrus 60 to 90 days postpartum (anestrus), repeaters, pregnant animals ($\geq$18 days post AI/mating) and those that were reported in-heat at the time of sample collection were included. Breeding history of each animal in the experimental group was taken. Reproductive status was confirmed on the farm by qualitative on-farm P4 assays and animals were grouped accordingly as anestrus, in-heat, diestrus and pregnant (1st trimester, 2nd trimester and 3rd trimester).

4.3.2. Sample Collection

Blood Collection and Serum Separation

Blood was collected in 10 ml plain vacutainer tubes (BD Vacutainer®, BD, Plymouth, UK) from the jugular vein of dry cows and heifers twice at eleven-day intervals. Blood

was allowed to clot at room temperature for 24-48 hr after collection and serum was separated. Serum was stored in 5ml capacity sterile plastic transport tubes (Alpha Laboratories, Hampshire, UK) and stored at -80^{o}C until assayed.

Milk Collection

Milk (10-20ml per animal) was collected into plastic centrifuge tubes (Alpha Laboratories) twice at eleven-day intervals from lactating dairy cows. Samples (foremilk) were taken manually from all four quarters after discarding the first four milk drops. Milk was collected only from cows with a clinically healthy udder and teats. Samples were frozen at -80^{o}C until assayed.

Saliva Collection

Saliva (5ml from each animal) was collected from dairy cows and heifers twice at 11-day intervals using a 7ml capacity plastic transfer pipette directly from the buccal commissures by aspiration. In cases when insufficient saliva was collected, animals were provided with some green feed or hay/straw to stimulate salivation, and in some cases saliva dropping out of the mouth was collected directly to the sterile, plastic transport tubes (Alpha Laboratories). Samples were transported from the farms to the laboratory in a cooling box with ice and samples were centrifuged at 4000g for 10 min at 20^{o}C to remove any contaminating food particles. In some cases, samples were frozen and centrifuged, multiple times until food particles were completely removed. Samples were stored at -80^{o}C until assayed. Collection procedures were based on previous reports (Gao et al., 1988; Kanchev et al., 1988).

Urine Collection

Urine (5ml/cow) was collected when cows urinated normally or following stimulation through massaging the ventral commissure of the vulva. Samples were frozen at -80^{o}C until assayed.

4.3.3. Experimental Design

Steroid Hormone Extraction

Whole milk, saliva and urine were used without steroid extraction (intact) for P4 measurement. Steroids were extracted from serum for both P4 and estradiol measurements. Extraction procedures were as follows: 120µl (for P4 measurement) or 250µl (for estradiol) of serum, standards or controls were added into 16 x 100mm glass tubes. 2ml (for P4) or 3ml (for estradiol) diethyl ether (Sigma-Aldrich, St. Louis, MO, USA) was then added to each glass tube and steroids were extracted on a muli-vortexer (IKA® Vibrax VXR basic, Staufen, Germany) for 5 min. Extracted samples were frozen in an ethanol (VWR International, Fontenay-sous Bois, France) plus dry ice bath, and the ether layer decanted. Extracted samples were dried overnight in a fume cupboard, or dried down rapidly under a stream of nitrogen in a hot block (Dri-Block® DB-3, Techne Ltd, Cambridge, UK) at 40°C for a maximum of 1hr. Extracted samples were reconstituted by vortexing with charcoal-stripped serum (volume equal to original volume of sample) and assayed immediately or stored short-term at 4°C until assayed.

P4 Determination in Serum, Whole Milk, Saliva and Urine

Quantitative in-house laboratory ELISA that was developed by Dr. Forbes Howie (MRC Centre for Reproductive Health, The University of Edinburgh, UK) was adapted to quantify the level of P4 in serum, whole milk, saliva and urine. All samples, standards and controls were first thawed at 4°C, and then warmed to room temperature. Whole milk and saliva samples were further warmed in a tube warmer (Dri-Block, Bibbi Scientific Ltd., Stone, Staffordshire, UK) at 40-50°C for 40 min to homogenise the milk and to break down the mucin present in saliva.

ELISA was performed by coating 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) with 100µl of primary antibody (rabbit anti-P4, AbD Serotec, Kidlington, UK) per well at a dilution of 1:1000 in coating buffer (100mM Sodium Bicarbonate, pH 9.6), covered with parafilm incubated overnight at 4°C and then washed 3 times with wash solution (BioWhittaker® PBS, Lonza, Verviers, Belgium) + 0.05%

Tween 20 (Tween® 20, Sigma-Aldrich, Inc., St. Louis, MO, USA). Standards, samples and controls (50µl per well) were added to each well, followed by 50µl of secondary antibody, Progesterone 3 - HRP conjugate (Meridian Life Sciences, Inc., Memphis, USA) 1:500 in assay buffer. Plates were incubated at room temperature for 2 hr on a microtitre plate shaker (IKA®, Schuttler MTS4, IKA Labortechnik, Staufen, Germany), then washed 5 times and 100µl of substrate solution (3,3,5,5-Tetramethylbenzidine/ TMB; EMD Millipore Corporation, Temecula, CA, USA) was added to each well. Plates were incubated at room temperature without shaking for serum and milk samples, or at 30°C on an Orbital Incubator (Stuart®, Stone, Staffordshire, UK), all in the dark. After 20 min, once a blue colour was developed, the reaction was stopped by adding 100µl of $2NH_2SO_4$ solution (Sigma-Aldrich Company Ltd., Dorset, UK). Finally, plates were read on a plate reader at 450nm.

Standard curves were prepared with a total of 8 different concentrations (40.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.613, 0.0 ng/ml) to measure P4 concentration in serum and whole milk, or 8.0, 4.0, 2.0, 1.0, 0.5, 0.25, 0.125, 0.0 ng/ml, to measure P4 in saliva. Samples, standards and controls were included in duplicate. Inter- and intra-assay CV were calculated from two controls of low and high P4 in duplicate in each of eight assays. The inter-assay CV for low and high pools respectively were 17.4 and 13.1% for serum, 2.6 and 7.1% for milk, and 8.6 and 12.7% for saliva P4, and the intra-assay CV were 8.9 and 11.6% for serum, 2.0 and 11.2% for milk, and 11.7 and 4.7% for saliva P4. Cross-reaction with other steroids was: estrone: 0.17%, estradiol: 0.28%, estriol: 0.18%, DHEA: 0.02%, testosterone: 0.36%, DHT: 0.15%, 17α-hydroxyprogesterone: 2.9%, androstenedione: 0.14%, 11-deoxycortisol: 0.46%, corticosterone: 0.18%, cortisone: 0.04% and cortisol: 0.04%.

Urine P4 ELISA: The same ELISA protocol as used for saliva was used to determine P4 in urine, without extraction. The P4 level in each urine sample was divided by the creatinine level in the same sample to adjust for dilution effects. Creatinine was determined using the creatininase/creatinase specific enzymatic method described by Bömer et al. (1979) utilising a commercial kit (Alpha Laboratories Ltd.) adapted for use

on a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd., Welwyn Garden City, UK). Within run precision was CV < 3% while intra-batch precision was CV < 5%.

Estradiol Measurement in Serum

Estradiol was measured using Estradiol Sensitive ELISA kit according to the manufacturer's instructions (Demeditec Diagnostics GmbH, Kiel, Germany). Steroids were extracted prior to assaying. Wells were precoated with a polyclonal anti-estradiol antibody; 100 µl of each standard (0, 3, 10, 50, 200 pg/ml of estradiol), control and samples were added into appropriate wells. Then, 200µl enzyme conjugate was added into each well, thoroughly mixed for 10 seconds on a plate shaker and plates were incubated for 4 hr at room temperature. Plates were washed 3 times with 400µl per well of wash solution and 200µl of TMB was added to each well, and then plates incubated for 30 min at room temperature. The reaction was stopped by adding 100µl stop solution. Finally, plates were read on plate reader at 450nm. Inter- and intra-assay CV were determined as for P4, and were 15.2 & 18.1% and 13.6 & 13.5% for high and low pools of controls, respectively. Cross-reactivity of anti-estradiol antibody was reported as 0.2% for estrone, 0.05% for estriol and <0.001% for a range of other steroids (Appendix 4) (Demeditec Diagnostics GmbH).

4.3.4. Statistical Analysis

Data was analysed using GraphPad Prism 6. Data was checked for normal distribution using D'Agostino-Pearson Test, and analysed using one-way ANOVA. Post hoc Dunn's multiple comparisons test was used to compare P4 level between pregnant cows and pregnant heifers, between 1st, 2nd and 3rd trimester pregnancies within cows and heifers, between in heat and diestrus cows and heifers and between diestrus and pregnant animals.

4.4. RESULTS

4.4.1. P4 Profiles

Laboratory serum P4 ELISA results were considered as gold standard to classify and confirm reproductive status. The P4 level in milk, saliva and urine was measured once cattle were classified into their respective reproductive status following serum P4 determination. Three different assays were developed for three different sample types, serum, milk and saliva/urine. Extraction of steroids was performed for serum, to avoid any solvent recovery interference. Standards and controls went through the extraction processes at the same time as the unknown samples. Extraction was deemed unnecessary for saliva/urine assay. Extraction of steroids for milk proved unsuccessful; therefore, P4 measurements had to be done without extraction.

Serum P4

The mean levels of P4 in serum strongly correlated with the level in milk ($r = 0.6368$, $P < 0.0001$) (Figure 4.1a), however there was no significant correlation between serum and saliva P4 or between serum and urine P4 levels (Figure 4.1 b, c and d). Serum P4 measurement in crossbred dairy cattle in different reproductive states is indicated in Figures 4.2, 4.3 and 4.4. Cattle with P4 levels of ≥1ng/ml in either of the tests (measured twice at 11-day intervals) were considered to have functional ovaries; hence, animals were regarded as normally cycling. In contrast, cattle with low P4 level (<1ng/ml) in both tests had non-functional ovaries (no CL or follicle detected per rectum) and were considered as anestrus. Cattle that were observed in-heat and had a low P4 level (P4 <1ng/ml) were considered in-heat or follicular phase. All other animals that had a history of insemination and serum P4 ≥1ng/ml at both tests, together with per rectal palpation findings, were considered pregnant. The P4 levels (Mean±SEM) of three cows that had ovarian follicular cysts was measured in matched serum and milk, and were 0.37 and 0.13 ng/ml respectively.

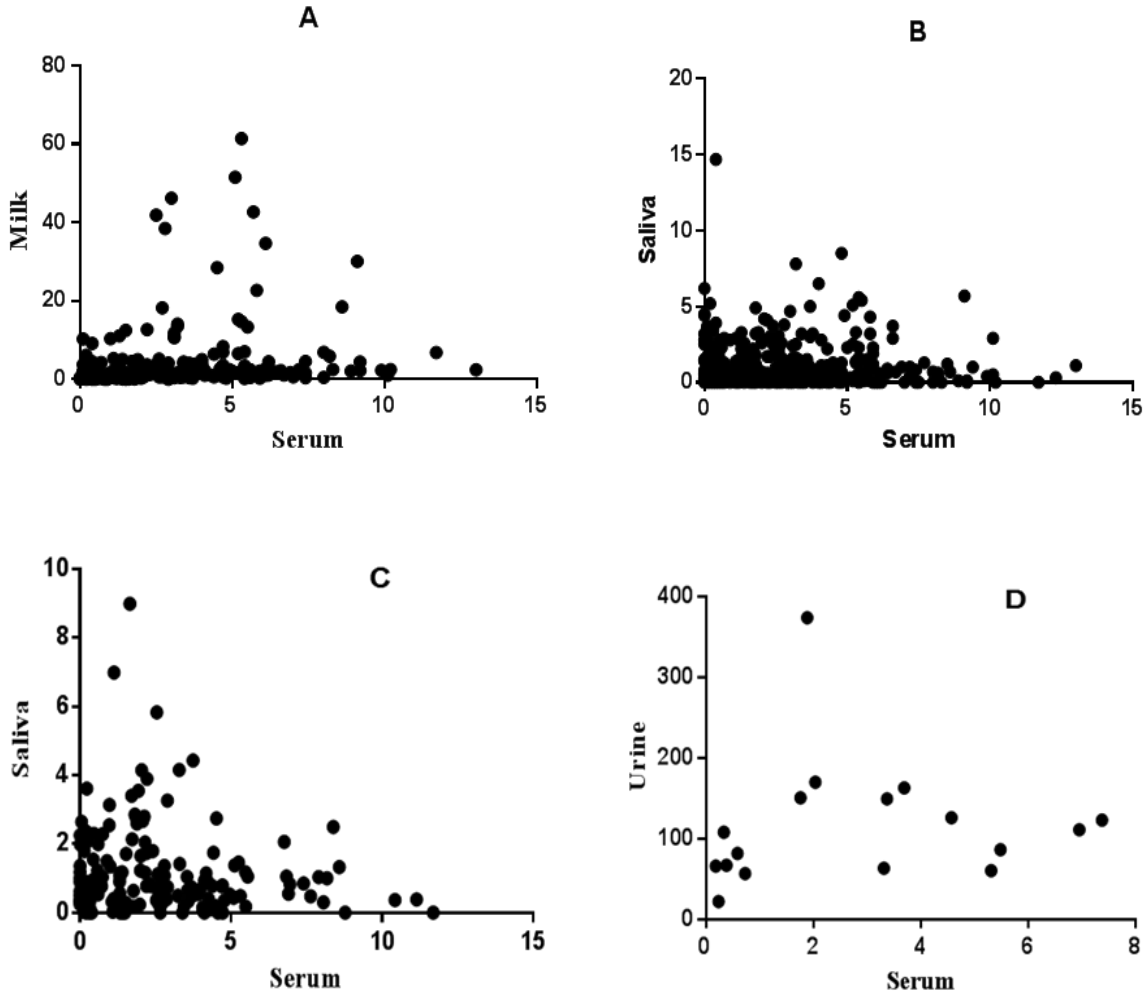

Figure 4.1: Correlation of serum vs milk (A) and serum vs saliva (B) P4 concentrations in cows, serum vs saliva P4 in heifers (C) and serum vs urine P4 in cows (D).

A: ($r = 0.6368$, $P < 0.0001$, $n = 232$); B: ($r = 0.02439$, $P = 0.6329$, $n = 232$); C: ($r = -0.02213$, $P = 0.7791$, $n = 104$); D: ($r = 0.3284$, $P = 0.1979$, $n = 30$). $P < 0.05$ is significant; r: correlation coefficient.

The mean serum P4 in cows and heifers either in-heat or anestrus was below 1ng/ml (Figure 4.2 & 4.3). There was similar serum P4 levels in cows and heifers that were either anestrus, in-heat or in their 3rd trimester pregnancy. Significantly higher ($p < 0.05$) P4 was recorded in heifers than in cows in diestrus and in the 1st trimester; whereas significantly higher P4 was detected in cows compared with heifers in 2nd trimester. While most

normally cycling cattle had <1ng/ml of P4 in one of the tests (first or second test), 22 cattle (10 cows and 12 heifers) had P4 levels ≥ 1ng/ml in both the tests. In contrast, two pregnant cows (1st and 2nd trimester, each) had low (< 1ng/ml) serum P4 concentration, whereas none of the pregnant heifers had <1ng serum P4. The overall mean P4 level in pregnant cows was significantly higher ($p < 0.05$) than in pregnant heifers. Cows that were in 1st trimester of pregnancy had significantly lower ($p < 0.05$) serum P4 levels than heifers at the same stage of pregnancy. In contrast, significantly higher ($p < 0.05$) serum P4 was detected in cows compared with heifers during 2nd trimester. P4 concentration in serum was similar between pregnant and diestrus heifers (Figure 4.3), however pregnant heifers had significantly higher ($p < 0.05$) P4 in saliva than had diestrus heifers. In contrast, there was significantly higher ($p < 0.05$) serum and milk P4 concentration in pregnant than diestrus cows (Figure 4.2).

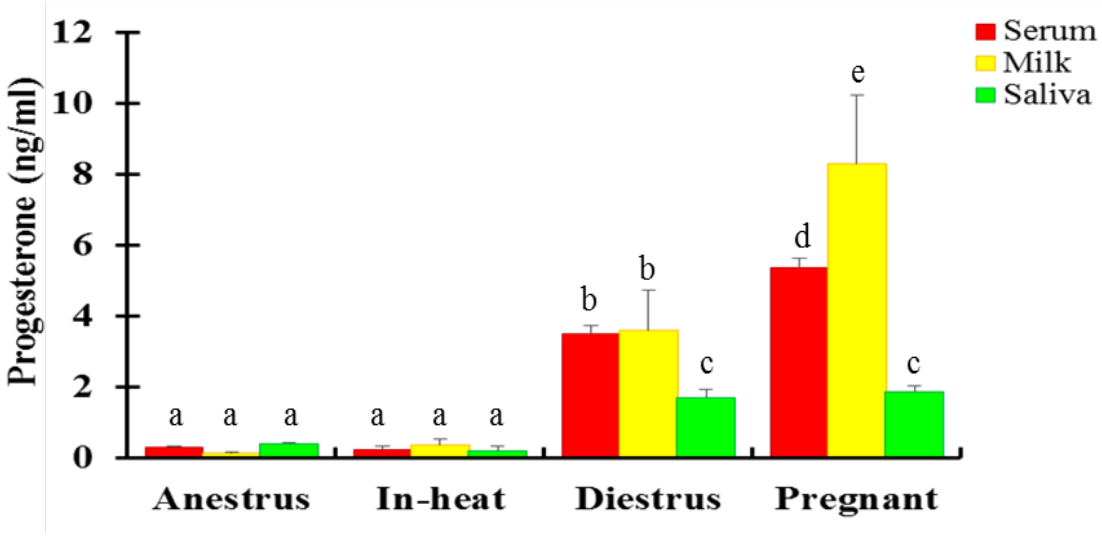

Figure 4.2: P4 concentration in serum (red), milk (yellow) and saliva (green) at different reproductive stages in crossbred dairy cows (Mean±SEM, ng/ml).

N=232: 45 anestrus; 7 in-heat; 66 diestrus; 114 pregnant). Of 7 cows in-heat, two were included following induction of estrus using a combination of CIDR-PGF2α-eCG (Chapter 6). Bars with different letters are significantly different, $p<0.05$; two-way ANOVA.

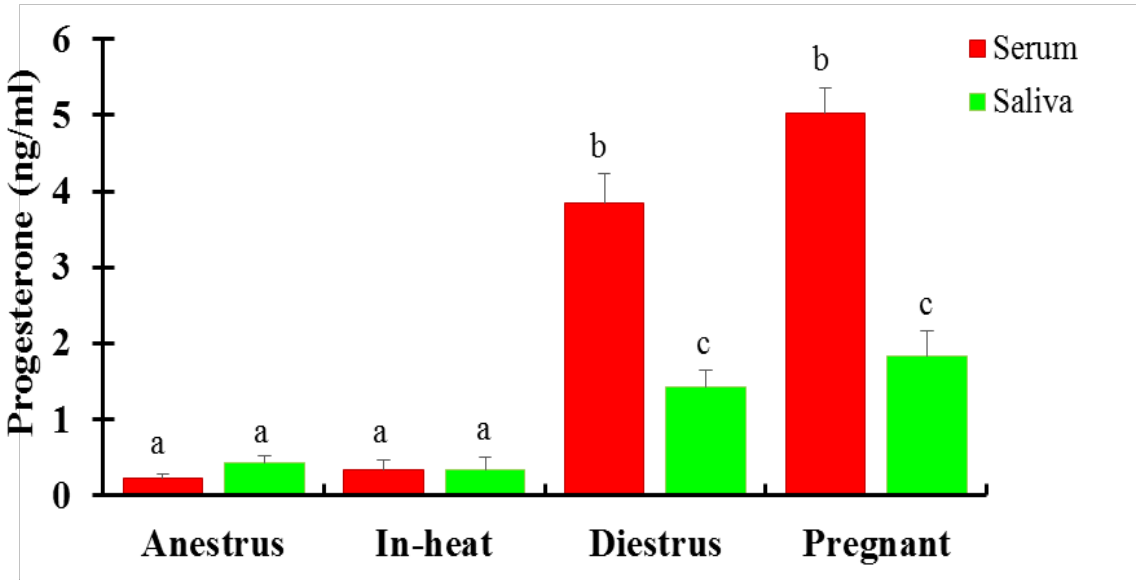

Figure 4.3: P4 concentration (Mean±SEM, ng/ml) in serum and saliva in crossbred dairy heifers in different reproductive states.

N=104: 18 anestrus; 5 in-heat; 38 diestrus; 43 pregnant); Bars with different letters are significantly different, $p<0.05$; two-way ANOVA.

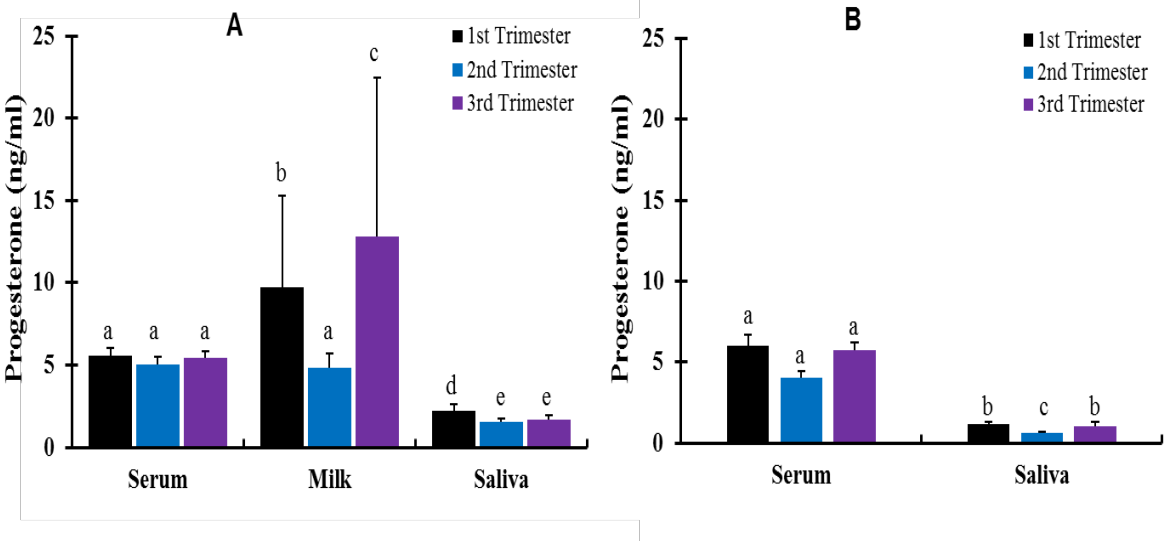

Figure 4.4: Effect of pregnancy stages on P4 concentration (Mean±SEM, ng/ml) in crossbred cows (A) and heifers (B).

Pregnant cows, n=114: 41, 36 & 37 in 1st, 2nd and 3rd trimesters, respectively; pregnant heifers, n=43: 12, 19 & 12 in 1st, 2nd and 3rd trimesters respectively. Bars with different letters are significantly different, $p<0.05$; two-way ANOVA.

Milk P4

Milk P4 concentration was determined in 200 lactating cows. Similar to serum, the level of P4 in milk was low in cows that were anestrus and in-heat, 0.16 and 0.14 ng/ml, respectively (Figure 4.2). The P4 levels in diestrus cows and heifers were similar (Figure 4.2). The overall mean P4 in milk was significantly higher ($p < 0.05$) in pregnant cows compared to those in diestrus (Figure 4.2). Cows that were in their 3rd trimester of pregnancy had significantly higher ($p < 0.05$) milk P4 than cows in other stages of pregnancy. Milk P4 in cows at 2nd trimester pregnancy was lower than those at 1st and 3rd trimester (Figure 4.4). Eight pregnant crossbred cows (one in 1st trimester, six in 2nd trimester and one in 3rd trimester) had milk P4 levels < 1ng/ml.

Saliva P4

The P4 level in saliva in anestrous cows and heifers was low, and similar to cattle in-heat. It was also similar to the levels in serum and milk (Figure 4.2 & 4.3). Similarly, cows in-heat had similar saliva P4 levels to heifers in-heat. Diestrus cows and heifers had significantly higher ($p < 0.05$) saliva P4 levels than those in anestrus or in-heat. Conversely, diestrus cows/heifers had significantly lower ($p< 0.05$) mean P4 concentrations in saliva than pregnant cows. A significantly lower ($p < 0.05$) P4 level was detected in saliva than in serum and milk in diestrus and pregnant cattle. Pregnant cows had a similar overall mean P4 to pregnant heifers. Pregnant cattle had low saliva P4 level (0.13ng/ml) in their second trimester of pregnancy. Significantly higher ($p < 0.05$) saliva P4 was detected in cows in 1st trimester than the 2nd and 3rd trimester (Figure 4.4), with similar P4 between 2nd and 3rd trimesters. The P4 levels in saliva were significantly lower ($p < 0.05$) than in serum in both cows and heifers and in milk in cows at all stages of pregnancy (Figure 4.4). In heifers, P4 was significantly higher ($p<0.05$) in 1st and 3rd trimester compared with 2nd trimester, with similar P4 levels between 1st and 3rd trimesters (Figure 4.4).

Urine P4

The mean urine concentration of P4 in pregnant cows was 119.24 ng/mg of creatinine. A significantly higher ($p< 0.05$) P4 level was detected in pregnant cows than in cows that were anestrus and in estrus (Figure 4.5).

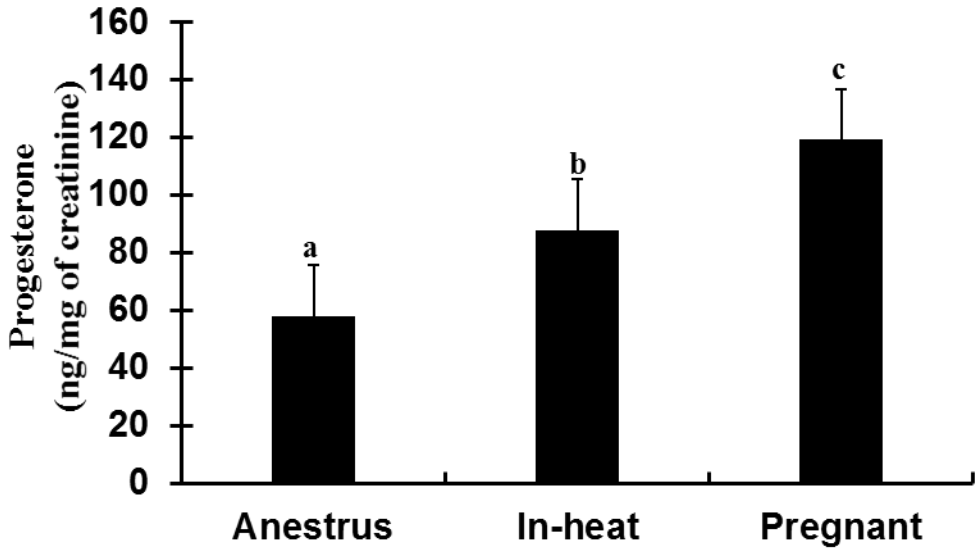

Figure 4.5: Urine P4 profile (Mean±SEM, ng/mg of creatinine) in crossbred dairy cows at different reproductive conditions; samples were collected from cows in and around Mekelle, Ethiopia.

N=30: 8 anestrus, 4 in-heat and 18 pregnant. Bars with different letters are significantly different ($p<0.05$); One-way ANOVA.

4.4.2. Serum Estradiol Profile

Serum estradiol concentration was estimated using a laboratory ELISA test (Figure 4.6A & B). Anestrus and diestrus cattle had significantly lower ($p < 0.05$) estradiol concentration compared with cattle in other reproductive stages, and the level was similar between cows and heifers. A significantly higher ($p < 0.05$) estradiol concentration was measured in cows which were in estrus than cows in other reproductive states. Pregnant heifers in term trimester had significantly higher ($p<0.05$) estradiol levels than heifers in-heat and in other reproductive states. A significantly higher ($p<0.05$) difference was found in serum concentration of estradiol in cows than heifers that were in estrus. In contrast, a

significantly higher ($p < 0.05$) estradiol level was detected in pregnant heifers than in cows in their first trimester. In both cows and heifers, higher estradiol concentrations were recorded during term pregnancy in contrast to other stages of pregnancy (Figure 4.6B).

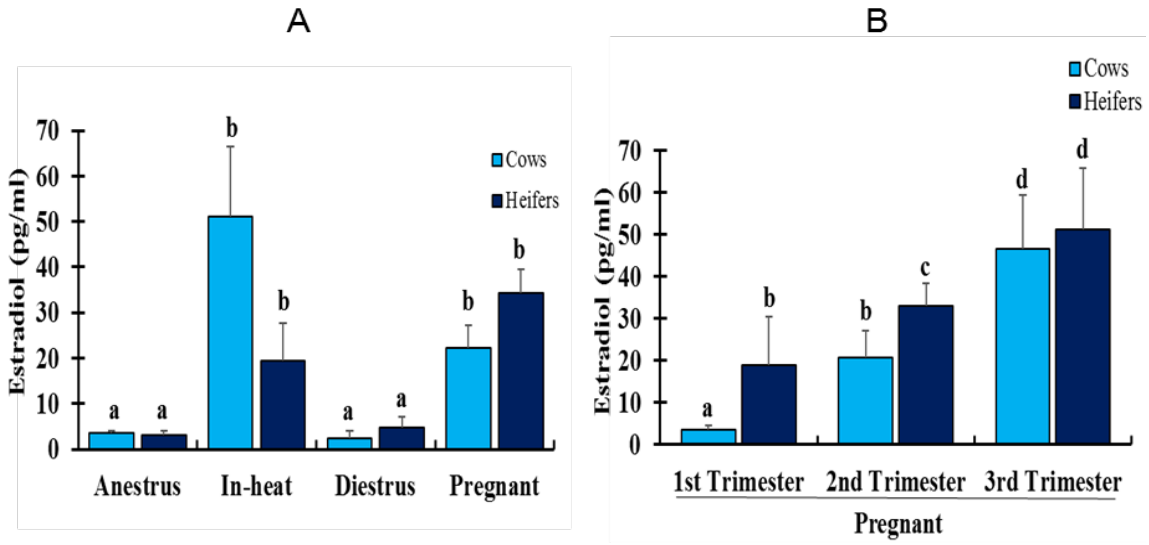

Figure 4:6: Diagrammatic representation of the mean serum estradiol concentration (Mean±SEM, pg/ml) at different reproductive states (A) and based on stages of pregnancy (B) in crossbred dairy cows and heifers.

N= 172: 100 cows; 24 anestrus, 5 in-heat, 14 diestrus, 54 pregnant (21 first, 16 second and 17 third trimester); 77 heifers; 19 anestrus, 5 in-heat, 14 diestrus, 39 pregnant (8 first, 22 second and 9 third trimester). Of 5 cows in-heat, 2 were included following induction of estrus using combination of CIDR-PGF2α-eCG (Chapter 6). In each panel, bars with different letters are significantly different ($p<0.05$); two-way ANOVA.

4.5. DISCUSSION

P4 detection in non-pregnant cycling and anestrous cattle

In the present study, cattle in estrus had low concentrations of P4 (< 1ng/ml), which is in line to previous reports in serum (Balakrishnan et al., 1986; Vadhanakul et al., 2008; Naik et al., 2013; Suthanthirakannan and Rameshkumar, 2014), plasma in Zebu cows (Alam and Ghosh, 1994) and in milk (Pope et al., 1976; Moore and Spahr, 1991; Alam and Ghosh, 1994). In contrast, Rajamahendran et al. (1993) reported that 32 (4.8%) cows that were submitted for breeding had milk P4 ≥1ng/ml, that was in line with other reports (Nebel et al., 1987; Singh et al., 2006; Ambrose and Colazo, 2007; Barui et al., 2015). Saliva P4 concentration in cattle in estrus in this study was higher than in previous reports in Friesian/ Jersey cows (Gao et al., 1988). It has been demonstrated that P4 levels during the follicular phase fluctuates from 0.025 to 0.08ng/ml (Kanchev et al., 1988). Although P4 levels are low when cows/heifers are in estrus, a low concentration of P4 does not necessary mean the animal is in estrus, as anestrous cattle with small ovaries without palpable follicles or CL, also have low levels of P4. However, it has been proposed that high P4 accurately confirms that the animal is not in estrus, even when animals exhibit behavioural symptoms of estrus (Nebel, 1988). This suggests inaccurate estrus detection (Kafi et al., 2007). The use of on-farm or laboratory P4 assays had played a significant role in identifying cycling (in-heat and those in the luteal phase) cattle. This increases the chance of breeding cattle in-heat and properly detecting when animals in the luteal phase subsequently come into estrus.

The present study has determined the P4 concentration in urine. Urinary creatinine concentration was used as a reference to determine P4 levels (Scommegna and Chattoraj, 1967; Jones and Erb, 1968), based on previous reports suggesting urinary creatinine excretion is constant in individual animals (Albin and Clanton, 1966; Hodgen et al., 1967). However, a recent report has shown that P4 metabolites (such as PdG) concentration can be measured for monitoring reproductive status of dairy cows without the need for creatinine adjustment (Yang et al., 2004). Yang et al. (2004) has shown that the correlation

between the urinary PdG and serum P4 was higher without adjustment than when adjusted by urinary creatinine concentration, which is in line with our finding whereby the level of P4 in serum and in urine was not correlated. Furthermore, studies on ovarian function in women using urinary steroid assays have shown that the correlation between serum steroid and their metabolite levels in urine was improved when not adjusted for creatinine levels (Denari et al., 1981). The P4 profile in urine of cows that were in heat, in the present study, was similar to a previous report in North American Bison cows (Kirkpatrick et al., 1991). The concentration of P4 (steroid) metabolite varies widely among different animal species (Yang et al., 2004).

The P4 level is measured, in the present study, in cycling cattle in diestrus. However, as the specific date of the previous ovulation or estrus was not available, P4 level of cattle at unknown stage of the estrous cycle, but with the existence of a palpable CL per rectal palpation was measured. Some animals had high P4 levels ($\geqslant$1 ng/ml) in either of the samples collected twice at 11-day intervals with no breeding history postpartum, reflecting the animals were normally cycling. Hence, of the two samples tested per animals, the one with the higher P4 concentration was taken for analysis in diestrus cattle, assuming it is comparatively closer to the level when the animal is in the middle of her estrous cycle. Some cycling cattle had high P4 levels in both samples collected at 11-day interval. The reason for cows/heifers having high P4 levels for an extended period could be due to a prolonged luteal phase (Ranasinghe et al., 2011). A prolonged luteal phase is one of the common reproductive problems of dairy cows, occurring mostly from calving to 90 days postpartum (Lamming and Darwash, 1998; Shrestha et al., 2004).

Ovarian cyclicity was determined based on the fluctuation of P4 levels in two samples collected 11 days apart with at least one of the samples having a P4 concentration $\geq$ 1ng/ml. That indicates the presence of a CL. The level of P4 in non-cycling (anestrous) cattle is low due to the absence of CL. In the present study, crossbred cattle that did not have CL during per rectal palpation and was confirmed by quantitative laboratory ELISA to have serum and milk P4 levels <1 ng/ml, in two samples collected at 11-day intervals, were considered as true anestrus. This correlates with the commonly accepted threshold

of 1.0ng/ml for ovarian function (Delwiche et al., 2001). However, several other studies used different P4 thresholds to distinguish luteal activity. A threshold of 0.5 ng/ml was used to determine non-luteal cows (Romagnolo and Nebel, 1993), while a threshold of 3 ng/ml was used to indicate luteal activity in the analysis of milk P4 profiles (Lamming and Darwash, 1998). The circulating P4 profile of crossbred cattle during luteal phase (diestrus) in the present study was similar to previous reports in HF cows in Thailand that used a serum RIA (Vadhanakul et al., 2008). Conversely, serum P4 levels in diestrus cattle in the present study was higher than a previous report in crossbred cows in India (Balakrishnan et al., 1986). In contrast, lower milk and serum P4 was recorded in diestrus cattle in the present study than reports in HF cows in the Netherlands (Roelofs et al., 2006) and Punganur cows in India (Naik et al., 2013). Higher serum P4 levels recorded in the present study in diestrus heifers than in diestrus cows was in agreement with a previous report after administration of CIDR (Nascimento et al., 2012). The P4 concentration in diestrus cattle in this study was higher than previous reports in Friesian /Jersey cattle (Gao et al., 1988; Kanchev et al., 1988).

P4 determination in pregnant cattle

Progesterone is an essential hormone for the maintenance of pregnancy, however, the minimum concentrations of P4 required is not clearly understood (Lucy, 2001). The P4 concentration recorded in the present study in crossbred cattle in early stage (first trimester) of pregnancy was similar to previous reports in crossbred cattle as well as local breed (Zebu) cattle in other part of Ethiopia (Lobago et al., 2009) and in Zebu cows in Bangladesh (Alam and Ghosh, 1994). In contrast, crossbred cows and heifers in the present study had higher serum P4 during their early pregnancy than HF cows in Pakistan (Muhammd et al., 2000).

Milk P4 recorded in crossbred cattle in early pregnancy in the present study was similar to previous reports in Zebu cows in Bangladesh (Alam and Ghosh, 1994). Conversely, comparatively lower milk P4 was recorded during early pregnancy in the present study than in previous reports (Karen et al., 2014). P4 is soluble in milk fat, which is the reason

for its higher level in milk than in blood (Noakes et al., 2009). The mean saliva P4 concentration detected in pregnant crossbred cows in this study was >1ng/ml, which was higher than in previous reports (Gao et al., 1988; Kanchev et al., 1988). In sheep, a mean P4 concentration of 1.8ng/ml has been reported (Needham, 2007), which is similar to the value in pregnant cows in this study. The P4 level in saliva during first trimester of pregnancy was not significantly higher than second and significantly higher than third trimester and this was significantly higher in cows than in heifers. The higher P4 in cow saliva than in heifers may be because cows have comparatively well developed reproductive physiology associated with previous calving and have larger ovaries with large CL. Previous reports have shown a positive correlation between the size of CL and P4 level (Ali and Fahmy, 2007).

Low milk P4 (< 1ng/mL) detected in pregnant cows in this study is in agreement with previous reports from British Colombia whereby 135 (29%) cows in early pregnancy (50-60 days post-service) showed low milk P4 level (Rajamahendran et al., 1993). Furthermore, Bulman and Lamming (1978) reported that a pregnant cow calved successfully despite its milk P4 level between 8 and 16 days after insemination being basal.

Several previous studies have shown application of analysis of urinary steroid metabolites to determine pregnancy status in various species (Liskowski and Wolf, 1972; reviewed by Lasley and Kirkpatrick, 1991; Yang et al., 2004; Volkery et al., 2012), including human (Vaikkinen et al., 2015). Both bovine blood and bovine CL tissue can convert P4 to 20β-hydroxypregn-4-en-3-one (Short, 1958) that can be determined in urine. It has been reported that 5β-pregnane-3α,20α-diol detected in the urine of late pregnant cows (Heitzman and Thomas, 1965). Determination of the level of pregnanediol, which is an inactive metabolite of P4, has also been used as an indirect way of measuring P4 to diagnose pregnancy (Yang et al., 2004). The higher P4 level detected in the urine of pregnant cows than in anestrus or cows in estrus, in the present study, reflects the formation and persistence of matured CL. The P4 profile in urine in this study was lower

than a previous report in North American Bison cows (Kirkpatrick et al., 1991), which may be due to breed difference.

Collecting blood samples for serum/plasma steroid hormone measurement requires venipuncture; hence, it is stressful and painful to the subject (Wong, et al., 1990; Yang et al., 2004). Furthermore, the challenges and the risk (to experts) collecting blood samples are formidable, particularly when working in farms where proper restraining facilities are not available. Unfortunately, almost all of the farms where the present study was conducted had no cattle crush for restraining the animals. Therefore, determining P4 or estradiol from serum samples for regular follow up of reproductive status of cattle is not safe for both the animals and the experts, particularly when someone approaches aggressive cattle. Though this particular study directly helped farmers involved in the study, as it was conducted at the field level, there were some farmers reluctant to provide the second blood samples, even some refrained from being included in the study. When asked why, they did not want their animals to feel pain and stressed, particularly those they thought were pregnant.

Milk is routinely used for determining reproductive status in lactating dairy cattle (Romagnolo and Nebel, 1993; Roelofs et al., 2006). A previous study showed that the changes in P4 concentrations in milk closely followed those in the blood/plasma during the reproductive cycle in cows (Laing and Heap, 1971). This supports our finding whereby the mean P4 value in serum had a strong correlation with the P4 level in matched milk. Unlike the present findings that agreed with a recent report (Sathe, 2012), previous reports demonstrated a positive correlation in the concentration of P4 in blood and saliva (Gao et al., 1988; Kanchev et al., 1988). However, it has been proposed that the most significant problem with milk P4 measurements is the presence of considerable amount of lipids, which interfere with the assay (Hoffman and Hamburger, 1973; Inaudi et al., 1982). Sampling has also been proposed to affect P4 concentration in milk (Hoffman and Hamburger, 1973). Additionally, it has been reported that storage conditions can affect P4 level in bovine milk whereby frozen samples were reported to have higher P4

concentration compared to fresh samples (Domènech et al., 2011). Furthermore, the availability of milk is limited to lactating cattle only.

Saliva and urine could be used as alternative samples to accurately determine the reproductive status of dairy cattle. Both saliva and urine can be collected through non-invasive methods, also from dry cows and heifers, hence avoiding the animal welfare problems when blood is collected or other inconvenience from using milk samples. Saliva has additional advantages due to its ease of storage, as it can be stored at -20^{o}C for over 6 months, at 48^{o}C for 7 days, or at room temperature for 48 hr with no significant change in steroid levels (Huang et al., 1981). Furthermore, urine has several advantages over saliva, serum and milk: it can be collected in large volume that enables multiple assays (Collins et al., 1979; Kesner et al., 1992; Lasley et al., 1994) and provides integrated hormone measurements without the confounding effects of pulsatile secretion (Munro et al., 1991). Additionally, the availability of steroid metabolite in higher concentration in urine (by two-to four-fold or more) than that of parent sex steroids in blood give greater advantage to the use of urine (reviewed by Lasley and Kirkpatrick, 1991; Yang et al., 2004) or in women (Shideler et al., 1989). Although the P4 level in urine in the present study did not correlate with its level in matched serum, based on previous reports that have shown urine P4 profiles correlated with serum profiles (reviewed in Lasley and Kirkpatrick, 1991; Yang et al., 2004). Urine might be a more reliable body fluid to use for monitoring reproductive status of dairy cattle. Furthermore, in the present study, collecting saliva was equally challenging as collecting blood in most cattle; whereas, urine was easily collected from both cows and heifers by the farmers themselves. This gives favours the use of urine in the future when a simple and rapid on-farm Dipstick method is developed, and can be used by farmers themselves.

Determination of serum estradiol

Circulating concentrations of estradiol during the preovulatory period can influence the establishment and maintenance of pregnancy by altering the uterine environment (Miller and Moore, 1976; Miller et al., 1977). Perry et al. (2014) reported a positive correlation

between follicle size and peak estradiol concentration, and greater peak estradiol concentration in cows exhibiting estrus than not exhibiting estrus. This is in line with the present finding whereby significantly higher estradiol levels were detected in cattle exhibiting estrus compared to anestrous or cycling (diestrus phase) cattle. The production of increased level of estradiol by the preovulatory follicles depends on the ability of the theca interna cells to produce androstenedione and the ability of granulosa cells to convert androstenedione to estradiol (Ginther et al., 1996). Theca interna cells convert pregnenolone to androstenedione, which can then be converted to estradiol by the granulosa cells (Fortune, 1986). Additionally, estradiol is proposed to induce FSH/ LH receptor expression in granulosa cells (Richards et al., 1976) and increase the stimulatory action of FSH on aromatase activity (Zhuang et al., 1982). Contrary to the positive effect of estrogen in reproductive function, several previous reports have suggested that estrogen present in cows' milk may be responsible for the development of cancer in estrogen-responsive organs (Ganmaa and Sato, 2005; Farlow et al., 2009). This opens future direction to study the estrogen profile in milk at different reproductive stages of crossbred as well as local breed cows in Tigray as well as other parts of Ethiopia.

Estradiol profiles recorded in crossbred heifers in estrus, in the present study, were in agreement with previous reports in Sahiwal heifers in estrus following treatment with CIDR in India (Singh et al., 2006), in Swedish dairy heifers (Båge, 2002) and in HF heifers following administration of PGF2α in USA (Coe and Allrich, 1989). In contrast, the estradiol level recorded in heifers in estrus in the present study was comparatively higher than reports in crossbred beef heifers in USA (Perry et al., 2007). Conversely, crossbred heifers that were in estrus in the present study had lower serum concentration of estradiol than previous reports in HF heifers in estrus induced by PGF2α in USA (Coe and Allrich, 1989) and in Bali heifers in Indonesia (Airin et al., 2014). The reason for lower estradiol profiles in the present study than in previous reports may be due to breed difference. The estradiol level in crossbred dairy cows in estrus in the present study, was similar to previous reports in crossbred, normally cycling or repeat-breeding cows in India that were in estrus (Barui et al., 2015) and in Sahiwal cows in India that were in estrus induced by

CIDR administration (Singh et al., 2006). In contrast, the present study recorded higher circulating estradiol in cows during estrus than previous reports in Punganur cows in India (Naik et al., 2013). Conversely, the estradiol profile in cows in estrus in the present study was lower than a previous report in Bunaji cattle in Nigeria (Opara et al., 2006).

Serum concentration of estradiol in anestrous cattle or cycling cattle in the diestrus phase of the estrous cycle, in the present study, was in agreement with a previous report in postpartum crossbred beef cows with calves weaned >30 days and reported to have ovulatory-sized follicles (>10 mm) but failed to exhibit standing estrus following Co-Synch protocol (Perry et al., 2014). Conversely, the estradiol profile in serum in crossbred cattle in our study was lower than previous reports in postpartum (20-40 days) crossbred Angus beef cows with calves allowed to suckle without restriction throughout the experiment in USA (Perry et al., 2014), in Punganur cows in diestrus (in 12th day of the estrous cycle) (Naik et al., 2013) and in anestrous or cycling (diestrus) Bunaji cattle in Nigeria (Opara et al., 2006). The higher estradiol level in pregnant heifers than in heifers in estrus or other reproductive conditions (anestrus or diestrus) was in agreement with a previous report in Bunaji cattle in Nigeria (Opara et al., 2006). However, the overall estradiol profile in serum in pregnant cattle in the present study was lower than a previous report (Opara et al., 2006). The reason behind this could be breed difference.

A quantitative ELISA technique can be applied in the Ethiopian research laboratories and veterinary clinics to identify pregnant dairy cattle and assess ovarian function. However, unlike the on-farm ELISA, application of quantitative ELISA at field (farm) level is not possible due to its high cost and the requirement for skilled farm personnel (Gordon, 2005; Mekonnin et al., 2015a).

In conclusion, the present study confirmed that a quantitative ELISA technique could be used to accurately determine the steroid profile and monitor the reproductive status of dairy cattle. This enables the detection of cycling animals and hence could lead to successful breeding, with pregnancy diagnosis as early as 18-days post service. Lastly, the

measurement of sex steroids in various sample types should provide a basis for further related studies in crossbred, as well as local breed cattle in the tropics and other regions.

CHAPTER 5: RAPID ON-FARM PROGESTERONE TEST TO MONITOR REPRODUCTIVE STATUS OF CROSSBRED (HOLSTEIN FRIESIAN X ZEBU) DAIRY CATTLE

Abstract:

Ethiopia maintains an extensive livestock population although reproductive performance of cattle and their breeding management are unsatisfactory. Currently, the sole reliable diagnostic tool is per rectal palpation, which is inaccurate for early pregnancy, and can cause embryonic and fetal loss. Hence, an alternative approach to monitor reproductive status is required. This study evaluated simple, cost-effective and rapid alternative monitoring approaches, on-farm diagnostic methods, to determine milk and serum progesterone (P4) and evaluate reproductive status of crossbred (HF x Zebu) dairy cattle. A total of 319 dairy cattle from 47 dairy farms were included in the study. Overtly healthy animals with body condition scoring of 2.5-4 with postpartum anestrus over 60 days, repeat-breeders (repeaters), pregnant and in-heat animals were included. Milk from cows and blood from cows and heifers was collected twice at 11-day intervals. Milk samples were analysed immediately at the farms. Serum was separated and stored in 5ml plastic transport tubes 24 to 48 hr after blood was collected. Serum samples were analysed immediately in the laboratory. P4 was measured by on-farm qualitative tests (enzyme-linked immunosorbent assay/ELISA tests: Target P4 and Dipstick (P4 Rapid)). Findings were referenced to per rectal palpation. Reproductive history of 319 animals reported by the farmers as in-heat 10 (3.1%), anestrus 118 (37.0%), repeaters 20 (6.3%) and pregnant 171 (53.6%), was evaluated using qualitative on-farm P4 ELISA and per rectal palpation, and overall findings were, in-heat 10 (3.1%), anestrus 77 (24.2%), repeaters (follicular cyst) 9 (2.8%), normally cycling 69 (21.6%) and pregnant 154 (48.3%). Among 118 animals reported anestrus only 72 (61.0%) had true anestrus while the remaining 1 (0.9%), 39 (33.0%) and 6 (5.1%) were confirmed as, repeaters (follicular cyst), normally cycling and pregnant, respectively. Similarly, of 171 animals with a history of pregnancy, P4 tests determined reproductive status as 4 (2.3%) anestrus, 1 (0.6%) repeater, 19 (11.1%) normally cycling and 147 (86%) pregnant. Significantly more ($p<0.05$) animals were

reported as anestrus by farmers (118/319, 37%) compared with detected by field P4 ELISA (72/319, 22.6%). Findings from on-farm P4 tests were validated using quantitative laboratory P4 ELISA, and similar results were obtained. The sensitivity and specificity of on-farm and laboratory P4 ELISA tests for diagnosing pregnancy were 88.6 & 99.4 % and 98.1 & 100%, respectively. Target P4 and Dipstick tests are equally effective methods to assess reproductive status. Application of these tests by veterinarians, AI technicians and low-income smallholder farmers offers a practical means to monitor reproductive status in cattle and improve breeding management to the benefit of the sector.

Key words: Breeding Management, Crossbred Cattle, On-farm Assays, P4, Reproductive Status.

5.1. INTRODUCTION

Ethiopia maintains a large livestock population, but production and reproductive performance of its livestock is relatively poor. Poor genetic performance of the local cattle breeds, poor nutrition, poor management, infertility, reproductive disorders and diseases, lack of veterinary professionals and AI technician, are some of the major constraints in the dairy industry in the country (Bitew and Prasad, 2011; Lobago, 2007; Mureda and Mekuriaw, 2007). Ethiopian livestock are genetically diverse, mainly as a result of natural selection influenced by environmental factors. Accordingly, indigenous livestock are better conditioned to withstand feed and water shortages, disease challenges and harsh climates. However, their milk and meat productivity is very low (Duguma et al., 2012).

Poor practice and lack of experience in the field of AI and pregnancy diagnosis have been among the main problems contributing to poor reproductive performance and infertility in cattle, resulting in loss of milk production, reduced calf crop and replacement heifers, unnecessary feeding and additional management costs. This can lead the owners to inappropriate management practices, and has been linked to a prolonged calving interval, abortion and mistaken selling of pregnant cows (Lobago, 2007).

Estrus detection is poorly managed in many farms. The most common method of estrus detection in Ethiopian dairy cattle production systems is visual inspection. In most cases, cattle remain unobserved when they come into estrus, particularly as this occurs in the evening, leaving a perception that they are anestrus, consequently leading to potential economic loss. Per rectal palpation is cheap for pregnancy detection, and remains the only practiced method of diagnosis (Lobago, 2007). Nevertheless, inaccurate diagnosis of pregnancy at an early stage and loss of embryos/fetuses are a common occurrence (Franco et al., 1987). In Ethiopia, per rectal palpation is usually carried out after 60 days post-service by AI technicians or animal health professionals.

Fertility of dairy cattle can be evaluated by detecting progesterone (P4) levels in milk or serum/plasma. Determining the level of P4 is a useful tool to detect abnormalities of ovarian function leading to subfertility or infertility, such as a long period of postpartum

anestrus, a lengthy anestrus period between cycles which might be due to delayed ovulation, presence of ovarian follicular cysts or absence of ovarian function, and a persistent CL (briefly reviewed by Royal et al., 2000a; Booth et al., 1979; Eddy and Clark, 1987; Rajamahendran et al., 1993; Ribadu et al., 1994; Jeffcoate and Ayliffe, 1995; Douthwaite and Dobson, 2000; Kafi et al., 2012). However, laboratory RIA and ELISA tests require procedures that can take over 24 hours, skill and high cost of chemicals and associated equipment that hinders their accessibility to evaluate fertility status of dairy cattle at the farm level. Rapid, simple and cost effective P4 tests have been developed and used to evaluate reproductive status by using monoclonal anti-P4 antibodies coupled with the employment of an amplification system (Worsfold et al., 1987; Nebel, 1988; Rajamahendran et al., 1990; Simersky et al., 2007; Osman et al., 2012).

In view of the problems outlined above, an alternative approach to monitor reproductive status is needed. The aims of the present study were to trial simple, cost effective, appropriate and rapid on-farm diagnostic methods as tools to evaluate the reproductive status of crossbred (HF x Zebu) dairy cattle. This would alleviate the problem/doubt of accuracy of monitoring the reproductive status using per rectal palpation; it could potentially avoid the need of per rectal palpation, and in the long term enable farmers to monitor fertility to properly manage their animals themselves.

5.2. SPECIFIC AIMS

- ✓ To determine whether rapid on-farm P4 kits are able to assess reproductive status of HF cows in a commercial farm, Edinburgh, UK, prior to the actual field assessment of reproductive status of crossbred dairy cattle in Ethiopia; this was intended to develop familiarity with the test procedures and interpretation of results prior to the field study in Ethiopia.
- ✓ To trial simple, cost effective, suitable and rapid on-farm diagnostic methods and to evaluate the reproductive status of crossbred (HF x Zebu) dairy cattle in and around Mekelle, Tigray, Ethiopia.

5.3. MATERIALS AND METHODS

5.3.1. Study Area

Initially the proposal was presented to key stakeholders and livestock experts and approved by The Government of National State of Tigray Bureau of Agriculture and Rural Development, Tigray, Ethiopia, and reviewed by The University of Edinburgh Animal Research Ethics Committee, UK. Mekelle is the largest city in northern Ethiopia, and is the capital city of Tigray. It is the sixth largest city in Ethiopia located at 39° 29` E and 13° 30` N at an altitude of 2000m above sea level. The climate of the study area is representative of Ethiopian highlands. The mean annual rainfall of the study area is 628mm and is associated with the north and south oscillation of inter-tropical conversion zone. The rainfall is bimodal with short rainy seasons occurring from March to May and from the middle of September to February. The annual minimum and maximum temperatures are 12°C and 30°C, respectively (BoPED, 2011).

Prior to the actual field study in Ethiopia, a preliminary trial of rapid on-farm P4 (Target P4 and Dipstick (Rapid P4) kits was carried out to assess reproductive status of HF cows in a commercial farm, Langhill Dairy Farm, Edinburgh, UK. Edinburgh is the capital city of Scotland and located at 55° 57′ 07″ N, 3° 11′ 47″ W at an altitude of 66m above sea level (http://dateandtime.info/citycoordinates.php?id=2650225). Edinburgh has a temperate, maritime climate, which is relatively mild. Winter daytime temperatures rarely fall below freezing; whereas, summer temperatures are normally moderate, rarely exceeding 22°C (https://en.wikipedia.org/wiki/Edinburgh#Areas).

5.3.2. Study Animals

A total of 334 dairy cattle (6 HF cows from the British farm and 328 crossbred (HF X Zebu) cattle: 226 cows and 102 heifers from 47 farms in and around Mekelle, Tigray Ethiopia) were included in the study. HF cows were included for preliminary qualitative rapid on-farm P4 analysis. The HF cows were managed either indoors or outdoors. Crossbred dairy cattle were managed indoors and owned by smallholder or organised

commercial dairy farms, and their management and feeding practices are described by Hailu et al. (2015).

Selection of Study Animals

HF Cows:

Six dairy cows at Langhill Dairy Farm, Edinburgh, UK with reproductive status confirmed using an Easy Scan ultrasound scanner, were included. The study evaluated and validated two different P4 kits, Target P4 and Rapid P4 for assessing reproductive status and evaluated the effectivness of the tests using P4 standards prepared with a commercial whole milk (purchased from a supermarket) at different P4 concentrations. Of these 6 cows, three (3) were pregnant (one in 1st trimester/ 42 days of pregnancy; one in 2nd trimester/101 days of pregnancy; and, another one in 3rd trimester/ 191 days of pregnancy); whereas, the other three cows were non-pregnant, among which two had follicles on their ovaries (follicular phase), and the other cow had an ovarian follicular cyst.

Crossbred Dairy Cattle (Cows/heifers):

Overtly healthy animals were included in the study. Animals were examined for any external physical injuries/deformities. Only those having body condition scoring of 2.5-4 were included in the study (DEFRA, 2001). According to breeding history and farmers' perception, animals which had never come into heat 60 to 90 days postpartum/ repeaters, pregnant animals ($\geq$18 days post AI/mating) and those which were reported in-heat at the time of sample collection were included. Breeding history of experimental animals were recorded individually. Data recorded included age, number of calving, date of last calvings, return to estrus after calving, whether the animals were repeat-breeding after insemination, or the animals were checked for presence of pregnancy by veterinarians/ AI technicians and their findings. Reproductive history of study animals as per farmer's/farm manager's/attendant's perception/breeding records (some) was immediately recorded before sample collection.

5.3.3. Sample Collection

Milk Sample Collection

HF Cows:

Whole milk was either purchased from the supermarket to prepare P4 standards and evaluate rapid on-farm P4 kits in determining the P4 levels in known concentrations of P4 standards, or collected (10-20ml) twice at 11-day intervals using plastic centrifuge tubes (Alpha Laboratories, Hampshire, UK) from 6 HF cows at Langhill Dairy Farm, Edinburgh, UK. All samples were transported immediately in an insulated box on ice packs to MRC Centre for Reproductive Health, Immunoassay Laboratory, The University of Edinburgh, Edinburgh, UK and analysed. These cattle were included only for validation of the on-farm P4 kits prior to the actual field study in crossbred dairy cattle in Ethiopia.

Crossbred Dairy Cows:

Milk (10-20ml per animal) was collected into plastic centrifuge tubes (Alpha Laboratories, Hampshire, UK) twice at 11-day intervals from lactating dairy cows. Samples were taken manually from all four quarters after discarding the first four milk drops. Milk was collected only from cows with clinically healthy udder and teats. Samples were immediately processed at the farms, except in the case of inadequate working conditions, such as excessive wind, and dust which could affect the results. In that case, samples were transported using an icebox to the Biochemistry Laboratory, College of Veterinary Medicine, Mekelle University, Ethiopia for analysis.

Blood Collection and Serum Separation

Blood was collected in 10ml plain vacutainer tubes (BD Vacutainer®, BD, Plymouth, UK) from the jugular vein of lactating and dry cows and heifers. Blood was allowed to clot at room temperature. Serum was separated and stored in 5ml capacity plastic transport tubes (Alpha Laboratories, Hampshire, UK) 24 to 48 hr after blood was collected. Serum samples from dry cows and heifers were immediately analysed using Target P4. Serum from both lactating and dry cows as well as heifers was then stored at -20^{o}C before

shipping to the United Kingdom for validation of findings from on-farm ELISA. Samples were stored at -80^{o}C prior to being assayed.

5.3.4. On-farm P4 Tests

Preliminary Assessment of Reproductive Status of HF Cows in Edinburgh, UK Using On-farm P4 Tests

Target P4

The P4 levels in HF cows in Langhill Dairy Farm, Edinburgh, UK were analysed qualitatively and findings from on-farm P4 tests were compared with ultrasound scanning results, and with Dipstick findings. Test procedures are discussed in Section 2.5.1.

Dipstick (P4 Rapid)

Milk P4 levels in HF cows in Langhill Dairy Farm, Edinburgh, UK and in milk P4 standards (in milk bought from a supermarket) were analysed using Dipstick kits. The test procedures and result interpretation are outlined in Section 2.5.1.

Rapid On-farm Assessment of Reproductive Status in Crossbred Dairy Cattle in Tigray, Ethiopia

Target P4

The P4 level was measured using Target P4 ELISA test according to the manufacturer's instructions (Target, Biometallics Inc., New Jersey, USA). Two drops of fresh whole milk/serum samples were added to each antibody-coated cup and incubated for 2 min at room temperature. Cups were washed twice with wash buffer before allowing all wash buffer to drain. One drop of HRP was added to each cup and incubated for 1 min at room temperature. Cups were washed again with buffer and allowed to drain completely. Two drops of substrate (mixture of buffered hydrogen peroxide solution and buffered tetramethyl benzidine solution/aqueous methanol) were then added to each cup. Results were recorded 9 min after adding substrate, based on a standard colour grade chart. White colour indicated high P4 level and the cows/heifers diagnosed as either pregnant or in mid-

cycle (diestrus); bright/dark blue colour indicates low P4 level and non-pregnancy. Light/faint blue indicates no definite diagnosis, so, a second test was required.

Dipstick (P4 Rapid)

The P4 level was measured using Dipstick (P4 Rapid) kit (Ridgeway Science Ltd, Gloucestershire, UK), according to manufacturer's instructions. The same milk samples used above were also used for the Dipstick. The P4 level was determined by adding 0.5ml of raw whole milk samples (without additive) in test tubes (supplied by manufacturers) using a fresh plastic pipette for each cow. The Dipstick provided with the kit was placed into a test tube containing sample and left for 5min. Results were recorded based on the appearance of one or two lines. The appearance of two dark lines indicates low P4, hence the animal is either in-heat, anestrus or suffering from follicular cyst/s whereas, a single line indicates high P4, hence the animal is either pregnant, diestrus or suffering from luteal cyst/s.

5.3.5. Per Rectal Palpation in Crossbred Dairy Cattle

Immediately after the P4 level in the second sample was measured, P4 test results were combined with per rectal palpation findings, and results were interpreted. The reproductive tract and ovaries were examined using per rectal palpation to determine pregnancy status (presence of fetus, uterine asymmetry, membrane slip, uterine fluid, placentome, CL, size of reproductive tract), size of ovaries, and presence of any abnormalities or pathological condition such as ovarian cysts, non-functional ovaries, pyometra or other problems. On-farm P4 along with per rectal findings were recorded separately for each individual in a follow up format (Appendix 5).

5.3.6. Quantitative Laboratory P4 ELISA to Validate On-Farm P4 Test Results

The quantitative laboratory (in-house) ELISA has been described in Section 2.14. The P4 levels in whole milk were measured without extracting the steroids; however, steroids were extracted from serum as described in Section 2.11. The cross-reactivity tests were

carried out and the inter- and intra-assay coefficient of variance (CV) calculated as described in Sections 2.15 and 2.16, respectively.

Based on the level of P4 in serum, study animals were classified as normally cycling when they had P4 ≥1 ng/ml on either the first or the second sample collected at 11-day intervals; non-cycling (anestrus) if cattle (≥ 60 days postpartum cows or heifers over two years old) had P4 <1ng/ml in two tests 11-day apart; pregnant if cattle had insemination history of ≥18 days and had P4 ≥1ng/ml twice at 11-day intervals (Tamadon et al., 2011; Hommeida et al., 2005); repeaters (follicular cyst) when they had P4 <1 ng/ml accompanied by per rectal palpation finding of a follicle/s at least 2.5cm in diameter (Osawa et al., 1994; Braw-Tal et al., 2009); luteal cyst when P4 ≥1 ng/ml twice at 11 days interval accompanied by per rectal palpation finding of CL at least 2.5cm in diameter (Braw-Tal et al., 2009). All these laboratory findings were combined with breeding history and per rectal palpation to avoid diagnostic bias to compare with qualitative on-farm P4 ELISA tests.

5.3.7. Statistical Analysis

Data was analysed using two-tailed Fisher's exact test. Sensitivity, specificity, and predictive values were analysed (Medicalc® statistical software 2015), with a $p < 0.05$ considered significant.

5.4. RESULTS

5.4.1. Preliminary Assessment of Reproductive Status of HF Cows

P4 levels were qualitatively measured using Dipstick and Target P4 tests, and reproductive status of HF cows determined based on milk P4, either based on blue/white colour formation (Target P4) or based on the number of lines formed and the intensity of the test line (Dipstick). Reproductive status of cows that was first confirmed using ultrasound scanning was compared with Target P4 findings (Figure 5.1). Target P4 accurately determined pregnancy in 3 HF cows, with similar findings with ultrasonography. However, Target P4 indicated lower P4 (2.1-5ng/ml, light blue) (Figure 5.1 D, Left) in pregnant cow during first test, which became high in a test 11-day apart (Figure 5.1 D, Right).

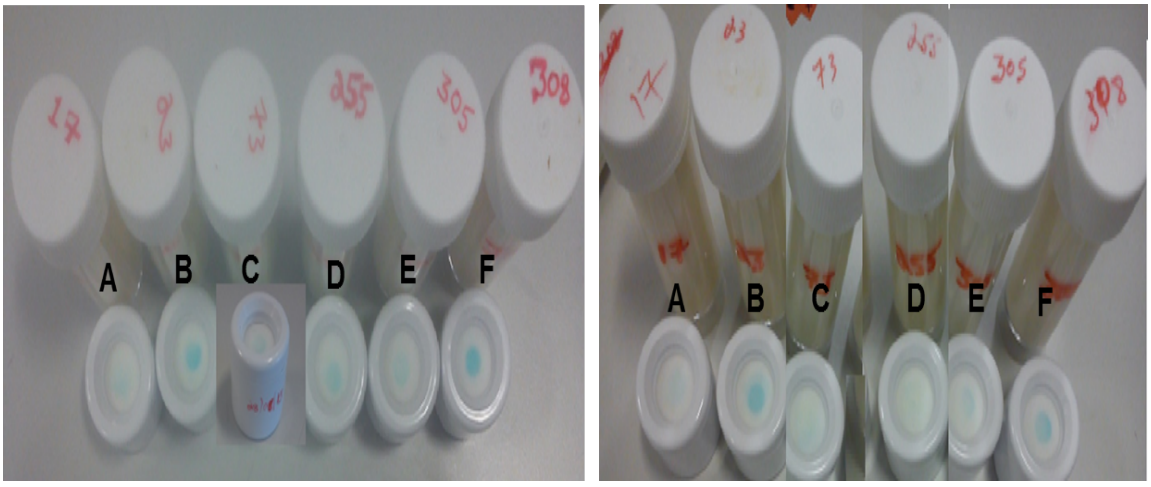

Figure 5.1: Target P4 test result in milk samples collected from HF cows in Langhill Dairy Farm, Edinburgh, UK twice at 11-day intervals.

1st test result (Left): cows A, D & E that were confirmed pregnant (42 days, 101 days and 191 days, respectively) using ultrasound scanning at first P4 test had moderate P4 levels (P4 level 2.1-5ng/ml, light blue). Cows that were at follicular Phase (B and F) had low milk P4 (bright blue, P4 <1ng/ml). A cow that had a follicular cyst (C), when checked with ultrasonography, had low P4 level (light blue colour, P4 < 1-2ng/ml) at first test. ***2nd test result (****11 days apart****) (Right):*** cows A, D & E had high P4 (no test colour/light, P4 >5ng/ml); cows (B and F) had low P4 (bright blue, P4 <1ng/ml); cow C (with follicular cyst based on scanning) had high P4 level (light colour, P >5ng/ml).

Similar to the ultrasonography, both Target P4 and Dipstick tests confirmed non-pregnancy in three non-pregnant HF cows. However, one cow that had a follicular cyst, confirmed by ultrasonography and had low P4 level during first P4 test, had high P4 (>5ng/ml) at the second test 11 days apart.

Assessment of reproductive status of HF cows was performed using Dipstick kits, and findings are indicated in Figure 5.2. Dipstick was also applied to analyse known concentrations of P4 (20, 10, 5, 2.5, 0.6125 ng/ml) prepared with charcoal-stripped milk, and samples with 20ng/ml, 10ng/ml and 5ng/ml showed a single line (high P4 level). The switch from two to one lines occures between 2.5ng/ml and 5ng/ml, whereby 2.5ng/ml showed two lines, with test line being light blue, whereas 0.6125ng/ml showed two dark lines indicating that the P4 level was low (Figure 5.3). The overall 1st and 2nd (11 days apart) test results of Target P4 and Dipstick, in reference with ultrasonography are summarised in Table 5.1.

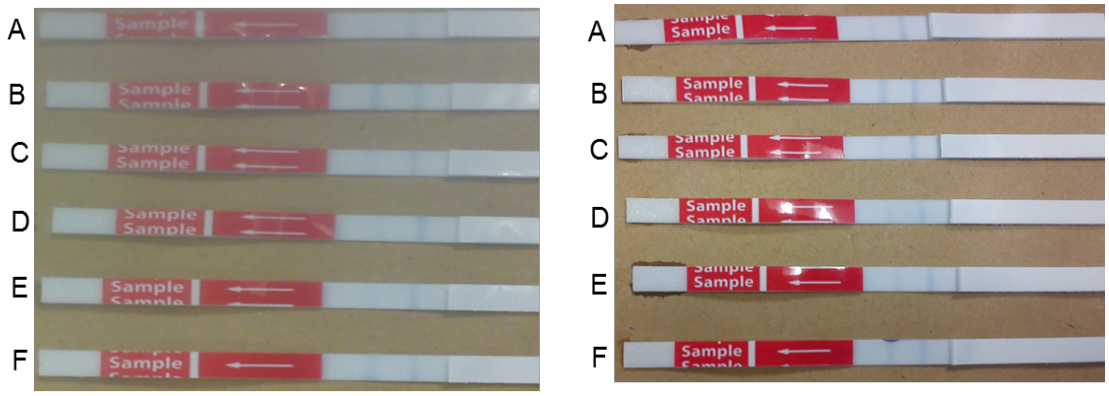

Figure 5.2: Findings with Dipstick method from milk collected first day (Left) and 11 days later (right) from HF cows in Langhill Dairy Farm, Edinburgh, UK.

At 1st day test (Left), results indicated only one line (test line white/light blue colour, high P4 concentration, ≥ 5ng/ml) were obtained in three cows that were pregnant, A, D & E (A, 3 month pregnancy; D, 6 month pregnancy; and, E, 2 months pregnancy); in two other cows that were in the follicular phase (B and F), and a cow having a follicular cyst (C), two test lines, with dark test line (low P4 <1ng/ml), were recorded. Eleven days later (Right), similarly high P4 (>5ng/ml) was determined in three pregnant cows (A, D & E). Two non-pregnant cows that were in follicular phase (B & F) had low P4 (two dark lines) the same as first test. However, a cow with follicular cyst confirmed using ultrasonography and low P4 during 1st test (C) had high P4 level (>5ng/ml) 11 days apart.

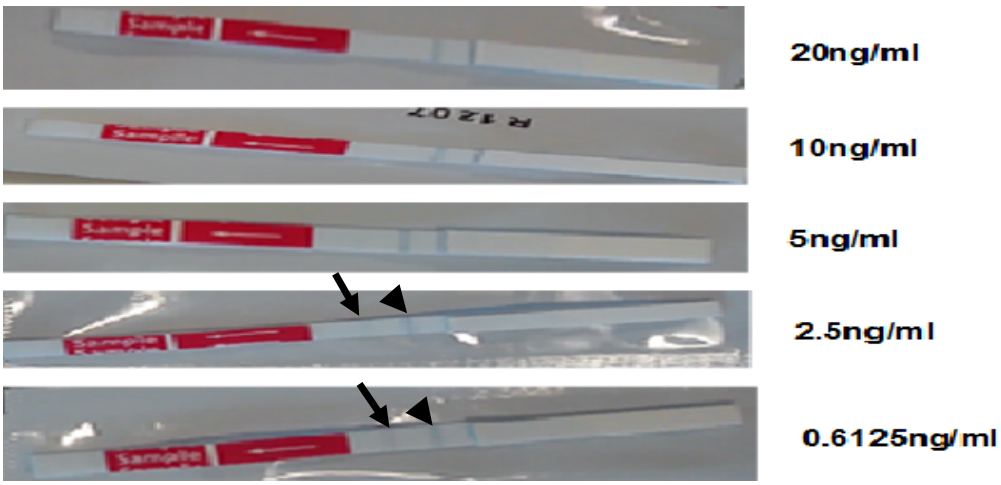

Figure 5.3: Photographs of Dipstick test results over a range of known concentration of P4 (ng/ml) prepared with charcoal-stripped milk standards; arrows indicate position of test line; arrowhead indicates reference line.

Table 5.1: Overall Dipstick vs Target P4 results in determining pregnancy in HF cows Edinburgh, UK.

Cow ID	Reproductive Status					Overall result
	Ultrasound scanning (1st day only)	Dipstick		Target P4		
		1st Day	11 days apart	1st Day	11 days apart	
17	Pregnant, 42 days	Pregnant (1 line)	Pregnant (1 line)	Pregnant (faint blue colour, P4 2.1-5ng/ml)	Pregnant (light colour, P4 >5ng/ml)	Pregnant
23	Not pregnant, on follicular phase	Not pregnant (dark test line, 2 lines)	Not pregnant (dark test line, 2 lines)	Not pregnant (bright blue, P< 1ng/ml)	Not pregnant (bright blue, P< 1ng/ml)	Not pregnant (Anestrus)
73	Follicular cyst (not pregnant)	Not pregnant (dark test line, 2 lines)	Cycling (luteal phase) (1 line)	Not pregnant (light blue, P4 1-2ng/ml)	Cycling (white colour, (P4 > 5ng/ml)	Not pregnant (Cycling)
255	Pregnant, 42 days	Pregnant, (light blue test line, two lines)	Pregnant, (light blue test line, two lines)	Pregnant, (Light blue, P4 1-2ng/ml)?	Pregnant, (white colour, P4 >5ng/ml)?	Pregnant
305	Pregnant, 42 days	Pregnant (1 line)	Pregnant, (light blue test line, two lines)	Pregnant (Light blue, P4 1-2ng/ml)?	Pregnant, (light colour, P4 >5ng/ml)?	Pregnant
308	Not pregnant, on follicular phase	Not pregnant (dark test line, 2 lines)	Not pregnant (dark test line, 2 lines)	Not pregnant (bright blue, P4 <1ng/ml)	Not pregnant (bright blue, P4 <1ng/ml)	Not pregnant (Anestrus)

5.4.2. Rapid On-farm P4 Test Findings in Crossbred Dairy Cattle

Breeding history and BCS were recorded prior to sample collection (Figure 5.4 Left) and blood was collected from jugular vein (Figure 5.4 Right). Reproductive status evaluated using qualitative on-farm P4 ELISA tests were compared and referenced with the findings of per rectal palpation conducted on the same day as second samples (Table 5.2). The P4 levels in milk (lactating cows, Figure 5.5) and in serum (dry cows or heifers) (Figure 5.6) were determined using rapid on-farm P4 tests and test results were recorded based on blue colour formation in Target P4 (Figure 5.5, top panel) or number of lines formed and intensity of the test line in Dipstick test (Figure 5.5, bottom panel). In some cases, when expected results were not obtained, tests were repeated.

Figure 5.4: Representative pictures showing recording breeding history of animals and BCS prior to sample collection (Left), and blood collection from jugular vein (Right).

Among 328 animals at the start of the study, 9 were excluded due to a variety reasons, such as mastitis, loss of BCS due to stress after first blood collection. Reproductive history of 319 animals (224 cows and 95 heifers) reported by the farmers as on-heat 10 (3.1%), anestrus 118 (37.0%), repeaters 20 (6.3%) and pregnant 171 (53.6%), was evaluated by qualitative field ELISA and per rectal palpation, and findings were, in-heat 10 (3.1%), anestrus 77 (24.2%), repeaters (follicular cyst) 9 (2.8%), normally cycling 69 (21.6%) and

pregnant 154 (48.3%) (Table 5.3). Significantly more ($p<0.05$) animals were reported as anestrus by farmers (118/319, 37%) compared to the number detected by field P4 ELISA (72/319, 22.6%) (Table 5.3).

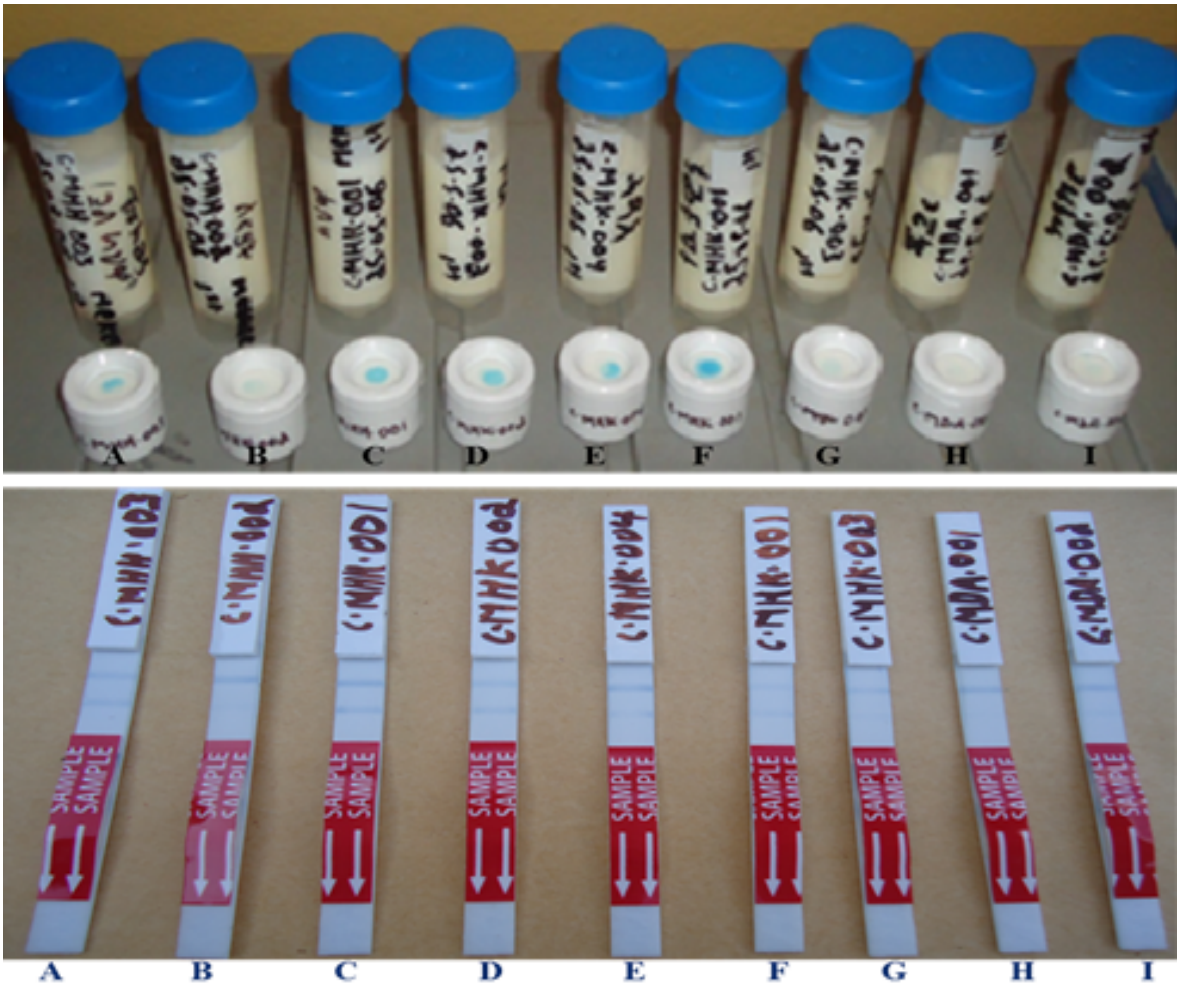

Figure 5.5: Representative photographs of on-farm milk P4 assays in crossbred cattle in Ethiopia.

Top panel, Target P4 in milk; white, high P4 level (pregnant/diestrus, B, G, H & I); blue, low P4 level (anestrus/in-heat, A, C, D, E & F), and bottom panel, Dipstick in milk; one line, high P4 level (B, G, H & I); two lines, low P4 level (A, C, D, E & F). Same samples were used for both tests.

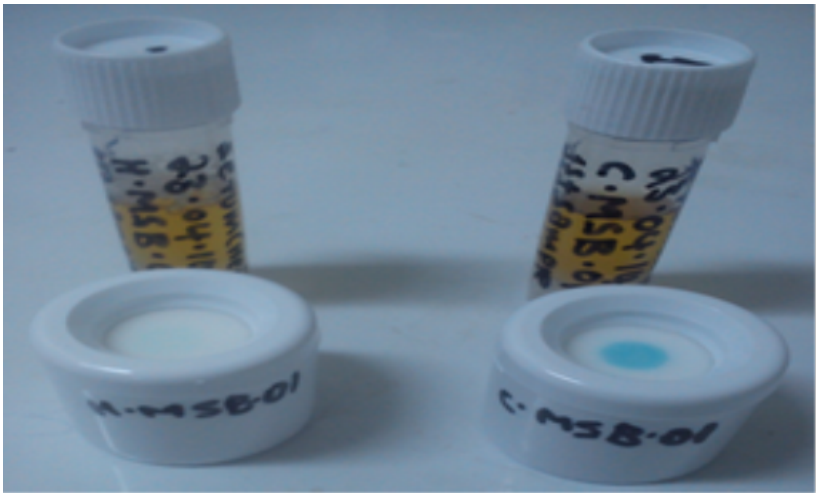

Figure 5.6: Representative photograph of serum P4 measured using Target P4 in crossbred dairy cattle; left, pregnant heifer with high P4 (white); right, anestrous cow with low P4 (blue).

Anestrus was the major reproductive problem recorded in the study area (Table 5.3). There was a statistically significant difference ($P< 0.05$) between reported anestrus (76/224, 33.9%) and clinical anestrus confirmed by P4 tests (47/224, 21%) in cows. Among 224 dairy cows which were grouped as in-heat 5 (2.2%), anestrus 76 (33.9%), repeater 14 (6.3%) and pregnant 129 (57.6%) according to their reproductive history (farmer's perception), true anestrus confirmed by P4 tests was 49 (21.9%). Out of 95 heifers which according to the farmer's perception (report) were in-heat 5 (5.3%), anestrus 42 (44.2%), repeater 6 (6.3%) and pregnant 42 (44.2%), true anestrus confirmed by P4 tests were 28 (29.5%). The overall true anestrus in cows and heifers was 77 (24.2%). Among 76 (33.9%) cows reported anestrus, P4 tests confirmed true anestrus, normally cycling and pregnant as 47 (61.8%), 26 (34.2%) and 3 (4.0%), respectively (Table 5.4). Similarly, among 42 (44.2%) heifers reported anestrus only 25 (59.5%) of them were confirmed true anestrus, whereas, the remaining were repeaters (follicular cyst) 1 (2.4%), normally cycling 13 (31.0%) and pregnant 3 (7.1%) (Table 5.5).

Figure 5.7: Per rectal examination of reproductive status of crossbred dairy cattle in Elshaday Dairy Farm, Wukro, Ethiopia.

Table 5.2: Interpretation of results obtained from P4 measurements in crossbred dairy cattle, in and around Mekelle, Tigray, Ethiopia.

1st P4 level	2nd P4 level	Rectal palpation findings	Result interpretation
Low	Low	Small ovaries without palpable structure	Non-functional ovaries (Anestrus)
High/Low	**Low**/High	Palpable structure present on the ovaries	Functional ovaries (Normally cycling)
Low	Low	Presence of follicles at least 2.5 cm in diameter.	Follicular cysts (Repeater)
High	High	Palpable CL, fetal membranes, uterine fluid, uterine asymmetry and fetus present	Pregnant
High	High	Palpable CL over 2.5 cm in diameter present and no history of insemination	Luteal cyst (s) (Anestrus)

Samples were collected twice at 11-day intervals (1st and 2nd) and instantly analysed at the field. Per rectal palpation was carried out immediately after second sample was collected.

Table 5.3: Overall reproductive status of crossbred dairy cattle (cows and heifers) reported by the farmers versus actual reproductive status according to P4 tests and rectal palpation.

Reproductive history		Reproductive status according to P4 tests and Rectal Palpation					
				Cycling			
Status	Number	In-heat	Anestrus	*Follicular cyst (Repeaters)	Normally Cycling	Pregnant	Total
In-heat	10	10 (100)	0	0	0	0	10 (100)
Anestrus	118	0	72 (61.0)	1 (0.9)	39 (33.0)	6 (5.1)	118 (100)
Repeater	20	0	1 (5)	7 (35)	11 (55)	1 (5)	20 (100)
Pregnant	171	0	4 (2.3)	1 (0.6)	19 (11.1)	147 (86)	171 (100)
Total	319	10 (3.1)	77 (24.2)	9 (2.8)	69 (21.6)	154 (48.3)	319 (100)

*Repeaters: animals with follicular cysts were considered as repeaters. Figures in parentheses are percentages. N=319.

Surprisingly, 5.1% (6/118) of reported anestrous animals (3 cows and 3 heifers) were confirmed pregnant. Furthermore, one cow that was reported by the farmer as anestrus was diagnosed by P4 tests and per rectal palpation to have a follicular cyst (Table 5.3 and 5.4). Per rectal palpation revealed that anestrous animals had small ovaries without palpable follicles or CL, small reproductive organs, or pyometra.

Reported history of pregnancy of crossbred dairy cattle was similar with combined P4 tests and per rectal palpation findings, whereby out of 171 animals reported by owners as pregnant, 147 (86%) were confirmed pregnant. The remaining reported pregnancy ended up as anestrus 4 (2.3%), follicular cyst (repeater) 1 (0.6%) and normally cycling 19 (11.1%) (Table 5.3). Among 129 cows reported pregnant as per farmers' perception, P4 tests were confirmed as 1.6% anestrus (2/129), 0.8% (1/129) repeaters (follicular cysts), 11.6% (15/129) normally cycling and 86.0% (111) pregnant (Table 5.3). Similarly, greater number of heifers were confirmed pregnant when diagnosed by per rectal palpation than P4 tests whereby among 42 heifers reported by owners as pregnant, 36 (85.71%) were confirmed actually pregnant by combined P4 tests and per rectal palpation. Among 36 clinical pregnancies in heifers, only 22 (66.1%) were confirmed pregnant by P4 tests alone, whereas all 36 (100%) were correctly diagnosed by per rectal palpation, providing

false negative pregnancies in 14 heifers. Similar pregnancy results were found when Target P4 and Dipstick were used. The Dipstick method did not work with serum samples, and no test result was obtained in two pregnant cows in third trimester, that was confirmed by per rectal examination (Figure 5.7).

Table 5.4: Reported reproductive status of crossbred dairy cows versus overall findings using Target P4 in milk and serum, Dipstick in milk and rectal palpation.

History (reported)		**Overall on-farm P4 ELISA and rectal palpation results**					
Reproductive status	Number of Cows	In-heat	Anestrus	*Repeaters	Normally cycling	Pregnant	Total
In-heat	5	5 (100)	0	0	-	0	5 (100)
Anestrus	76	0	47 (61.8)	0	26 (34.2)	3 (4.0)	76 (100)
Repeater	14	0	0	6 (42.9)	7 (50.0)	1 (7.1)	14 (100)
Pregnant	129	0	2 (1.6)	1 (0.8)	15 (11.6)	111 (86.0)	129 (100)
Total	224	5 (2.2)	49 (21.9)	7 (3.1)	48 (21.4)	115 (51.3)	240 (100)

*Repeaters: Animals with follicular cysts were considered as repeaters. Figures in parentheses are percentages. N=224.

Table 5.5: Overall reproductive status of crossbred dairy heifers using Target P4 test in serum and rectal palpation.

History (reported)		**Overall Target P4 ELISA and Rectal Palpation Results**					
Reproductive status	Number of heifers	In-heat	Anestrus	*Repeaters	Normally cycling	Pregnant	Total
In-heat	5 (5.3)	5 (100)	0	0	-	0	5 (100)
Anestrus	42 (44.2)	0	25 (59.5)	1 (2.4)	13 (31.0)	3 (7.1)	42 (100)
Repeater	6 (6.3)	0	1 (16.7)	1 (16.6)	4 (66.7)	0	6 (100)
Pregnant	42 (44.2)	0	2 (4.8)	0	4 (9.5)	36	42 (100)
Total	95 (100)	5 (5.2)	28 (29.5)	2 (2.1)	21 (27.4)	39 (41.0)	95 (100)

*Repeaters: animals with follicular cysts were considered as repeaters. Figures in parentheses are percentages. N=95.

5.4.3. Validation of Results Using Laboratory P4 ELISA

Field P4 test results were validated by quantitative laboratory P4 ELISA in serum. There was no difference in the number of animals recorded in each group between on-farm (qualitative) and quantitative laboratory P4 ELISA tests (Table 5.6). However, of 77 animals diagnosed anestrus by the on-farm P4 tests, 65 (84.4%) were confirmed true anestrus using quantitative laboratory P4 ELISA, which was not significantly different. Among 154 cattle diagnosed by qualitative on-farm P4 tests, quantitative laboratory P4 ELISA confirmed 152 (98.7%) of them as pregnant and the remaining 2 (1.3%) as normally cycling.

Table 5.6: Comparison between on-farm (qualitative) vs laboratory (quantitative) P4 ELISA findings.

Method	In-heat	Anestrus	*Repeaters	Normally cycling	Pregnant	Total
Reported reproductive history	10	118	20	-	171	319
On-farm P4 assays	10 (3.1)	77 (24.2)	9 (2.8)*	69 (21.6)	154 (48.3)	319 (100)
Laboratory P4 assay	10 (3.1)	65 (20.4)	6 (1.9)*	86 (27)	152 (47.7)	319 (100)

Figures in parentheses are percentages. *Repeaters: cattle with ovarian follicular cysts. Findings from on-farm and laboratory P4 assays were combined with per rectal palpation results.

Three false negative (low serum P4 <1 ng/ml) and no false positive pregnancies were recorded in laboratory P4 ELISA, while 18 false negative and 1 false positive pregnancies recorded by the field P4 ELISA tests. False negative pregnancy was significantly higher ($p < 0.0001$) in on-farm compared to laboratory P4 ELISA (Figure 5.8) which is due to pregnancy was missed in large number (14/36, 38.9%) of heifers. The three false negative pregnancies by laboratory P4 ELISA were recorded in animals that were in third trimester, which was confirmed by per rectal palpation. Among 18 false negative pregnancies (Figure 5.8), 14 (77.8%) were recorded in heifers at different stages of pregnancy when P4 was determined in serum, whereas three were in lactating cows in late trimester, and

one in a dry cow that was in first trimester of pregnancy. Per rectal palpation also missed pregnant cow in the third trimester. Furthermore, among 9 cattle confirmed positive for follicular cyst/s by qualitative ELISA along with per rectal palpation (size of the follicle/s), only 6 (66.7%) were confirmed positive by laboratory ELISA, whereas the remaining 3 (33.3%) were free of follicular cysts. Hence, of 20 animals with history of repeat-breeding, only 6 (30%) had follicular cysts (Table 5.3 and 5.6). The sensitivity of qualitative on-farm P4 ELISA (Target P4 /Dipstick) and quantitative laboratory P4 ELISA to diagnose pregnancy in cattle were 88.6 and 99.1%, respectively, and specificity was 99.4 and 100%, respectively. Positive and negative predictive values are indicated in Table 5.7.

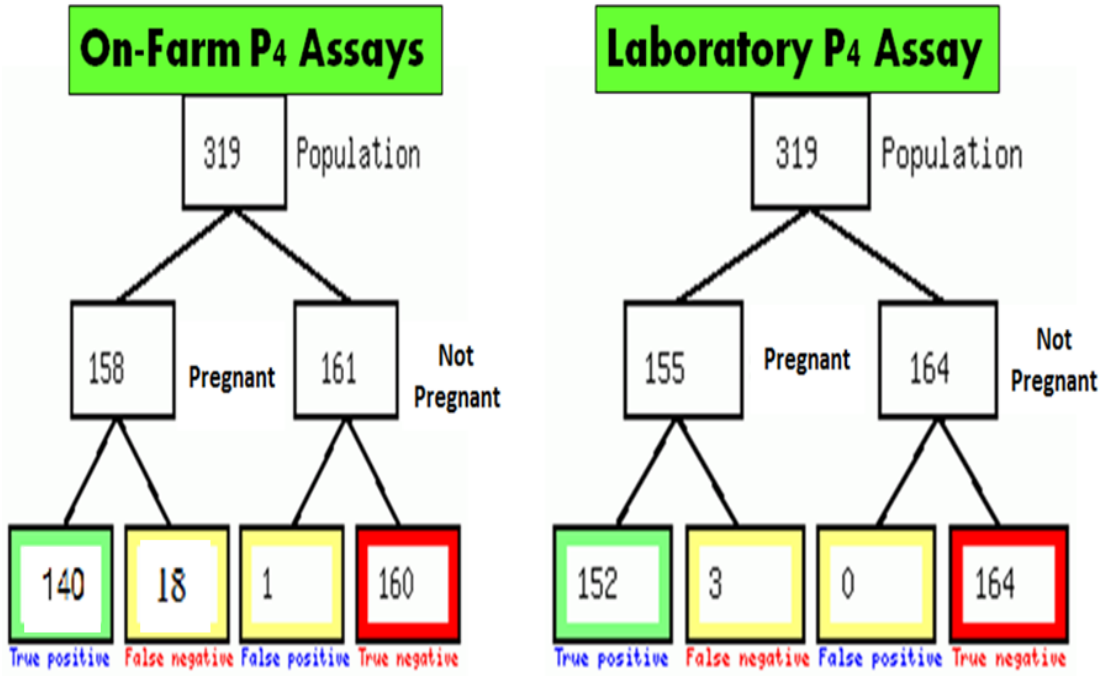

Figure 5.8: Diagrammatic expression of comparison of pregnancy diagnosis between qualitative on-farm and quantitative laboratory P4 assays.

$p = 0.0646$ for true positive; $p < 0.0001$ for false negative pregnancies between on-farm vs laboratory P4 tests; analysed using MedCalc®.

Table 5.7: Sensitivity, specificity, and predictive values of qualitative on-farm and quantitative laboratory P4 ELISA tests for pregnancy diagnosis in lactating crossbred dairy cattle.

Diagnostic methods	Sensitivity (%)	Specificity (%)	+PV (%)	-PV (%)
On-farm P4 ELISA (Target P4/Dipstick)	88.6	99.4	99.3	90
Laboratory P4 ELISA	98.1	100	100	98.2

+PV= Positive Predictive Value, -PV= Negative Predictive Value; analysed using MedCalc®; N=319.

5.5. DISCUSSION

Rapid on-farm P4 in HF cows

A preliminary study was carried out to qualitatively measure milk P4 in HF cows using two different rapid on-farm P4 kits (Target P4 and Dipstick) and assess reproductive status that was confirmed using ultrasonography. Both Target P4 and Dipstick tests accurately determined reproductive status of HF cows, with similar findings as ultrasonography in agreement with previous reports (Simersky et al., 2007; Osman et al., 2012). Target P4 indicated lower P4 (2.1-5ng/ml, light blue) in pregnant cows during first day test, whereas high P4 was detected in the second test 11 days later, which could be due to storage temperature or thawing problems. Target P4 results can be affected more than Dipstick by storage temperature, and unless proper storage and thawing is carried out, false results may be obtained. This gives the advantage of Dipstick over Target P4 in field conditions, where fridges and cooling boxes are not available.

Similar to the ultrasonography, both Target P4 and Dipstick tests confirmed non-pregnancy in three cows. However, one cow that had an ovarian follicular cyst when examined using ultrasonography immediately before the first P4 test and had a low P4 level during the first test, showed high P4 (>5ng/ml) at the second test 11 days later, which could be due to rupture of the follicular cyst by manual pressure during ultrasound scanning, concequently a CL might have developed, causing a rise in P4 level (Ijaz et al., 1987).

The P4 tests are capable of accurately determining pregnancy. This was the case for both Target P4 and Dipstick, which was similar with ultrasonography. This indicates that on-farm P4 tests can reliably replace ultrasound scanning. Both on-farm P4 kits showed similarly high accuracy in determining pregnancy and non-pregnancy. As qualitative on-farm P4 tests are not able to quantify the exact levels of P4 in milk or serum, determination of the concentration of P4 standards in charcoal-stripped milk using these on-farm tests enabled us to easily record and interpret test results in actual samples.

Rapid on-farm P4 in crossbred cattle

Qualitative on-farm P4 tests (Target P4 and Dipstick) were used to assess reproductive status of crossbred dairy cattle, which was combined with per rectal palpation findings. Results were validated using quantitative laboratory P4 ELISA tests in serum. The use of P4 assay for early pregnancy diagnosis, as well as determination of other reproductive status of cattle, for example cattle with normal ovarian cycle, in-heat, or non-functional ovaries (anestrus), is a common and essential tool for farmers/ veterinarians/ farm managers in making early decisions to cull or rebreed their cattle (Eddy and Clark, 1987; Gao et al., 1988; Dionysius, 1991; Lamming and Darwash, 1998; Kafi et al., 2012).

Both Target P4 and Dipstick tests are based on an ELISA test. However, it was noted that Dipsticks do not work in serum samples, but in milk, whereas Target P4 test can be used in both milk and serum samples, with more accurate test results in milk than in serum. Therefore, Dipstick is of no practical use to diagnose reproductive status of dry cows and heifers. Clearly, blood samples have to be collected from dry cows and heifers for serum separation by veterinary professionals, so the use of Dipstick will be limited to lactating cows if farmers themselves need to assess reproductive status of their animals. However, Dipstick has advantages with regard to storage conditions, as it can be stored at room temperature compared to Target P4 that requires a cooling box or refrigerator for storage. Furthermore, the time required for Dipstick (5 min) is three times less than for Target P4, relatively cheaper and procedures are comparatively simple. For this reason, most farmers were eager to conduct the test themselves. This was in agreement with a recent report on the reliability of the test performed on 63 cows (Järvinen et al., 2014). Despite these minor differences, farmers, farm attendants, farm managers, veterinary professionals and AI technicians expressed great interest in the tests. The accuracy of the two P4 tests in diagnosing anestrus, repeat-breeding (follicular cyst, assisted by per rectal palpation on detecting the size of follicle) and pregnancy was similar. The P4 tests were found to be much better than per rectal palpation in diagnosing cyclicity of (normally cycling) animals. These methods could potentially assist or replace per rectal palpation, as the established reliable method of diagnosing reproductive status (Lobago, 2007; Tefera,

2011). As these rapid on-farm P4 tests are very simple, livestock owners could conduct the test using milk samples without any professional involvement.

In our recent survey (Chapter 3), it has been shown that cows in the study area have long postpartum anestrus (114.5 days) and calving interval (35.3 months), and heifers were older at first service and first calving. This supports previous Ethiopian studies (Negussie et al., 1998; Mureda and Mekuriaw, 2007; Nuraddis et al., 2011; Dinka, 2012; Kumar and Tkui, 2014). In the present study we assessed reproductive status of cattle using qualitative on-farm P4 tests, and animals having low P4 level in milk (lactating cows) or serum (dry cows and heifers), determined twice at 11-day intervals, were considered as having non-functional ovaries, hence considered anestrus. Findings were similar to previous studies (Dionysius, 1991). In contrast, animals having high P4 levels in the first test and low in the second tests, accompanied by the presence of CL/follicle, were considered as normally cycling. These findings are in agreement with previous reports using plasma P4 tests (Zduńczyk et al., 2002). The incidence of true anestrus was comparatively lower than previous studies (Fischer et al., 1998). Per rectal palpation revealed that, anestrous animals had small ovaries without palpable follicles or CL, and small reproductive tract, similar to a previous report (Roche et al., 1992), and pyometra.

When cows/heifers are in-heat, P4 concentration is very low, and gradually increases following ovulation. If the level of P4 in milk or serum is > 1ng/ml at the time of insemination there is high chance cattle will not conceive (Ambrose and Colazo, 2007). The profitability of dairy farming depends on accurate heat detection and timely breeding. Currently, detection of heat in Ethiopian dairy farming systems is not satisfactory (Belihu, 2002). This is due to visual observation being the only means of heat detection. In the present study, a large percentage of cattle reported by the farmers as anestrus were confirmed normally cycling, as evidenced by the qualitative rapid on-farm P4 tests. Reported anestrus in cows was significantly higher ($p < 0.05$) than true anestrus confirmed by on-farm P4 tests. The remaining cows reported anestrus were normally cycling, which could be due to silent estrus (Gundling et al., 2012) or poor estrus detection. Previous

studies have shown more estrus detection errors in dairy farms with larger dairy herd size compared to farms with smaller herd size (Kafi et al., 2007).

The efficiency of on-farm P4 tests on identifying cattle in-heat was similar to that of quantitative laboratory P4 ELISA tests. Cattle presented with signs of estrus were all confirmed by both on-farm and laboratory P4 ELISA tests. However, this does not necessarily mean that the efficiency of farmers in detecting estrus is 100%, because 33% of animals that were reported as non-cycling (anestrus) were confirmed by on-farm P4 tests as normally cycling, showing poor estrus detection. Estrus detection using P4 assay in the present study agrees with previous reports (Rajamahendran et al., 1990; Ambrose and Colazo, 2007). True estrus detection is necessary for successful AI and conception. In the present study, since there were few animals in estrus, the likelihood of estrus detection failure was low. Larger scale studies of estrus detection efficiency by farmers using rapid on-farm P4 tests are required. Successful widespread introduction of this technology could have a significant impact on conception rate.

Incidence of repeat-breeding in crossbred dairy cattle in the study area was high. One of the major reproductive problems affecting fertility of crossbred dairy cattle in Ethiopia is repeat-breeding (Fischer et al., 1998; Gizaw et al., 2007; Dinka, 2012; Mandefro and Negash, 2014; Benti and Zewdie, 2014; Mekonnin et al., 2015b). Repeat-breeding is generally defined as a cow or heifer repeatedly returning to estrus after 3 or more services, while having normal duration of estrous cycles (17-25 days) associated with true estrus and without anatomical abnormalities or infections (Zemjanis, 1980). Incidence of repeat-breeding in the present study were in agreement with previous reports (Fischer et al., 1998; Mandefro and Negash, 2014). However, incidences of repeat-breeding in the present study were higher than studies in crossbred cattle in other parts of Ethiopia (Bitew and Prasad, 2011) and in Bangladesh (Al-Maruf et al., 2014). Conversely, this study showed lower repeat-breeding incidence compared with 10.3% in indigenous Borana breed cows, in Borena Zone, Ethiopia (Benti and Zewdie, 2014), 15% in crossbred dairy cows in East Showa, Ethiopia (Esheti and Moges, 2014) and 63% in Goa, India (Chakurkar et al., 2008).

Of 20 animals with a history of repeat-breeding, 7 (35%) were diagnosed by the on-farm P4 tests along with per rectal palpation to have follicular cysts. Higher incidence of repeat-breeding in the present study could also be due to factors such as genetic, negative energy balance, retained placenta, metritis/pyometra, infectious diseases, early embryonic mortality, poor semen quality, improper insemination and timing of insemination (Pathiraja et al., 1988; Rekwot et al., 1999; Sreenan et al., 2001; Mondal, 2015). Unlike our finding whereby less than half of reported repeat-breeding cases were caused by follicular cysts, previous reports have shown that repeat-breeding was caused entirely by follicular cysts (Friggens and Chagunda, 2005). Ovarian follicular cysts significantly affect reproduction in dairy cattle, resulting in significant economic loss (Calder et al., 1999). Animals with follicular cysts can be treated with either P4 (Calder et al., 1999) or GnRH (Osawa et al., 1994).

Identifying pregnancy status of dairy cattle as early as possible is very important to reduce the calving interval, and enable farmers to rapidly identify non-pregnant cows/heifers and rebreed or treat them. However, one of the challenges in the present study of assessing pregnancy status of animals was that few farmers assumed non-return to estrus as an absolute indicator of pregnancy. Consequently, these farmers were reluctant to use pregnancy diagnosis. Although, return to estrus from 18 to 24 days post service is usually considered by the farmers the easiest and cheapest method for determining non-pregnancy (Fricke, 2010), a previous study has shown that estrus detection efficiency is estimated to be less than 50% on most dairy farms (Senger, 1994). Fortunately, the majority (98.7%) of animals in the present study reported as pregnant due to non-return to estrus or previously diagnosed per rectum by AI technicians post-service were confirmed by on-farm P4 tests as pregnant. However, few animals that were assumed pregnant and kept in a proper feeding regime were diagnosed as non-pregnant. As most farms in the study area have only 1-5 head of cattle, the loss from keeping non-pregnant animals and assuming they are pregnant should be considered as very significant when the small holder farmer pays for extra feed, extended calving interval, loss of milk, loss of calf crop, salary for farm attendant/s, and most importantly loss of his/her income for living.

The reproductive history and actual reproductive status of animals determined by on-farm P4 tests were similar for pregnancy. Findings were in agreement with previous reports (Rajamahendran et al., 1993). Diagnosing pregnancy in cows 18 days post service was similar to reports that used on-farm determination of P4 for pregnancy detection, although longer than that required when using a microwell strip reader (Simersky et al., 2007). False negative pregnancy could be due to large numbers of animals in the third trimester, and this stage is a time when P4 level could be low in milk as well as serum. Furthermore, less sensitivity of the on-farm test to determine low level of P4 in serum than milk reflected the higher percentage of false negative pregnancy in heifers than cows. Consequently, P4 measurement in milk and serum, using both quantitative and qualitative methods was low leading to false negative pregnancy diagnosis. The accuracy of on-farm P4 assays used in the present study (88.6%) in determining pregnancy is comparable with previous reports (Ropstad and Refsdal, 1985; Kaker et al., 1993; Srilatha et al., 2016). In contrast, the accuracy in the present findings is better than other reports (Stevenson and Call, 1986; Osman et al, 2012). On the other hand, the present finding had lower accuracy of determining pregnancy than a previous report (Rajamahendran et al., 1990). The accuracy of on-farm P4 assays used in this study in determining non-pregnancy (99.4%) is in agreement with previous and recent reports (Ropstad and Refsdal, 1985; Elmore, 1986; Takeuchi et al, 1987; Srilatha et al., 2016). In contrast, the present finding has shown better accuracy of on-farm P4 kits in determining non-pregnancy than previous reports (Wimpy et al., 1986; Nebel et al., 1987; Kaker et al., 1993; Osman et al, 2012).

The similarity between on-farm and laboratory P4 ELISA in accurately diagnosing pregnancy would make on-farm P4 tests preferable due to cost-effectiveness and simple test procedures. Rapid on-farm P4 assays are affordable means of determining reproductive status. Hailu et al. (2015) previously reported that an estimated loss of production cost of US $148.48 per cycle from single cow for extended periods due to reproductive problems, such as anestrus and repeat-breeding. The poor estrus detection in the study area, which is proven in the present study when large number of cattle reported by the farmers as anestrus, but confirmed by P4 tests as cycling, can be improved using

on-farm P4 kits used in the present study. In the present study, the cost of Dipstick was £37.5 (converts to US $47.07) per 25 tests; and, the cost of Target P4 was £45 (converts to US $56.49) per 20 tests. Considering the loss when a cow is not inseminated at the optimal voluntary waiting period postpartum in the study area (Hailu et al., 2015), on-farm P4 is highly beneficial to accurately determine reproductive cyclicity and early pregnancy diagnosis, as early as 18 days post insemination. Laboratory P4 ELISA kits, that uses a 96-wells plate, costs approximately US $225. It also requires laboratory equipment, such as plate readers, centrifuges, plate shakers, which are unaffordable, even by organised dairy farms in the region and other part of the country. Furthermore, unlike laboratory P4 ELISA where 96-wells plate was used for determination of reproductive status in cows on large sample size, on-farm P4 assays can be performed to determine reproductive status of even one cow in a smallholder farm, without wasting of the kit. It is not feasible to use laboratory P4 ELISA in individual smallholder farms owning 1 to 5 cows in the country.

Breeding management was poor in some farms in the study area to the extent that pregnant animals were not recorded, but reported anestrus. Furthermore, a cow confirmed by on-farm P4 tests and per rectal palpation as having a follicular cyst was reported by the farmers as pregnant. This animal must have repeatedly come into estrus (Mondal, 2015), however never detected in estrus.

Determining reproductive status using milk or serum P4 tests could be a very important tool for breeding management. Accurately detecting the P4 level could also reduce the economic loss from abortion caused by luteal regression following administration of PGF2α to pregnant cows in error, during estrus synchronisation. Therefore, caution should be taken when administering PGF2α to animals recently inseminated and having high milk/serum P4. Earlier detection of reproductive status enables earlier provision of proper breeding management and health care intervention in the farm. Assuring proper breeding management significantly influence overall fertility and resumption of ovarian activity, consequently increasing the income from dairy production (Opsomer et al., 1996).

Where pregnancy diagnosis and/or assessment of reproductive status is carried out solely using per rectal palpation, the method is not as efficient as required due to inefficiency of AI technicians, the time to carry out the examination and the possible side effects (Lobago, 2007). It has been reported that some veterinary students had no training in per rectal palpation practices (Tefera, 2011), so an alternative method of examination is crucial to enhance the reproductive performance of dairy cattle. The present study showed that the use of Target P4 and Dipstick methods can reliably assess reproductive status of crossbred dairy cows and heifers, and their use could be very important in providing simple, accurate, cost effective monitoring for the Ethiopian farming community.

In conclusion, insight into the reproductive history of animals and their actual reproductive status, the breeding management in the study area was not satisfactory. The accuracy of on-farm P4 tests in determining pregnancy and non-pregnancy was high. Target P4 and Dipstick are equally effective methods to assess reproductive status. Farmers, AI technicians and farm managers are enthusiastic about the technology. Therefore, application of Target P4 or Dipstick is recommended to veterinarians, AI technicians and, more importantly, low income smallholder farmers, to effectively monitor the reproductive status of animals.

CHAPTER 6: MANIPULATION OF FERTILITY IN CROSSBRED DAIRY CATTLE USING A CONTROLLED INTERNAL DRUG RELEASE DEVICE COMBINED WITH PROSTAGLANDIN AND EQUINE CHORIONIC GONADOTROPHIN

Abstract:

Simplifying and improving our understanding of the protocols for inducing or synchronising estrus is important for improving management of dairy cattle. This study evaluated the estrus response and conception rate of crossbred (HF X Zebu) dairy cows (n=75) and heifers (n=47) assigned to a 10-day CIDR device - PGF2α - eCG based estrus synchronisation protocol. Animals were assigned to three groups (a) anestrus (n=62), (b) repeat-breeders (n=11) and (c) silent estrus (n=46), according to their reproductive history, per rectal palpation, and progesterone concentrations determined by on-farm ELISA in milk (lactating cows) or serum (dry cows and heifers). For each animal, a CIDR device (containing 1.38g progesterone) was inserted into the vagina and allowed to remain in-situ for 10 days. On Day 8 after device insertion, a dose of 500µg of PGF2α was administered intramuscularly (IM). On Day 10 (device removal), 500 IU of eCG was given IM. Animals were inseminated or mated 48 and 72 hr post device removal. Pregnancy was assessed 20-24 days post insemination by measurement of progesterone in milk/serum and pregnancy was confirmed by per rectal palpation and/or ultrasonography 44-90 days post insemination. Overall estrus response and conception rates were 97.5% and 78.3%, respectively, with no significant differences in parity, pre-treatment reproductive status and farming system (smallholder vs organised commercial farms). Conception rate after induced/synchronised estrus was 82.9% and 72.1% in cows and heifers, respectively, with an overall calving rate of 94.4%. In conclusion, the present study has shown high estrus response and conception rate in crossbred dairy cattle using this CIDR-PGF2α-eCG based estrus synchronisation protocol. Application of this protocol is highly recommended to enhance fertility of dairy cattle in the study area, and this could readily be applied to other regions.

Key words: Cattle, CIDR, Conception Rate, Estrus, On-farm ELISA, Synchronisation.

6.1. INTRODUCTION

Ethiopia possesses the tenth largest cattle population in the world, and the largest in Africa. Despite this, productivity and reproductive performance of indigenous and crossbred (HF X Zebu) cattle is poor (Kumar and Tkui, 2014; Kumar et al., 2014). Efficient reproduction underpins a profitable dairy industry (Dickson et al., 2012) and producing one calf per year per cow is an important step in achieving this. Cows must get pregnant and maintain the pregnancy with parturition 270 days post service/AI, and wait for a period of 40-50 days (voluntary waiting period) to be successfully inseminated again (Båge, 2002; Dubuc, 2011; Perez-Marin et al., 2012). This results in one calf per year per cow with regular availability and increased milk production in the dairy farm, in addition, the provision of replacement heifers. However, there are a number of factors affecting reproduction in dairy cattle, particularly in the tropics, where reproductive performances as well as milk production of local (zebu) and crossbred, including HF in the tropics is regarded as poor. The main reasons are poor nutritional status, estrus detection, AI technique or breeding management and postpartum uterine disease and calf suckling (reviewed in Abdel-Rahman and Alemam, 2008; Dubuc, 2011; Tschopp et al., 2014). Anestrus and repeat-breeding are the major problems affecting the reproductive performance of crossbred dairy cattle in Ethiopia (Mandefro and Negash, 2014; Mekonnin et al., 2015b). Both anestrus and repeat breeding lead to a longer calving interval. In smallholder farms, most cows including crossbreeds, fail to calve every 12 to 13 months after the first calving (Shiferaw et al., 2003; Mekonnin et al., 2015b). Puberty in crossbred (Zebu x HF) heifers is usually 24 months (Duguma et al., 2012), and heifers are reported to be older at first calving (Kumar and Tkui, 2014; Kumar et al., 2014, Mekonnin et al., 2015b).

A successful breeding programme must incorporate efficient and accurate detection of estrus, proper semen handling techniques, and timely AI (Walker et al., 1996). Failure of proper estrus detection is the most common problem in dairy cattle breeding programme (Walker et al., 1996; Hansar et al., 2014) resulting in loss of lifetime milk yield, a decrease in number of calves born per lifetime, excessive days open, and an increase in reproductive

culling (Walker et al., 1996). As the accuracy and efficiency of estrus detection declines, it is important to incorporate estrus synchronisation/induction and TAI into the breeding management programme (Nebel and DeJarnette, 2015). Estrus synchronisation can avoid or reduce the need for estrus detection and increases the fertility and productivity of cattle (Larson et al., 2006). Furthermore, estrus synchronisation facilitates planning of AI, shortens first day service in postpartum cows, reduces the calving interval and helps to maintain uniform calf crops and replacement heifers (Larson et al., 2006; Lamb et al., 2010).

Current worldwide research is focusing on the development of methods that effectively synchronise estrus by decreasing the period of time over which estrus is detected, hence facilitating the use of fixed time AI (Patterson et al., 2011). Progesterone (P4)-based estrus synchronisation protocols are more advantageous than others. The CIDR-based estrus synchronisation protocols have been widely studied (Macmillan and Peterson, 1993; Williams et al., 2011; Siregar et al., 2015), and are more preferable than using prostaglandin (PGF2α) alone, due to ineffectiveness of PGF2α to induce cyclicity/estrus in animals that do not have CL, nor does it exert an effect on follicular wave (DeJarnette, 2015). Most successful estrus synchronisation protocols have been developed in *Bos taurus* cattle, however in *Bos indicus* breeds, utilisation of the same synchronisation protocols results in low to unacceptably low pregnancy rates (Williams et al., 2011; Yelich and Bridges, 2012).

It has been demonstrated that CIDR can be maintained in the vagina for a longer period of 14 days (Powell et al., 2012), however, intravaginal devices maintained over 12 days reduces fertility by causing aged follicles (Maurer et al., 1975; Macmillan and Peterson, 1993; Gordon, 1999). Promising developments have been shown by reducing the time of keeping CIDR devices in the vagina to 4 (Palomares et al., 2015) or 5 days (Bridges et al., 2008; Williams et al., 2011; Powell et al., 2012), although estrus response and conception rates are not consistent even after the use of the standard 7-day CIDR regime (Broadbent et al., 1993; Kim et al., 2007; Dickson et al., 2012) which could be due to environmental, management, breed and other factors. A recent report showed a similar pregnancy success

when CIDR was maintained for 5 days, 6 days and 14 days in heifers (Bridges et al., 2014). Moreover, another recent study that compared between 7 and 9 days CIDR inserts on estrus response and fertility in dairy cattle has shown a similar effect between the two protocols (Romano and Fahning, 2013). Others have suggested that keeping intravaginal devices for a period of 10 to 12 days has no negative effect on fertility and recommended for use in controlled breeding programme in cattle (Maurer et al., 1975; Broadbent et al., 1993; Gordon, 1999).

In view of the poor estrus detection and reproductive performance of crossbred dairy cattle in Ethiopia (Alemayehu and Moges, 2014; Kumar and Tkui, 2014; Kumar et al., 2014; Hailu et al., 2015; Mekonnin et al., 2015a; Mekonnin et al., 2015b), it is necessary to establish an estrus synchronisation programme that can work at any reproductive condition (anestrus and cycling) as a management tool to enhance their fertility and productivity.

6.2. SPECIFIC AIMS

The objective of the present study was to evaluate the reproductive performance of crossbred dairy cattle following treatment with a 10-day CIDR in combination with PGF2α and eCG.

6.3. MATERIALS AND EXPERIMENTAL DESIGNS

6.3.1. Study Area

The proposal was presented to key stakeholders and livestock experts and approved by The Government of National State of Tigray Bureau of Agriculture and Rural Development, Tigray, Ethiopia. It was conducted from November 2013 to December 2014 in and around Mekelle, the largest city in northern Ethiopia, and the capital city of Tigray, which is located at 39° 29`E and 13° 30` N at an altitude of 2000 m.a.s.l. The climate of the study area conforms to that of Ethiopian Highlands. The mean annual rainfall is 619mm, and is bimodal with a short rainy season occurring from March to May and another from the middle of September to February. The annual minimum and maximum temperature is 11.8°C and 29.9°C, respectively (BoPED, 2011).

6.3.2. Study Animals

Crossbred dairy cattle owned by smallholder farms and organised commercial farms were included in this study. Cattle in both farm types were fed grass hay or straw, concentrate composed of wheat by products, crop residues and local beer residue (Attela). However, cows in organised commercial farms were supplemented with green fodder, such as fresh grass, alfalfa, elephant grass, leucaena and sesbania, they received regular watering; and, the farms had better breeding records and housing conditions. A total of 122 dairy cattle (75 cows with a mean age of 6.6 years and >60 days postpartum and 47 heifers with a mean age of 3.1 years) from smallholder farmers and organised commercial farms, with an average BCS of 3.1 (on a scale of 1-5) (DEFRA, 2001) were assigned in the study. Reproductive organs of the study animals were assessed for genital tract pathology using per rectal palpation, and only apparently health animals were included in the study. Study animals were assigned into three groups prior to treatment as (a) anestrus (non-cycling, n=67), (b) repeat-breeders (normally cycling and observed estrus, but never conceived despite multiple inseminations, n=11), and (c) silent estrus (cycling but no visible estrus signs, n=44) according to their reproductive history, two equivalent on-farm P4 assays,

Dipstick (P4 Rapid, Ridgeway Science Ltd, Gloucestershire, UK) in milk (cows) or Target P4 ELISA (Biometallics Inc, New Jersey, USA) in serum (heifers and dry cows) and per rectal palpation. Progesterone assays were carried out twice at 11-day intervals immediately after breeding history was recorded, which was immediately followed by per rectal examination of ovarian and uterine conditions (Mekonnin et al., 2015a).

6.3.3. Study Design

The treatment regime is schematically illustrated in Figure 6.1. The perineal region of each animal was thoroughly washed with water, an Eazi-Breed CIDR™ (CIDR®, Pfizer Ltd, Kent, UK) device, containing 1.38g of progesterone, was inserted into the vagina and allowed to remain in-situ for 10 days. On the 8th day after device insertion, a dose of 500μg PGF2α (Estrumate, Schering-Plough Animal Health Corp, Summit, Germany) was administered intramuscularly (IM). On the day of device removal (Day 10), 500 IU eCG (Intervet UK Ltd, Walton, UK) was injected IM. The estrus response was compared according to parity (cows vs heifers), pre-treatment group (anestrus, repeat-breeders and silent estrus) and farming systems (smallholder vs organised commercial farms). Animals were inseminated using frozen semen or naturally mated twice at 48 and 72 hr post device removal. Animals were examined for the presence of pregnancy 20-24 days (average of 21 days) post insemination/mating using either on-farm milk progesterone test (using Dipstick in lactating cows), or using on-farm serum progesterone test (using Target progesterone kits in dry cows and heifers). Pregnancy was confirmed by per rectal palpation/ ultrasonography (KX5200V, Kaixin®, Xuzhou Kaixin Electronic Instrument Co., Ltd, Xuzhou, Jiangsu, China) 44-90 days post insemination/mating. Conception rate was compared according to the variables above and between breeding methods (AI vs natural mating).

6.3.4. Statistical Analysis

Data were analysed using Fisher's exact test and Chi-square test using GraphPad Prism (GraphPad Software, Inc, CA, USA). Differences were considered significant when $p < 0.05$.

6.4. RESULTS

The overall estrus response and conception rate in the present study was 97.5% and 78.3%, respectively. There was a similar estrus response between cows and heifers (Table 6.1). Although, the estrus response of cattle to CIDR - PGF2α - eCG treatment, based on observed estrous behaviour recorded by the farmers, was 97.5%, the remaining animals 3/122 (2.5%) that did not exhibit external symptoms, were deemed fit for insemination as their cervices were open, as evidenced by the AI gun easily passing through to deposit semen in the uterine body. This brought the estrus response to 100%. Subsequently, three (2.5%) animals (two cows and one heifer) did not retain their CIDR. Of 119 animals in estrus, 115 (96.6%) were inseminated or naturally mated, however four (3.4%) were not bred due to various reasons including animals being unable to be inseminated or mated.

Table 6.1: Estrus and conception status of cows and heifers treated with CIDR-PGF2α-eCG combination synchronisation protocol.

Observations	Treated animals		
	Total*	Cows*	Heifers*
No. treated	122 (100)	75 (100)	47 (100)
No. eliminated as dropped CIDR	3 (2.5)	2 (2.7)	1 (2.1)
No. in estrus	119 (97.5)	73 (97.3)	46 (97.9)
Missed insemination/mating	4 (3.4)	1 (1.4)	3 (6.5)
Number mated/ inseminated	115 (96.6)	72 (98.6)	43 (93.5)
No. conceived at induced estrus	90 (78.3)	59 (82.9)	31 (72.1)
No. calved	85 (94.4)	56 (94.9)	29 (93.5)

*Values in parentheses are percentages.

The conception rate in cows and heifers was 82.9% and 72.1% respectively, which was not significantly different ($p > 0.05$) (Table 6.1). Similar conception rates were found between anestrus, repeat-breeders and normally cycling-but silent estrus/unobserved by the farmers (Table 6.2). Natural mating resulted in a higher conception rate than AI, but

the difference was not significant (Table 6.3). The farming system did not affect estrus response or conception rate (Table 6.4).

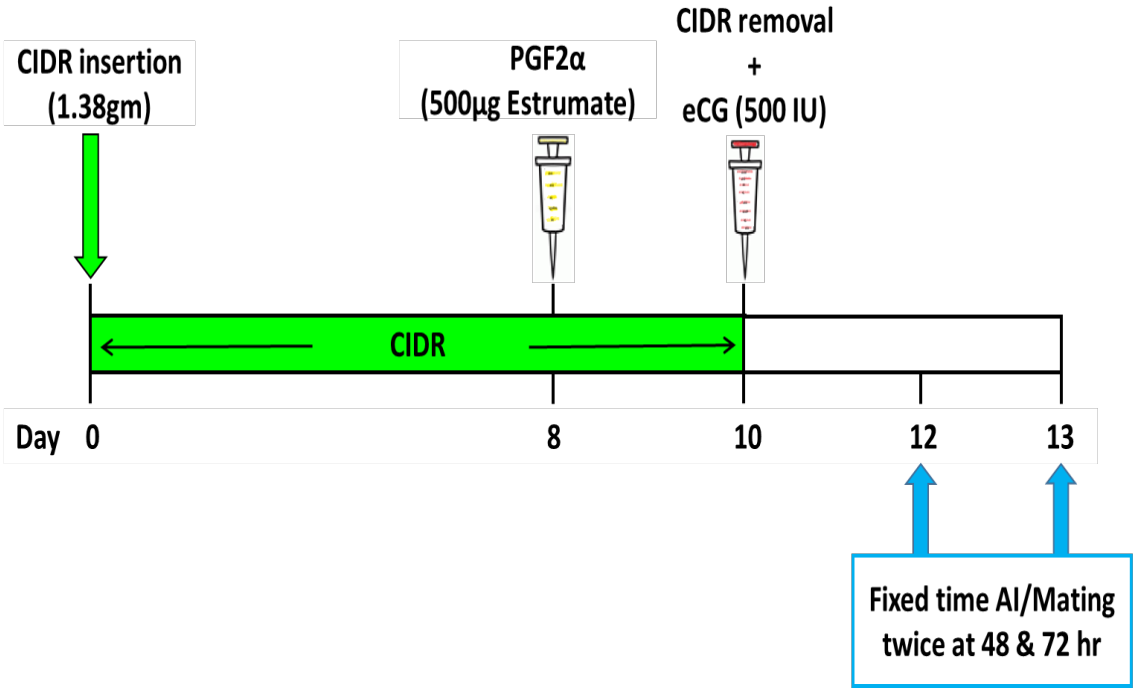

Figure 6.1: Schematic representation of treatment regime.

Two cows and one heifer gave birth to twin calves, while the remaining deliveries were singletons. Of 90 pregnant cows/heifers 85 (94.4%) successfully calved, and there was no effect of parity (Table 6.1), pre-treatment reproductive status (Table 6.2), breeding method (Table 6.3) or farming system (Table 6.4). Pregnancy loss occurred in 4/90 (4.4%) animals: three cows (3/59, 5.1%) and one heifer (1/31, 3.2%). These losses occurred in one cow in a smallholder farm with the remaining losses of two cows and one heifer in organised commercial farms where animals were bred by AI. There was also death of a pregnant cow (1/90, 1.1%) in one of the organised commercial farms due to lumpy skin disease.

Table 6.2: Fertility status of cows and heifers treated with CIDR-PGF2α-eCG protocol, on the basis of pre-treatment reproductive history and status.

Observation	**Total***	**Anestrus***	**Repeat-breeders* (Observed estrus)**	**Silent estrus* (unobserved estrus)**
No. of animals mated/ inseminated	115	62 (53.9)	11 (9.6)	42 (36.5)
Conception rate (% pregnant/AI or mated)	90 (78.3)	48 (77.4)	8 (72.7)	34 (81.0)
No. calved	85 (94.4)	44 (91.7)	7 (87.5)	34 (100)

*Values in parentheses are percentages.

Table 6.3: Fertility status of cows and heifers treated with CIDR-PGF2α-eCG protocol, on the basis of farming system (scale).

Observations	Total	Smallholder farm	Organised farm
No. treated	122	34	88
No. eliminated	7	1	6
No. inseminated	115	33	82
No. pregnant	90 (78.3)	24 (72.7)	66 (80.5)
No. calved	85 (94.4)	22 (91.7)	63 (95.4)

Table 6.4: Fertility status of cows and heifers treated with CIDR-PGF2α-eCG protocol, on the basis of breeding methods.

Observation		**Breeding Method**	
	Total*	**AI***	**Natural mating/bull***
No. of animals mated/inseminated	115	95 (100)	20 (100)
No. conceived at induced estrus	90 (78.3)	72 (75.8)	18 (90)
No. calved	85 (94.4)	68 (94.4)	17 (94.4)

*Values in parentheses are percentages.

6.5. DISCUSSION

A simple procedure that uses solely CIDR inserts without any combination treatment on the initial day was performed in this study. This is unlike previous reports (Broadbent et al., 1993, Kawate et al., 2011) that used CIDR in combination with a Co-Synch or Ovsynch protocol (GnRH-PGF2α-GnRH combination protocol) whereby GnRH is administered simultaneously with CIDR on the initial day, which has been demonstrated to be effective. Progesterone delays the time of estrus after natural or induced luteolysis and extends the estrous cycle by preventing the occurrence of spontaneous estrus (Lucy et al., 2001). Administration of PGF2α on Day 8 of CIDR insertion was intended to cause regression of the CL and to bring the animals into estrus 1-3 days post treatment. The PGF2α reduces the risk of continued progesterone production by a functional CL after device removal (AHDB, 2014). eCG has both FSH and LH like activity in bovine (Racowsky, 1991), but with more FSH activity (Gospodarowicz, 1972). Therefore, administration of eCG on the day of device removal stimulates follicular development, final maturation of the dominant follicle, initiation of ovulation and CL formation (Racowsky, 1991; Sá Filho et al., 2009). Furthermore, in the present study three animals gave birth to twin calves, which could be due to administration of gonadotrophins, such as eCG that can cause superovulation (Tegegne et al., 1994).

In the present study, the retention of CIDR exceeded 97% which is comparable with previous reports (Broadbent et al., 1993; Grant, 2006), although, others reported no CIDR loss (Macmillan and Peterson, 1993). Animals that did not show external symptoms of estrus, but when inseminated had open cervices evidenced when the AI gun was easily and smoothly passed through the cervix is suggestive of silent estrus post CIDR treatment. In agreement with our finding, a previous report showed that 2% of CIDR-treated heifers had a palpable CL, although they were not observed in estrus (Broadbent et al., 1993). All animals that were reported by the farmers as anestrus, but confirmed cycling by the on-farm progesterone tests, showed external symptoms of estrus when subjected to the present estrus synchronisation protocol, which indicates poor estrus detection. Furthermore, all anestrous (except 3 with just their cervical os opened) and repeat-breeder

cattle, including those with silent/unobserved estrus showed external symptoms of estrus following estrus synchronisation. Administration of CIDR in anovulatory and anestrous cows/heifers re-establishes the hypothalamic responsiveness to estradiol produced from the dominant follicle. It also normalises the duration of the luteal phase (Garcia-Winder et al., 1986), and increases estradiol responsiveness in behavioural centres of the brain (Rosenberg et al., 1990; Fike et al., 1997) thereby increasing the proportion of cattle expressing behavioural estrus in conjunction with ovulation (McDougall et al., 1992; Gasser, 2013).

The high estrus response and conception rate in the present study is possibly due to the exclusion criteria such as unacceptable BCS, assessment of pre-treatment reproductive condition of animals as unfit for estrus synchronisation (having unhealthy and small sized reproductive tract and organs), and the use of double insemination or mating at 48 and 72 hr. Previous reports have shown that BCS and uterine infections are among the factors that reduce fertility (McNally et al., 2014). Nutritional limitations and poor response of dairy genotypes are the two common features observed in most developing countries (Tekeba et al., 2014). The nutritional status of a dairy cow can be assessed by the BSC (Nicholson and Butterworth, 1986). Previous reports have shown that BCS has significant effect on fertility in cattle (Buckley et al., 2003; Rhodes et al., 2003a; Roche et al., 2007; Alam and Sarder, 2010). Siddiqui et al. (2002) reported that indigenous Zebu cows with BCS 2.5-3 were considered more suitable for the induction of superovulation compared to cows with BCS of 4-4.5 that were more prone to ovarian cyst. This is the reason for exclusion of animals with BCS <2.5 in the present study. Despite the difference in reproductive status of animals (anestrus/cycling) prior to CIDR insertion, a high estrus response was found in both cows and heifers in the present study. This is in agreement with previous studies that reported a 100% estrus response using a 7-day CIDR and eCG injection in 30 anestrous Zebu breed cows (Singh et al., 2006), a 100% response in 73 crossbred cows with unknown ovarian cyclicity using a 7-day CIDR in combination with PGF2α and GnRH (Dickson et al., 2012) and a 100% response in anestrous Sahiwal (Zebu) cows using a 9-day CIDR in combination with eCG injection one day prior to

CIDR removal and hCG at the time of AI (Virmani et al., 2013). Conversely, the present study improved on other studies that reported 79.5% and 87.5% estrus response using 7-day and 9-day CIDR, respectively in combination with PGF2α in 128 HF/ crossbred dairy cows (Romano and Fahning, 2013), 86.7% using Ovsynch + CIDR in 55 repeat-breeder HF cows (Kim et al., 2007), 80% in 5 normally cycling Aceh cattle in Indonesia using CIDR-B + PGF2α (Siregar et al., 2015), and *Bos indicus* and other cattle breeds (Larson et al., 2006; Williams et al., 2011).

There were similar conception rates between anestrous, repeat-breeder and silent estrus crossbred dairy cattle in this study, with a high overall conception rate (78.3%) similar to reports in anestrous Zebu breed cows (70%) in India (Singh et al., 2006). The present study has shown a better conception rate than reports in crossbred cattle (40.9%) in the tropics (Dickson et al., 2012), in HF/crossbred cows (45.5%) (Romano and Fahning, 2013), in *Bos indicus* cattle (Williams et al., 2011) and in beef cattle in USA (Larson et al., 2006), and in beef heifers (54.5/55.5%) using 14-day/ 5-day CIDR protocols (Kasimanickam et al., 2015) that reported lower pregnancy rates, 54.5% and 55.5%, when using long- and short-term CIDR protocols, respectively. Moreover, the present study has shown better conception rate in repeat-breeder cows/heifers than previous reports in South Korea (Kim et al., 2007) and Japan (Kawate et al., 2011). Conversely, conception rate in the present study was lower than previous reports that used CIDR treatment in Aceh cattle (100%) in Indonesia (Siregar et al., 2015). However, their study was only in 5 cattle, so the higher conception rate may reflect their small sample size. Nevertheless, conception rate can be affected by factors such as nutrition, breed, postpartum interval, and geographic location (Gordon, 1996; Lamb et al., 2001).

The conception rate in cows (82.9%) was slightly higher, but not significant, than in heifers (72.1%) in the present study. Contrary to our findings, a previous report showed higher conception rate in heifers (67.9%) than cows (53.1%) (Colazo et al., 2004). The present study recorded lower pregnancy loss (5.1%) in cows compared with previous reports where the loss in cows was 20% when PGF2α/ progestins was used (Smith and Stevenson, 1995). Infectious diseases, and non-infectious factors such as poor

management systems, milk yield, parity, BCS, ambient temperature (Zobel et al., 2011) can cause pregnancy loss.

By way of direct comparison, the estrus response recorded in the present study was higher than our recent report that used single or double administration of PGF2α with simultaneous administration of GnRH (Mekonnin et al., 2016). The present study had a higher (78.3%) conception rate than our recent report in same study area when repeat-breeder crossbred dairy cattle were subjected to 20μg or 10μg GnRH (Buserelin acetate) (68%) (Hailu et al., 2015). Furthermore, the present study has shown a higher conception rate than was observed in the large-scale controlled reproduction programme in Tigray region that used PGF2α (31.5%) in local/ crossbred dairy cattle (Giday, 2014). This may be due to progesterone having a positive effect in inducing/ synchronising estrus in anestrus and cycling cattle, whereas PGF2α has no effect on anestrous cattle. AI technicians lack the experience and skills to effectively diagnose cyclicity of animals through palpation of the ovaries per rectum (Lobago, 2007; Ashebir et al., 2016), so non-cycling animals may have been included in the PGF2α-based breeding programme. In this current study, the on-farm progesterone assay confirmed the presence of CL, though our CIDR-based estrus synchronisation can be used at any stage of the estrous cycle, including in anestrous animals and prepubertal heifers.

Although farmers were advised to record animals in estrus, all animals were inseminated or naturally mated at a fixed time. This did not affect pregnancy regardless of pre-treatment reproductive status of animals, breeding method, farming system or parity. This was similar to a previous report in cows and heifers, although they reported comparatively lower overall pregnancy rate than the present findings (Lamb et al., 2010). Some farmers in the study area prefer to have their own bull to using AI services due to a number of reasons as discussed in our recent reports (Mekonnin et al., 2015b; Ashebir et al., 2016). Conception rate did not vary between breeding methods (AI vs bull) in the present study, yet a higher conception rate was found in animals bred with natural mating/bull than with AI, which could be due to improper insemination. We recently evaluated the quality of frozen semen (sperm motility and viability) in the study area, and it was within the normal

range (Ashebir et al., 2016). In the present study, double inseminations/mating at 48 and 72 hr have resulted in high pregnancy rate in all animal groups (anestrus, repeat-breeding and silent/unobserved estrus). In contrast to our findings, a previous study indicated that the use of double insemination did not bring any significant difference in pregnancy rates compared to single insemination in repeat-breeder dairy cattle in other treatment regime (Stevenson et al., 1990). However, it has recently been proposed that double insemination can improve pregnancy rate by 5-10% (AHDB, 2014).

There was a limitation of inclusion in this study due to the lack of a control group because of the large number of study cattle reared by smallholder farmers. The majority of smallholder farms, owning 1 to 5 head of cattle, including organised commercial farms, participated in this study for mutual benefit. They had either anestrus or repeat-breeding cows/heifers. These farms were not interested in their cattle being left untreated because they were aware of potential pre-existing infertility problems with their animals. Therefore, no control group could be included to compare with the treatment group on estrus response and conception rates. Hence, it was not possible to predict how many of these animals would have come into estrus without intervention. However, in previous studies, where control animals were used, lower estrus response and conception rate were recorded in control group compared with treated animals (Irmã et al., 2008).

Application of a progesterone (CIDR)-based estrus synchronisation programme is highly recommended to enhance fertility of dairy cattle. Furthermore, the expense of hormones required for estrus synchronisation (US $18.60/per animal: CIDR, US $11.16 + PGF2α, US $4.03 + eCG, US $3.41/animal) used in this study is less than the cost farmers spend feeding anestrous and repeater cows, with an estimated loss of production cost of US $148.48 per cycle from a single cow (Hailu et al., 2015) for extended periods of time. This excludes the labour costs. In anestrus cattle, the cost of this estrus synchronisation protocol can be further reduced to US $14.57, as PGF2α is not necessary in the absence of CL. Additionally, the prospect of reusing a used CIDR device (Abdallah and El Rahim, 2014) makes the protocol worthwhile. In most developed countries, in individual farms, cattle are bred during the breeding season following a routine mass estrus synchronisation. It

would be beneficial to implement this in Ethiopia, but would require centralised government funding.

In conclusion, the present study has shown a high estrus response, conception rate and calving rate in cows and heifers assigned to progesterone (CIDR) - PGF2α - eCG based estrus synchronisation protocol irrespective of pre-treatment reproductive condition, farming system and breeding methods. This study also confirms the effectiveness and affordability of the protocol. Thus, the protocol is recommended to enhance fertility of dairy cattle in the study area and other parts of the country and improve the livelihood of the farming community.

CHAPTER 7: MILK COMPOSITION AND EFFECT OF REPRODUCTIVE STATUS, MANAGEMENT, LACTATION STAGES, PARITY AND BREED ON MILK COMPOSITION IN CROSSBRED DAIRY COWS

Abstract:

The aims of the present study were to determine macronutrient composition of milk and assess the effect of reproductive status, farm (nutritional) management, stages of lactation, parity and breed on milk composition. Two hundred and forty-six (246) dairy cows, involving 184 crossbred dairy cows from farms in and around Mekelle, Ethiopia and by way of comparison, 62 HF cows (25 non-pregnant and 37 pregnant) from a commercial farm in Edinburgh, United Kingdom, were included in the study. Of 184 crossbred dairy cows, 76 (36 non-pregnant and 40 pregnant) were reared on smallholder farms under unregulated husbandry, while 108 (62 non-pregnant and 46 pregnant) reared on organised commercial farms under good husbandry practices. The HF cows were housed either indoors or outdoors. Milk from early milking was collected into individual plastic tubes after discarding the first four drops of milk (foremilk). Samples were frozen until analysed using a Miris DMA. The mean milk fat, protein, lactose, TS and SNF recorded were 2.36%, 3.46%, 4.37%, 10.39% and 7.82%, in crossbred dairy cows, compared with 5.05%, 3.71%, 4.72%, 13.68% and 8.43%, in HF cows, respectively. Macronutrients were significantly lower ($p<0.05$) in milk from crossbred cows than in milk from HF cows. Milk fat in both breeds was significantly affected by reproductive status, farm (nutritional) management and stages of lactation, but not by parity. In both cattle breeds, the milk fat content was significantly higher ($p<0.05$) in pregnant than non-pregnant cows. Milk protein content was significantly ($p<0.05$) affected by reproductive status (in crossbred) and stages of lactation (in both breeds), but not by farm management or parity. Reproductive status (in crossbred), stages of lactation (in both breeds) and parity (in crossbred) affected lactose levels, although farm management had no effect on lactose level in either breed. Lactose levels were significantly higher ($p<0.05$) in non-pregnant than in pregnant cows in early compared to late lactation, and in cows that had calved more than three times. Milk TS content was highly affected by reproductive status (both breeds), farm management (HF) and stages of lactation (both breeds), but not by parity. While stage of lactation significantly ($p<0.05$)

affected milk SNF content in crossbred cows, reproductive status, farm management and parity had no effect in both breeds. In conclusion, milk fat was the most affected macronutrient in both breeds and the overall macronutrient content in crossbred cows was low. Hence, application of proper nutritional and breeding management is required to enhance the nutritional value of milk production from dairy cattle in the study area.

Key words: Cattle, DMA, Lactation stage, Milk composition, Nutrition, Parity, Reproductive status.

7.1. INTRODUCTION

Dairy production is widely practiced in Ethiopia (Tegegne et al., 2013). Cows, sheep, goats, buffaloes as well as camels are sources of natural milk for human consumption (Santos and Lies, 2013). The main sources of milk in Ethiopia are the indigenous cattle (Zebu breed), accounting for 97% of the country's annual milk production (Tedla et al, 1991), with an average 1.85 L/head per day (Tegegne et al., 2013). The growing animal protein deficit in developing countries, associated with the ever-increasing human population, has resulted in importation of more productive European dairy cattle in an attempt to improve milk production (El-Wishy, 2013; Combellas et al., 1981). Since the late 1960s, when exotic breeds were first imported, this has been accompanied by crossbreeding of the local cattle, mainly with HF (Felleke and Geda, 2001; Ahmed et al., 2003).

A total of 1.5 million tonnes of milk are produced annually from cattle in the country (FAO, 2005). The average milk production of crossbred dairy cows is much higher than local breeds (Zebu), and is higher in urban (10.21-15.9 L/head per day) than peri-urban farms (9.5 L/head per day) (Tegegne et al., 2013). The importance of milk in the diet of Ethiopians differs according to the farming systems and the society and cultural expectations and norms (Yilma et al., 2011). Milk consumption in rural areas can be considered as a function of wealth or availability to a given household, unlike the urban areas where it is determined by the purchasing power of the household, the level of awareness on its nutritive value and availability (Yilma et al., 2011). A previous report has shown that of the total urban milk production, 73% is sold, 10% is used for household consumption, 9.4% is fed to calves and 7.6% is processed into butter and Ayib (cottage cheese) (Reda, 2001). Whereas, of the total rural areas annual milk production, 85% is used for household consumption and 7% is sold, while only 0.3% is used for wages in kind and the remaining 8% is used for other purposes (CSA, 2010).

Hand milking is the main method of milking in Ethiopia, and is carried out twice per day. Almost all crossbred and HF cows in Ethiopia are milked without being suckled by their

calves. However, local breed cattle are partially suckled prior to milking to stimulate milk letdown. Previous reports have shown that partial suckling has improved milk yield and lactation length in local breed cows compared to non-suckling cows (Tegegne et al., 1994; Kumssa and GebreYohannes, 1995; Yilma et al., 2006). However, this has the drawback of causing prolonged postpartum anestrus (Tegegne et al., 1994; Yilma et al., 2006) and reducing the milk fat percentage (Yilma et al., 2006).

There is rapid growth in milk and dairy consumption in many developing countries over recent years (FAO, 2005; Seifu and Doluschitz, 2014), which is due to a rapid increase in population size with a growing urbanisation (Seifu and Doluschitz, 2014). Tigray regional state (the study area) was reported to be the second region where milk is highly consumed after the Amhara Region in Ethiopia (CSA, 2010). In Eastern African countries, most dairy farmers in urban areas have little or no (zero-grazing) access to grazing land (Msangi et al., 2005, Cole et al., 2008) and depend mainly on purchased feeds and communal grazing lands. This leads to suboptimal levels of nutrition in dairy cows, especially during the dry periods (Gillah et al., 2012). Despite Ethiopia having a large cattle population, the demand for milk consumption is high compared to its production (Yilma et al., 2011). The increase in the demand in milk consumption and the poor feed supply to dairy cattle therefore raises the question whether the nutritional value of milk to consumers is of a sufficiently high standard. Furthermore, in Ethiopia, milk is produced mostly in sub-standard conditions, with no quality control in place prior to reaching the consumers (Gurmessa et al., 2015). About 95% of the marketed milk at national level is channeled through informal system (Yilma et al., 2011). Furthermore, a recent report has shown that the main challenges in milk collection and processing in Ethiopia are lack of chilled transport, lack of quality control by processor, lack of awareness on dairy technology, under-utilisation of processing capacity, lack of economies of scale in collection and processing, weak management of cooperatives, and lack of transparency, quality and financial results (Zijlstra et al., 2015). Since breed, feeding regimens, calving patterns and breeding practices differ, milk composition will also differ between countries and within

a country (Heck et al., 2009). These differences place a challenge on production of milk with optimum nutritional values.

In Ethiopia, organised milk collection and processing was introduced mainly in Addis Ababa, the capital city of the country, in the 1960s, when only one milk processing plant was functional, and processing and distribution was run by a government agency, Sholla Dairy (Yilma et al., 2011). This milk-processing unit is currently privatised. In early stages, the unit had raw milk collection points from close smallholder producers that further expanded to seven km radius from the city, and subsequently extended to cover a distance of about 150 km from Addis Ababa. The unit is now operating with 25 collection centres, where at these collection points milk is analysed with a field acidity (alcohol) test for freshness, for adulteration (addition of water), and for removal of cream with a lactometer reading (Yilma et al., 2011). Currently, there are more than five dairy enterprises across the country, including Sebeta Agro Industry (Mama), Family Milk and Zemen, that process milk. These dairy enterprises process and pack fresh milk collected for distribution to consumers in urban areas through agents and retailers in the form of homogenised, pasteurised or standardised (2.7-2.8% milk fat) milk packaged in half litre capacity plastic packets (Yilma et al., 2011).

The composition of raw milk determines the nutritional value of milk and dairy products (Heck et al., 2009). Bovine milk contains the nutrients needed for growth and development of the calf, and is a source of lipids, proteins, amino acids, vitamins and minerals (reviewed in Haug et al., 2007; Santos and Lies, 2013). Fresh cow milk contains approximately 3.5% protein (reviewed by Santos and Lies, 2013), 3.8% fat, 4.6% lactose, 12.5% TS, 8.7% SNF, 0.8% ash, and 87.5% water (Athar and Ali, 1986). However, the composition of milk, particularly fat content, can vary due to several factors, such as breed (Woodford et al., 1986; Hurley 2009; Arnould and Soyeurt, 2009), nutrition (Chilliard et al., 2000; Chilliard et al., 2001; Forsbäck et al., 2010), age (Gurmessa and Melaku, 2012), disease (Ogola et al., 2007), stages of lactation (Fox and McSweeney, 1998; Yilma et al., 2006) and milking technique (McDonald et al., 1995).

7.2. SPECIFIC AIMS

- To determine the nutritional composition of milk using Miris dairy milk analyser in crossbred dairy cows in Ethiopia.
- To evaluate the effect of reproductive status, stages of lactation, farming (nutritional) system, parity and breed, with reference to HF cows in Edinburgh, United Kingdom, on macronutritional composition of milk.

7.3. MATERIALS AND METHODS

7.3.1. Study Animals and Farms

Milk samples were collected twice at 11-day intervals from a total of 246 cows (crossbred dairy cows and pure HF cows) having BCS of 3.1 (on a scale of 1-5).

Crossbred dairy cows:

One hundred and eighty-four (184) crossbred dairy cows from farms in and around Mekelle, Ethiopia were included in the study. Of these, 76 cows (36 non-pregnant and 40 pregnant) were reared by smallholder farms with unregulated husbandry, while the remaining 108 cows (62 non-pregnant and 46 pregnant) were reared by organised commercial farms with good husbandry practice. Cows were in their early (< 120 days post calving), mid- (120-210 days post calving) or late lactation (> 210 days post calving), classification was according to previous reports (Gizat, 2004) with a slight modification. Pregnant cows were in their 1st (1-3 months), 2nd (3-6 months) or 3rd (7-9 months) trimester. Cows in both types of farms were fed grass hay or straw, concentrate composed of wheat by-products, crop residues and local beer residue (Attela). However, cows in organised commercial farms were supplemented with green fodder, such as fresh grass, alfalfa, elephant grass, leucaena and sesbania, including regular watering, and they had better breeding records. Cows in both types of farm had a similar average milk yield of 15L/day. Crossbred cows were in either early, mid- or late lactation.

HF cows:

Sixty-two HF cows at a commercial farm in Edinburgh, United Kingdom were included in the study to compare findings from crossbred cows in Ethiopia. Among 62 cows, 25 were non-pregnant, while 37 were pregnant (in their 2nd and 3rd trimester), and cows were either in their early or late lactation. Cows were managed either indoors or outdoors. Cows managed indoors were provided with rations of grass silage, whole crop, Langhill total mixed ration meal (composed of maize, Hipro soybean, barley, rapeseed ext, wheat, soya hulls, D maize grains, D wheat grain, beet pulp/mill, megalact LI, limestone, salt, total

premix, calcined Mag and vitamin E) and molasses, and had BCS of 2.8 and produced an average milk yield of 38L/day. Cows under outdoor husbandry were left entirely outdoors grazing on grassland, except when they were brought inside for milking, at which time they were provided with a mixed ration meal. These cows had BCS of 2.5 and produced an average milk yield of 25L/day.

7.3.2. Milk Collection and Pretreatment

Milking procedure instructions suited to the study, were given to the farmers/ farm attendants prior to milk collection. Prior to milking, the udder and teats were carefully washed and dried and 10-20ml milk was collected per cow. Crossbred cows were hand milked and milk was collected after discarding the first four drops (foremilk). Milk collection was carried out in the afternoon (2pm-6pm). Milk was collected from HF cows in the same way, but also machine-milked milk was obtained from early milking to assess if milking technique affects milk composition. Samples from crossbred cows were heat treated at 56^{o}C for 30 min and frozen at -20^{o}C before being shipped to MRC Centre for Reproductive Health, Immunoassay Laboratory, The University of Edinburgh, Edinburgh, United Kingdom (UK) for analysis. This heat treatment was a requirement stated on the Import Authorisation of Animal Products for Study or Analysis [TARP(S)2014/23], according to the Trade in Animals and Related Products (Scotland) Regulations 2012, issued by the Agriculture, Food and Rural Communities Directorate of the Scottish Government. Milk from HF cows was treated similarly. All samples were kept at -80^{o}C until analysed.

7.3.3. Experimental Design

Frozen milk samples were thawed overnight at 4^{o}C and 1-4 hr at room temperature. Samples were then warmed at 40^{o}C for 5-10 min. Wash solution and deionised water were simultaneously thawed at 4^{o}C in a water bath. Macronutrient content of milk, in its component parts of fat, carbohydrate/lactose, protein, TS (TS includes proteins, lactose, minerals, acids, enzymes, vitamins, including fat) and SNF (SNF is TS minus fat) were analysed using a Dairy Milk Analyser (DMA) (Miris AB, Uppsala, Sweden). Prior to milk

composition analysis, samples were homogenised using a Sonicator (Miris). This homogenisation is used to break large fat globules into smaller, more equally distributed globules in emulsion. Procedures were according to the manufacturer's instructions. Milk (2.5ml per sample) was injected into the analyser (Appendix 6) and the macronutrient components in milk were analysed within one minute, and repeated twice per sample. The mean value was taken for subsequent analysis. The levels of macronutrient components of milk were also evaluated in relation to reproductive condition (non-pregnant vs pregnant), farming system, stage of lactation, parity and breed (crossbred cows in Ethiopia vs pure HF cows in the United Kingdom). Furthermore, milk composition was evaluated in pregnant cows between stages of gestation: 1st trimester, 2nd trimester and 3rd trimester.

7.3.4. Data Analysis

Percentage of milk components were analysed using Microsoft Excel. Comparison of findings between stages of lactation as well as between pregnancy stages was carried out by one-way ANOVA with Tukey's multiple comparison test. However, all other data were analysed using the Mann-Whitney U-test. All statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc, CA, USA). Differences were considered significant when $p < 0.05$.

7.4. RESULTS

The average milk fat, protein, lactose, TS and SNF recorded in crossbred dairy cows are detailed in Table 7.1. Milk composition of crossbred cows was compared with HF cows. Findings were compared between reproductive status (non-pregnant vs pregnant (Table 7.1), and between stages of pregnancy: 1st, 2nd and 3rd trimester, Table 7.2), farming systems (Table 7.1), lactation stages (early, mid and late lactations), parity and breed (crossbred vs HF). The macronutritional composition of milk in HF cows is detailed in Table 7.3.

Table 7.1: The overall macronutritional components of milk, findings based on farming system and reproductive status of crossbred dairy cows.

Milk components	Overall (n=184)	Farming system		Reproductive condition	
		Smallholder (n=76)	Organised commercial (n=108)	Non-pregnant (n=98)	Pregnant (n=86)
Fat (%)	2.36±1.69	2.64±1.53	2.16±1.77§	1.87±1.27	2.91±1.92*
Protein (%)	3.46±1.33	3.54±1.51	3.4±1.2	3.13±0.82	3.83±1.66*
Lactose (%)	4.37±1.2	4.21±1.192	4.48±0.87	4.59±0.8	4.11±1.18*
TS (%)	10.39±2.1	10.58±1.89	10.25±2.24	9.79±1.42	11.06±2.5*
SNF (%)	7.82±0.99	7.75±1.06	7.88±0.93	7.72±0.78	7.95±1.17

Mean ± standard deviation (SD). §$p<0.05$ compared with smallholder. *$p<0.05$ compared with non-pregnant.

i) Milk Composition Based on Reproductive Status

Crossbred cows: Milk fat, protein and TS were significantly higher ($p<0.05$) in pregnant than in non-pregnant cows. In contrast, lactose was significantly higher ($p<0.001$) in non-pregnant than pregnant cows. SNF content was similar between non-pregnant and pregnant cows (Table 7.1). Stages of pregnancy (1st, 2nd and 3rd trimester) did not affect fat, TS and SNF content of milk in crossbred dairy cattle (Table 7.2). However, significantly lower ($p<0.05$) milk protein was measured in pregnant cows at their 1st trimester than in the 2nd trimester. Similarly, milk protein was significantly lower ($p<0.05$) in pregnant crossbred cows at their 1st trimester than in the 3rd trimester. Lactose level was

significantly higher ($p<0.05$) in pregnant cows in 1st trimester than in 3rd trimester, however no difference was recorded between 1st and 2nd trimester as well as between 2nd and 3rd trimester (Table 7.2). In general, fat, protein, TS and SNF all increased during pregnancy, whereas lactose decreased during pregnancy in crossbred cows. In HF cows, milk protein, lactose and SNF contents were similar between non-pregnant and pregnant cows. However, significantly higher ($p<0.05$) milk fat and TS was measured in pregnant than in non-pregnant cows.

Table 7.2: Comparison of milk composition based on stages of pregnancy in crossbred dairy cows.

Milk components	Stages of pregnancy		
	1st trimester (n=38)	2nd trimester (n=32)	3rd trimester (n=16)
Fat (%)	2.56±1.32	3.08±1.95	3.44±1.87
Protein (%)	3.25±0.88	4.06±1.58*	4.76±2.6*
Lactose (%)	4.43±0.92	4.01±1.26	3.57±1.4*
TS (%)	10.44±1.42	11.35±2.58	11.97±3.91
SNF (%)	7.68±0.76	8.07±1.12	8.34±1.85

Mean ± standard deviation (SD). *$p<0.05$ compared to 1st trimester.

ii) Milk Composition Based on Farming systems

Significantly higher ($p<0.05$) milk fat was recorded in crossbred cows from smallholder farms than in those from organised commercial farms (Table 7.1), however similar milk protein, lactose, TS and SNF content were recorded between the two farming systems. In HF cows, similar milk protein, lactose and SNF content was recorded between cows managed indoors and outdoors. However, milk fat and TS were significantly ($p<0.05$) affected by the feeding and management system used, whereby higher contents were recorded in cows managed outdoors (Table 7.3).

Table 7.3: Milk composition of HF cows based on farming system and reproductive condition.

Milk components	Overall (n=62)	Farming system		Reproductive condition	
		In-door (n=24)	Out-door (n=38)	Non-pregnant (n=25)	Pregnant (n=37)
Fat (%)	5.05±1.59	4.25±1.77	5.56±1.24*	4.32±1.75	5.56±1.24§
Protein (%)	3.71±0.56	3.71±0.62	3.71±0.52	3.65±0.57	3.71±0.52
Lactose (%)	4.72±0.41	4.82±0.51	4.65±0.31*	4.78±0.50	4.65±0.31
TS (%)	13.68±1.81	12.99±1.97	14.12±1.58*	12.95±1.88	14.12±1.58§
SNF (%)	8.43±0.64	8.54±0.78	8.36±0.54	8.43±0.73	8.36±0.54

Mean ± standard deviation (SD). *$p<0.05$ compared to indoor. §$p<0.05$ compared to non-pregnant.

iii) Milk Composition Based on Lactation Stages

Crossbred cows: There was an increase in protein and TS, and a decrease in lactose, as lactation stage increases. There was a statistically significant difference ($p<0.05$) in milk fat between early and mid-lactation as well as between early and late lactation; in both cases, the fat content was lower in early lactation. Whereas, no difference was found between mid- and late lactation in milk fat percentages. Similar findings were obtained between early and mid-lactation in milk protein content. However, the protein content of milk during early and mid-lactation was significantly lower ($p<0.05$) than in late lactation. Lactose content was similar between early and mid-lactations as well as between mid- and late lactations, although significantly lower lactose levels were recorded in late than in early lactation. Significantly lower ($p<0.05$) TS was detected in early than in late lactation, however similar findings were recorded between early and mid-lactations as well as between mid and late lactations. SNF content of milk was similar between early and mid-lactations. However, significantly higher ($p<0.05$) SNF was obtained in late lactation than in early, and in late lactation compared to mid-lactation (Table 7.4).

HF cows: Macronutritional components of milk were evaluated between early and late lactation. Significantly higher ($p<0.05$) milk fat, protein and TS was recorded in late compared to early lactation, however lactose levels were significantly higher ($p<0.05$) in

early lactation than in late lactation. SNF content was similar between early and late lactation.

iv) Milk Composition Based on Parity

Parity did not affect milk fat, protein, TS and SNF content in crossbred cows, however lactose content was significantly higher ($p<0.05$) in cows that had calved more than three times compared with those with a parity of 1 to 3 (Table 7.4). In HF cows, parity had no effect on milk fat, protein, lactose, TS and SNF (Table 7.5).

Table 7.4: Milk composition of crossbred dairy cows based on lactation stages and parity.

Milk components	Lactation stage			Parity	
	Early (n=40)	Mid (n=44)	Late (n=100)	1-3	>3
Fat (%)	1.72±1.14	2.6±1.63*	2.51±1.84*	2.43±1.87	2.23±1.264
Protein (%)	2.81±0.53	3.01±0.60	3.91±1.59*¤	3.6±1.55	3.18±0.69
Lactose (%)	4.77±0.38	4.54±0.83	4.13±1.20*	4.25±1.16	4.6±0.65§
TS (%)	9.51±1.23	10.34±1.64	10.76±2.44*	10.48±2.43	10.2±1.27
SNF (%)	7.58±0.65	7.54±0.82	8.05±1.11*¤	7.85±1.06	7.78±0.83

Mean ± standard deviation (SD). *$p<0.05$ compared with early lactation; ¤$p<0.05$ compared with mid-lactation. §$p<0.05$ compared with parity of 1-3.

Table 7.5: Milk composition of HF cows based on stages of lactation and parity.

Milk components	Lactation stage		Parity	
	Early (n=12)	Late (n=40)	1-3 (n=50)	>3 (n=12)
Fat (%)	4.17±2.37	5.61±1.22*	5.13±1.65	5.01±1.29
Protein (%)	3.29±0.23	3.75±0.49*	3.73±0.53	3.62±0.66
Lactose (%)	5.00±0.28	4.63±0.31	4.77±0.35	4.57±0.55
TS (%)	12.66±2.31	14.19±1.54*	13.84±1.82	13.40±1.68
SNF (%)	8.29±0.32	8.38±0.53	8.50±0.59	8.19±0.77

Mean ± standard deviation (SD). *$p<0.05$ compared with early lactation.

v) The Effect of Breed on Milk Composition

Crossbred dairy cattle had an overall significantly lower ($p<0.05$) milk fat, protein, lactose, TS and SNF compared to HF cows (Table 7.6).

Table 7.6: Comparison of overall macronutritional components of milk between crossbred dairy cows (in and around Mekelle, Ethiopia) and HF cows (Edinburgh, United Kingdom).

Milk components	Breed		p-value
	Crossbred	HF	
Fat (%)	2.36±1.69	5.05±1.59	< 0.0001
Protein (%)	3.46±1.33	3.71±0.56	< 0.0001
Lactose (%)	4.37±1.2	4.72±0.41	0.0417
TS (%)	10.39±2.1	13.68±1.81	< 0.0001
SNF (%)	7.82±0.99	8.43±0.64	< 0.0001

Mean ± standard deviation (SD). $p<0.05$ was considered significant.

vi) The Effect Milking Methods, Heating and Storage on Milk Composition

Similar macronutritional composition of milk was found in HF cows collected manually (hand milking) and using machine (validation study, results are not shown). The effect on macronutritional composition of heating frozen milk at 56^{o}C for 30 min in a water bath was compared with frozen samples that were not exposed to heat (validation study, results are not shown). Heating had no effect on milk fat, protein, lactose, TS and SNF content. However, comparison of heat treated frozen milk samples with heat treated fresh samples showed that frozen milk samples had significantly lower ($p<0.05$) protein and SNF content compared with fresh whole milk (validation study, results are not shown).

7.5. DISCUSSION

In the present study, macronutrient content was measured in milk from crossbred dairy cows in Tigray Region, Ethiopia and HF cows Edinburgh, UK. The effects of reproductive status, farm (nutritional) management system, lactation stage, parity and breed on milk composition were evaluated. The overall milk fat, protein, lactose, TS and SNF in crossbred cows was lower than in HF cows. Milk protein, lactose and SNF content in crossbred dairy cows in the present study was comparable with a recent report in local (Borena) breed cows in other parts of Ethiopia (Gurmessa et al., 2015), however milk fat and TS content were lower in the present study. The reason for this low milk fat and TS could be due to local cows having a genetically higher milk fat content than crossbred or pure HF cows. Furthermore, in the present study, milk was collected from early milking, while Gurmessa et al. (2015) used milk from late milking from individual milk containers.

Milk protein and lactose levels in crossbred dairy cows in the present study was also comparable to previous reports in HF cows in the Netherlands analysed in 2005 (Heck et al., 2009), however the milk fat level was lower in crossbred cows in and around Mekelle Ethiopia than in HF cows in the Netherlands. Contrary to the Dutch milk composition data (Heck et al., 2009), the present study found higher milk fat, protein and lactose in HF cows in Edinburgh, United Kingdom. Fat content of milk can be affected by milking technique, as incomplete milking may leave a considerable amount of fat-rich milk in the udder (McDonald et al., 1995). Furthermore, the milk fat level in HF cows in the present study was also higher than in reports from indoor or outdoor managed HF/ Czech Fleckvieh cows in Czech (Frelich et al., 2009), and in HF cows in Spain with or without supplementing polyunsaturated fatty acids (Gonzalez et al., 2015).

Higher milk fat and TS was obtained in pregnant than in non-pregnant cows in both crossbred and HF breeds. This was in contrast to previous reports in crossbred cows in North-western Ethiopia that showed no effect of reproductive status on milk fat content (Gurmessa and Melaku, 2012) and in HF cows in USA that reported lower milk fat and protein levels in pregnant than in non-pregnant cows (Lee et al., 1997). Milk protein and

lactose content in both pregnant and non-pregnant, as well as milk fat in pregnant crossbred cows in the present study were comparable with previous reports in crossbred cattle in other parts of Ethiopia (Gurmessa and Melaku, 2012). In contrast, the present study showed lower SNF content in both pregnant and non-pregnant cows, also lower fat in non-pregnant cows.

Pregnancy affects milk production (Akers, 2006), leading to reduced milk fat and protein (Loker et al., 2009). This is due to hormonal changes resulting in regression of the mammary gland (Brotherstone et al., 2004; Akers, 2006) and resorption of nutrients for fetal growth (Bell et al., 1995). The effect of pregnancy increases as the fetus grows due to an increase in nutrient demand (Olori et al., 1997; Brotherstone et al., 2004; Loker et al., 2009). Lactose and protein content in milk in crossbred cows was affected by stages of pregnancy, which is in line with previous reports (Bell et al. 1995; Brotherstone et al., 2004). The present study has shown that stage of pregnancy had no effect on milk fat, TS and SNF content in crossbred dairy cows. On the contrary, Loker et al. (2009) demonstrated that milk and fat yields began to decline after about 4 months of pregnancy, and protein yield began to decline after about 2 months in Ayrshire, Jersey, Brown Swiss, and Guernsey breeds. Furthermore, other previous studies in HF and in buffalo cows have reported a lowering of milk fat levels as pregnancy advances (Sørensen and Østergaard, 2003; Khan et al., 2011).

During pregnancy, the energy requirement includes energy stored for consumption by the fetus, for fetal metabolism and for use by maternal tissues to support the conceptus (Khan et al., 2011). Energy requirements directly attributable to pregnancy were presumed to be close to zero (NRC, 2001) up to day 190 of gestation (Khan et al., 2011). Farm (nutritional) management did not affect milk protein, lactose and SNF content in either crossbred or HF cows, or TS in crossbred cows; however, it affected milk fat in both breeds, and TS content in HF cows, in the present study. The higher milk fat content recorded in crossbred cows in smallholder farms than in organised commercial farms could possibly reflect the nutritional intake. Although cows in both smallholder and organised commercial farms were managed indoors, unlike cows in smallholder farms,

cows in most organised commercial farms were supplemented with fresh green forage such as grass, alfalfa, elephant grass and sesbania. Milk composition changes when cows switch from a silage based diet to a fresh-grass based diet (Elgersma et al., 2004). Previous reports have suggested that cows managed under fresh pasture have lower milk fat content compared with cows exposed to silage (Elgersma et al., 2004; Couvreur et al., 2006), which is in keeping with the lower milk fat content in crossbred cows in organised commercial farms, although none of the study farms in Ethiopia supplemented silage. In contrast, HF cows grazing outdoors on pasture, in the present study, had higher milk fat than those managed indoors. Frelich et al. (2009) has shown the effect of nutrition on fatty acid concentration in cow milk fat between the indoor and the grazing period, whereby the level of long-chain fatty acids in the milk fat was higher in the grazing period than in the indoor period; whereas, medium-chain and saturated fatty acids content in milk fat was higher in indoors than outdoors.

The present findings are in keeping with the general observation that fat is the most sensitive component of milk to dietary changes (Jenkins and McGuire, 2006; Walstra et al., 2006; Heck et al., 2009). However, other reports have shown no effect of supplementing fat in the diet on milk fat content in cows (Chilliard et al., 2003; Gonzalez et al., 2015), but do show an effect on milk protein content and total milk yield (Gonzalez et al., 2015). Further to the nutritional effect, crossbred cows in smallholder farms might have higher Zebu than HF pedigree compared to cows in organised commercial farms, as AI using pure HF semen is more commonly used in organised commercial farms. Cows sired by HF bulls have lower levels of milk fat compared to offspring produced by Zebu sires (Barbosa et al., 2008). Lehnert et al. (2015) have shown an effect of parental cows and sires on milk fat percentage of their offspring.

The present study has shown significantly lower milk fat in early than mid- or late lactation, with similar findings between mid- and late lactations, in contrast to previous report showing higher milk fat content in early and late than mid-stage of lactation (Gurmessa and Melaku, 2012). Another study reported increased fat content in crossbred cows with advancement of lactation (Barbosa et al., 2008). The present findings whereby

milk protein obtained during early and mid-lactation was significantly lower than in late lactation, though no difference was obtained between early and mid-lactation in milk protein. In contrast, recent reports showed no effect of lactation stage on milk protein, lactose and SNF contents (Gurmessa and Melaku, 2012), whereas the present study has demonstrated that stage of lactation has an effect on lactose, TS and SNF content in crossbred dairy cows and stage of lactation significantly affected milk fat, protein, lactose and TS in HF cows; however, it did not affect SNF, unlike in crossbred cows. Dairy cows are in negative energy in early lactation and mobilise the body fats reserves to meet lactation demands (Garnsworthy and Topps, 1982), which would explain lower milk fat content in early lactation in the present study.

In agreement with a previous report from other parts of Ethiopia (Gurmessa and Melaku, 2012), parity did not affect milk fat, protein and SNF content in crossbred cows. In contrast, previous studies in HF cows have shown that the milk fat level was affected by parity whereby higher milk fat was detected in the first compared to the third and fifth calvings (Yoon et al., 2004). However, in the present study, significantly higher lactose was measured in cows that had calved more than three times.

Crossbred dairy cattle had an overall significantly lower milk fat, protein, lactose, TS and SNF compared to HF cows. Lactation stage significantly affected milk fat, protein, lactose and TS, but not SNF in HF cows. Similar to crossbred cows, milk fat, protein, and TS in HF cows was significantly higher in late compared to early lactation. In contrast, in both breeds, lactose was significantly higher in early than in late lactation. While similar SNF content was observed between early and late lactation in HF cows, significantly higher SNF was found in late than in early lactation in crossbred cows. Similar to crossbred dairy cows, parity had no effect on milk fat, protein, TS and SNF content in HF cows. However, unlike HF cows, parity had significantly affected lactose levels in crossbred dairy cows. Milk protein, lactose and SNF content was not affected by reproductive status in HF cows, however, significantly higher milk fat and TS was measured in pregnant compared with non-pregnant HF cows.

According to the International Organisation for Standardisation (ISO), the standard macronutritional content of pasteurised milk is 3.5% fat in whole milk, 1.5-3.5% fat in semi-skimmed milk, 0.5-1.5% in skimmed milk, 3.20% protein and 12.80% TS (Yilma et al., 2011). The milk protein and TS levels recorded in crossbred dairy cattle in the present study are within this ISO standard requirement, whereas the milk fat was below the standard quantity for pasteurised whole milk, it rather resembles semi-skimmed. Under similar handling and analysis with milk from crossbred dairy cows, the macronutritional composition of milk from HF cows in the present study was within the standard ISO requirement. Similar to a previous report that showed no effect of heat treatment on nutritional quality of milk proteins, following pasteurisation at 72^{o}C for 15 seconds, domestic boiling for 30 seconds of pasteurised milk and an ultra-high temperature process 135^{o}C - 150°C for a few seconds (Efigênia et al., 1997), the present study observed that heating milk at 56^{o}C for 30 min had no effect on its macronutritional composition. This deviation in the present study from the standard requirement of milk fat levels in crossbred dairy cattle requires further investigation. Furthermore, the genetic inheritance from crossbreeding with the Zebu breed should offer advantages to crossbreds than HF cows, which also warrants further investigation.

CHAPTER 8: GENERAL DISCUSSION

This study evaluated reproductive performance and prevalence of reproductive health problems (Chapter 3), and showed that a large number of cows and heifers were affected by anestrus and repeat-breeding. This is in agreement with previous reports reliant on questionnaire survey and/or individual cattle inspection using regular follow-up or rectal palpation (Mekonnen et al., 2010; Hadush et al., 2013; Moges, 2015). The prevalence of poor estrus detection (reviewed by Mukasa-Mugerwa 1989; Belihu, 2002) and silent estrus leads farmers to mistakenly consider their cattle as anestrus. Furthermore, non-return to estrus 21-days post service has been used as regular and straightforward assumption of pregnancy in most farms.

Due to lack of availability of diagnostic methods to detect early pregnancy in cattle, farmers use "non-return to estrus" to indicate if their animals are pregnant, as it is the easiest and cheapest method (Fricke, 2010), and mistakenly regard non-pregnant cattle that do not return to estrus as pregnant. The lack of an established hormonal profile in crossbred cattle in the study area as well as other parts of the country provided an important rationale to establish P4 and estradiol profiles at different reproductive stages (Chapter 4). In the present study, both cows and heifers that were reported as anestrus, repeaters or pregnant (Chapter 3) were included to confirm their actual reproductive status and to trial simple, cost effective and reliable diagnostic tools (Chapter 5). Furthermore, the reproductive performance of non-pregnant crossbred dairy cattle was assessed following treatment with CIDR-PGF2α-eCG combinations (Chapter 6). The main justification for a dairy industry is producing quality milk and subsequent consumer and farmer benefit. Evaluating milk composition is an important activity in the dairy industry and is used to provide consumers with appropriate nutritional information, and potentially informing farmers of relative value of their milk production. The last aim in this study was evaluating nutritional composition of milk and whether this is affected by reproductive status, farming systems, stages of lactation and parity in crossbred dairy cows, when compared to HF cows (Chapter 7).

8.1. SUMMARY OF FINDINGS

- The questionnaire survey on reproductive performance and problems of crossbred dairy cattle indicated that (Chapter 3):

- Crossbred heifers in the study area had later onset of puberty and calve later.
- Crossbred cows had longer postpartum estrus (day open) and calving interval.
- The major reproductive problems in the study area were anestrus, repeat-breeding, dystocia, RFM, endometritis and abortion.

- The study has successfully established serum, milk, saliva and urine P4 and serum estradiol profiles of these crossbred dairy cattle (cows and heifers) at different reproductive stages (Chapter 4).

- Higher P4 levels were detected in pregnant and diestrus cattle than in other reproductive states, whereas high estradiol was recorded in cattle that were in estrus.
- Estradiol levels significantly increased with increasing pregnancy stage.

- On-farm P4 tests have successfully assessed reproductive status of crossbred dairy cattle (Chapter 5):

- On-farm P4 tests effectively diagnosed cyclicity (cycling or anestrus) and pregnancy.
- The causes of repeat-breeding in 2.8% repeater cattle in this study was due to ovarian follicular cysts.
- Significantly more cows/heifers were confirmed cycling using on-farm and laboratory P4 assays, but were reported by the farmers as anestrus, indicating the presence of heat detection problem in the farms (large number of animals diagnosed with normal ovarian function, in contrast with reports by the farmers as anestrus).
- Reproductive history of pregnancy reported by farmers (also breeding records) was similar to the on-farm findings.
- Comparison of qualitative on-farm and quantitative laboratory P4 test was performed to confirm field results, and similar findings were found in assessing reproductive status, except that significantly more cycling cattle were considered anestrus using on-farm P4 tests, which indicate that on-farm P4 tests are unable to detect lower P4 levels in cycling cattle.

- On-farm P4 ELISA enabled pregnancy diagnosis as early as 18 days post service, whereby the first test was carried out 18 days post service and the second test 29 days post service.

- The sensitivity and specificity of on-farm P4 assays (88.6 and 99.4%, respectively) were similar to that of laboratory P4 assay (98.1 & 100%, respectively) in determining pregnancy or non-pregnancy.
- Estrus synchronisation using CIDR-PGF2α-eCG combinations resulted in high estrus response (97.5%) and conception rate (78.3%) (Chapter 6); these findings were similar between cows and heifers, smallholder vs organised commercial farms, cycling vs non-cycling (anestrous) cattle, and conception rate was similar between AI vs natural mating.
- Nutritional composition of milk was determined and significantly lower milk fat recorded in crossbred than in HF cows (Chapter 7). Lactation stage significantly altered milk composition in both breeds.

8.2. EVALUATING REPRODUCTIVE PERFORMANCE AND IMPROVING STRATEGIES IN CROSSBRED DAIRY CATTLE

Reproductive performance is a measure of the speed (frequency) at which cows get pregnant after the voluntary waiting period (Dayyani et al., 2013). The characteristics of good female cattle fertility are: returning to cyclicity soon after calving, exhibiting strong signs of estrus, having a high probability of conceiving when inseminated/mated at standing estrus, and having the ability to carry the conceptus to term (Zavadilová and Štípková, 2013). Fertility of dairy cattle can be affected by a number of factors such as, age, disease, breeding management, nutrition and genetic.

The average ages at first service recorded in crossbred heifers in the study area (Chapter 3) was in agreement with a recent report in the study area (Kumar and Tkui, 2014) and in other parts of Ethiopia (Mureda and Mekuriaw, 2007; Nuraddis et al., 2011; Dinka, 2012). The average age at first calving (35.3 months) in the present study was in line with recent reports in the study area (Weldeslasse et al., 2012; Kumar and Tkui, 2014), other parts of

the country (Haile-mariam et al., 1993; Kebede et al., 2015) and in Tanzania (Asimwe and Kifaro, 2007). Our finding has shown an earlier age at first calving than some previous reports in other parts of the country (Shiferaw et al., 2003; Tadesse et al., 2010), Malawi (Agyemang and Nkhonjera, 1990) and Zimbabwe (Masama et al., 2003), but later age at first service than in other reports (Negussie et al., 1998; Yifat et al., 2009). Late calving could be caused by management, fertility, or other health problems (Zavadilová and Štípková, 2013), and could be resolved through provision of proper breeding, and improvement in health and farm management.

The mean first postpartum estrus period (day open) in the study area was 114.6 (range 9 to 480) days (Chapter 3). Though this is in agreement with previous reports in the region (Negussie et al., 1998), and is better than other reports in HF/crossbred cattle in Kenya (149 days) (Wangdi et al., 2014) and in HF cattle in Sudan (167.8 days) (Gader et al., 2007), it is longer than the recommended voluntary waiting period of 50-80 days postpartum. A shorter first postpartum estrus period was reported in other parts of Ethiopia (Dinka, 2012), which may be due to better nutritional and reproductive health management. When the first postpartum estrus period is short, there is a high calf production per lifespan (Habib et al., 2010). Nutrition, season, age, parity, lactation, suckling and management could all influence the duration of postpartum anestrus (Lamming et al., 1981), consequently, affecting calving interval. A previous report from Kenya has shown that stall-fed cattle had a significantly shorter first postpartum estrus and required fewer services per conception than grazed animals (Mbugua et al., 1999). This indicates that provision of proper nutrition and optimum farm management is of paramount importance in tackling long first postpartum estrus period as well as calving interval.

Calving interval in crossbred cows in the study area (Chapter 3) was in agreement with previous reports (Dinka, 2012; Negussie et al., 1998). Furthermore, the calving interval in the UK over the past 20 years indicates a similar finding, that calving interval has increased by 24 days, from 386 to 410 days due to a reduction in first service conception rate and heat detection rates (Astley, 2015). In contrast, the present study has shown

shorter calving intervals than reports in other parts of Ethiopia (Haile-mariam et al., 1993; Shiferaw et al., 2003; Kumar and Tkui, 2014), Kenya (Muinga, 1992; Wangdi et al., 2014), Malawi (Agyemang and Nkhonjera, 1990) and Tanzania (Asimwe and Kifaro, 2007). The calving interval in the present study (Chapter 3) is long. However, it is better than reports in pure Zebu breeds that calve every two years (reviewed by Mukasa-Mugerwa, 1989; Honhold et al., 1992).

Anestrus, repeat-breeding, dystocia, RFM, endometritis and abortion were the major reproductive problems in the study area. Among 177 farms, 163 (92.1%) farms were affected by one or multiple reproductive problems. These problems are important factors for a delayed first postpartum estrus period and a longer calving interval. It is necessary to improve farm management to shorten the calving interval and enhance milk production to the benefit of the dairy sector. To achieve a calving interval of 365 days (one calf every 12-13 months), the calving to conception interval should not be more than 80-85 days (Peters, 1984). Inadequate nutrition is one of the causes of subfertility/infertility in dairy cattle (reviewed in Moellers and Riese, 1988). A previous report in Senegal has shown that supplementation of agro-industrial by-products, such as brewer's grains, molasses, groundnut cake, oyster shell and salt, improved reproductive performance, reducing the first postpartum estrus period and calving interval in Zebu cattle under tropical conditions (Sawadogo et al., 1998), and this may be an intervention strategy that could be considered in the present study area.

Profiles for P4 and estradiol were established in crossbred dairy cattle at different reproductive stages, and is demonstrated in Chapter 4. Crossbred cattle that were in estrus, in the present study, had low P4, in agreement with previous reports (Pope et al., 1976; Alam and Ghosh, 1994; Vadhanakul et al., 2008; Naik et al., 2013) and high estradiol levels comparable with other reports (Coe and Allrich, 1989; Singh et al., 2006). Diestrus and pregnant cattle had high P4, which was in agreement with previous reports (Alam and Ghosh, 1994; Muhammd et al., 2000; Lobago et al., 2009) and relatively low estradiol levels compared with cattle in estrus. Determining P4 levels in serum/milk can be useful to identify whether a cow is cycling or not. In the present study, serum/milk P4 levels in

anestrous cows was low (<0.5ng/ml), which was in agreement with a previous report (Arregu´ın et al., 1997). However, as cycling cows have low P4 during the follicular phase, two tests performed sequentially at 11-day intervals could reveal whether that animal is cycling or not.

The P4 test results in milk correlated with matched serum that proves laboratory P4 ELISA (Section 2.14.1) is suitable and is recommended for application in future related studies or applied for assessment of reproductive status of cattle at the veterinary clinics, AI centres and research centres in the country or elsewhere. Saliva samples do not contain proteins or fats that can interfere in the assays, compared with blood or milk samples (Kanchev et al., 1988), and can be collected both from lactating and non-lactating cattle (cows and heifers). In contrast, milk and serum have proteins and/or fats that can interfere with the assays. However, application of the same protocol that was used for serum and milk, in the present study (Section 2.14.1), to determine P4 in saliva and urine did not produce an acceptable correlation between P4 levels in saliva and urine and P4 levels in matched serum. Hence, a more sensitive ELISA kit should be used to validate the findings (P4 profiles) in saliva or urine. This study will form a basis for the study of hormonal profiles of local cattle breeds of Ethiopia and could be a valuable future field of study.

In the present studies (Chapter 4 and 5), cystic ovarian disease was one of the major causes of repeat-breeding, and was observed in 30% of animals that were presented with a history of repeat-breeding. Previous studies that used ultrasonography have suggested that follicles typically ovulate at 13-17 mm in diameter (Ginther et al., 1989). However, follicles that persist at 17mm in diameter or over may be regarded as cystic (Hatler et al., 2003). The diameter of the cyst may reach 25mm or more, and persist on the ovarian surface for 10 or more days (Roberts, 1971; Cook et al., 1991; Silvia et al., 2002; Youngquist and Threlfall, 2007). Ovarian cysts are defined as mature follicles that fail to ovulate at the appointed time during the estrous cycle (Peter, 2004), and the major cause of anovulation is insufficiency of LH pulses, which could be due to poor BCS and negative energy balance (Wiltbank et al., 2010). Cows that have a luteal cyst usually have serum P4 $\geq$ 0.5 ng/ml, while those with a follicular cyst have P4 $\leq$ 0.5 ng/ml (Farin et al., 1992).

In the present study (Chapter 4), we have determined the P4 levels of cows and heifers that had cystic ovaries (follicular cysts). Due to small number of cows and heifers with this problem, the data is not shown. However, the P4 levels (mean) of three cows that had ovarian follicular cysts, in the present study (Chapter 4), in matched serum and milk, were <0.5 ng/ml, which is in agreement with a previous report (Farin et al., 1992). None of the cows in this work were diagnosed with luteal cysts (Chapter 4 and 5). Hence, the cause of anestrus in the present study could be due to small reproductive tract and/or ovaries without palpable structures (Section 5.4.2) which is in agreement with a previous report (Montiel and Ahuja, 2005). When ovaries are without palpable structures (follicles or CL), follicles do not reach maturity to ovulate, which causes anestrus (Moro et al., 1994). Lack of a balanced diet (though animals with good BCS were included in Chapters 4, 5 and 6), and poor housing conditions (poor flooring and drainage) that cause stress (De Kruif, 1978), particularly in smallholder farms could be some of the factors causing anestrus. Other factors causing prolonged postpartum anestrus could be breed, age, number of calvings, milk yield, calving season, presence or absence of the bull, delayed uterine involution, dystocia and general health status (reviewed in Montiel and Ahuja, 2005).

Problem of anestrus and repeat-breeding can be avoided by improving nutrition (Beam and Butler, 1999; Ferguson, 1996). Improper nutritional management has an effect on ovarian activity (Webb et al., 2004). Poor nutrition, improper insemination and timing of AI, poor semen quality and early embryonic mortality could be causes of repeat-breeding. A recent report has shown the negative effect of dystocia on reproductive performance of HF cows (Hossein-Zadeh, 2016).

Addressing proper AI services, control and treatment of dystocia, retained fetal membrane, endometritis, and diseases and factors causing abortion can enhance reproductive performance by shortening the first postpartum estrus period and consequently the calving interval. AI is widely practiced in the study area, however due to various reasons, such as poor motivation or lack skill of AI technicians (Chebo and Alemayehu, 2012) or semen quality, as cows fail to conceive despite multiple inseminations, some farmers own breeding bulls. A recent study in the region evaluated

sperm motility and viability in frozen semen straws at field level and reported that both sperm motility and viability were within the normal range (Ashebir et al., 2016), which ensures the quality of semen. However, lack of experience of AI technicians in proper insemination and carelessness of proper timing (Chapter 3) requires better attention to effectively utilise AI technology and estrus synchronisation programme. This should improve the problem of repeat-breeding and shorten calving interval. A study in another part of Ethiopia that focused on local breed cattle reported that 63% of interviewed farmers do not use AI technology due to lack of knowledge, while others due to inaccessibility of AI services (9%), heat detection problem (7.2%) and small body size of local cows (3.6%).

Almost all AI technicians in the country are government employees, and, while most government workers have days off during weekends, AI technicians work throughout the whole week, without any additional incentives. Better management of AI technicians is encouraged by either increasing their salary or arranging other sorts of overtime payments. Furthermore, provision of continued professional development for AI technicians on determining the reproductive status of animals is a priority. In the present study, one reason for the large proportion of missed inseminations while the animals were in estrus was failure of AI technicians to turn up to meet appointments, or arriving late after being contacted by the farmers (Chapter 3). This causes delayed insemination that leads to repeat-breeding and longer calving intervals.

The P4 levels in the blood or milk reflect the presence and activity of CL on the ovary. Following ovulation, the P4 level increases for the first 4 to 6 days, which increases to its maximum level between 10 to 17 days. Determining the P4 levels is one of the methods used to monitor reproductive status of animals. The use of on-farm P4 assays enable farmers and farm managers to focus on breeding management of their cattle, particularly with regular follow up of animals in estrus and getting their animals diagnosed for presence of pregnancy as early as possible post service. Estrus detection problems lead to incorrectly assuming and managing cycling cows and heifers as non-cycling. Furthermore, animals that are inseminated but not pregnant, and due to improper follow up for estrous behaviour, will be assumed as pregnant due to non-return to estrus between

18-24 days post service. However, poor estrus detection and the presence of silent estrus in the farms may require additional aid for estrus detection or early diagnosis of pregnancy. In the present study, few cows, 24/171 (14%), were assumed pregnant while they were not, whilst some were ignored from being followed for return to estrus, which would have a significant impact on the calving interval. Assessment of the reproductive status of crossbred cattle using simple, cost effective and reliable qualitative on-farm P4 tests (Chapter 5) changed farmers'/farm managers' perception towards their animals' actual reproductive status. It also validated findings from the questionnaire survey, mainly of animals reported as non-cycling (anestrus), as most studies in Ethiopia use questionnaire surveys to assess reproductive problems and reproductive status such as anestrus or repeat-breeding (Hadush et al., 2013; Moges, 2015). The present study has successfully shown application of rapid on-farm P4 tests in determining reproductive status of crossbred dairy cattle (Chapter 5), which is in agreement to previous reports (Ropstad and Refsdal, 1985; Kaker et al., 1993). However, the drawback of either qualitative or quantitative P4 tests is the inability of determining the length of pregnancy, although this could be supported by improved breeding records. Nevertheless, the study (Chapter 5) has proven that rapid on-farm P4 tests could be used for rapid assessment of cyclicity or non-cyclicity as well as diagnosing pregnancy as early as 18-days post service.

Identifying the reproductive performance and status of dairy cows is an important part of farm management. Cattle that are anestrus for a prolonged period and more than the recommended voluntary waiting period should need to be properly diagnosed to enable appropriate measures to be taken. In the present study, we have demonstrated that estrus synchronisation or induction has improved the reproductive status of anestrous cows/heifers. Previous reports have shown that application of CIDR devices for 10-12 days improved fertility in cattle (Broadbent et al., 1993; Gordon, 1999). In the present study, both cycling and non-cycling cattle came into estrus (100%) following the treatment (Chapter 6). This improved the livelihood of the smallholder farmers, as well as improving profitability of organised commercial farms. As the study was carried out in individual farms that had small number of animals and very few animals (5 heifers and 5

cows) came naturally into estrus, no control group was used to compare the conception rate with the treatment group. This opens a future potential research direction to compare the conception rate between animals that come into estrus naturally and those treated with estrus synchronisation hormones.

The conception rate of cattle that were bred with natural mating was non-significantly higher than those bred by AI. This could be due to semen handling problem, thawing time or improper AI; although a recent study in the region reported that the sperm motility and viability of frozen semen evaluated at AI centres were within the recommended range (Ashebir et al., 2016). Therefore, the conception difference between cattle that were bred by AI may not be a result of semen quality. The calving interval in the present study was long (Chapter 3), which could be due to several factors like a longer postpartum estrus period, poor estrus expression, or detection, improper timing of AI, and reduced conception rate at first AI (Murugavel et al., 2003). Furthermore, a recent report that evaluated constraints of AI in other, neighbouring parts of the region, showed that time of insemination and heat detection problems were among the major constraints affecting conception rate (Samre et al., 2015). As estrus detection in most farms is not properly performed, application of fixed-time AI is important. Estrus synchronisation can avoid or reduce the need for estrus detection. Fixed-time AI protocol that synchronises growth of the follicle, regression of the CL and ovulation, is useful to improve reproductive performance, as all animals are inseminated whether they show estrus or not (Colazo and Mapletoft, 2014). Furthermore, application of fixed-time AI twice at 48 and 72 hr post CIDR removal could solve problem of insemination timings or delayed ovulation. In the present study, administration of eCG at CIDR removal was aimed to facilitate ovulation. Ovulation that occurs around 48 hr of eCG treatment would be covered by insemination at 48 hr post device removal; a delayed ovulation on the other hand would be covered by inseminating animals 72 hr post device removal. A previous study that compared reproductive responses of dairy cows following TAI showed that fixed-time AI at 48hr or 72hr post induction of luteolysis provided low pregnancy outcome (44.7 & 43.1 %, respectively) (Hillegass et al., 2008). This result might have been improved if animals

were inseminated twice at 48 and 72 hr post induction of luteolysis. A recent report on the effect of a modified GnRH-PGF2α combination estrus synchronisation protocol, from the College of Veterinary Medicine, Mekelle University, Ethiopia showed a high pregnancy outcome (70.5%) following fixed-time AI twice at 48 and 72 hr post GnRH administration (Mekonnin et al., 2016). This supports the present findings. Administration of GnRH at the onset of estrus increases LH surges and prevents delayed ovulation (Kaim et al., 2003). In the present study, this was supported by administration of eCG, which has LH- and FSH-like activity.

Our use of rapid-on-farm P4 ELISA to determine reproductive status of cattle (Chapter 5) prior to estrus synchronisation enabled us to exclude animals that had high P4 and history of recent insemination (breeding). Though animals with recent history of insemination had high P4 during the first P4 test, application of a second on-farm P4 test 11 days later enabled us to confirm whether they were clinically pregnant or not. Those with a fluctuating P4 in the first or second P4 tests carried out twice at 11-day intervals were considered normally cycling (non-pregnant) and included in the controlled breeding programme (Chapter 6). None of the cattle that were administered with the estrus synchronisation hormones were recorded with problems of abortion due to the treatment effect. Incorporating P4 tests in estrus synchronisation programmes can avoid the risk of abortion due to PGF2α administration. To improve reproductive performance of crossbred cattle in the study area as well as in other parts of the country, regular monitoring of the reproductive status of animals should be carried out. Animals with fertility problems, such as a prolonged postpartum anestrus period and repeat-breeding should be given appropriate treatment. The causes of repeat-breeding should be thoroughly examined to properly diagnose and enable appropriate treatment. The high conception rate in the present study shows no negative effect of the duration of keeping the CIDR devices. Concerns have been raised over application of CIDR devices beyond 7 days (Romano and Fahning, 2013). Considering the inconsistency in conception rates when using the standard 7-day CIDR (Broadbent et al., 1993; Kim et al., 2007; Dickson et al., 2012), the high conception rate recorded in the present study justifies the extra days the device was

maintained. This protocol shows a high estrus response and conception rates. Adoption of the protocol as a routine breeding management regime to improve fertility in the study area and in other part of the country should be encouraged.

Improving fertility of dairy cattle secures regular availability of a calf crop and replacement heifers, as well as availability of milk in the farms, to satisfy customers' demand. Milk is the best source of nutrients providing carbohydrate, fat, protein, vitamins and minerals, and water (Adesina, 2012). However, the composition of milk can be affected by various factors. The macronutritional composition of milk was determined for crossbred dairy cows in Tigray, Ethiopia, with reference to HF cows in Edinburgh, UK, and the effect of reproductive status, farm (nutritional) management, stages of lactation, parity and breed on milk composition was evaluated (Chapter 7). The macronutrient levels of milk in crossbred cows was significantly lower than in HF, which could be due to genetics (O'Mahony, 1988), breed (Adesina, 2012) and farm (nutritional) management (Smith et al., 2000; Zeleke, 2007). Milk fat in both breeds was significantly affected by reproductive status, farm (nutritional) management and stages of lactation, but not by parity. Previous reports have suggested that milk fat levels can be affected by genetics, physiological status of the cow breed (O'Mahony, 1988) and herd management (Zeleke, 2007). In both cattle breeds, the milk fat and TS content was significantly higher in pregnant than in non-pregnant cows. This is in agreement with the report that the physiological status of the cow affects milk fat levels (O'Mahony, 1988). In contrast to our findings, previous studies reported no effect (Gurmessa and Melaku, 2012) or lower milk fat and protein level in pregnant compared to non-pregnant cows (Lee et al., 1997).

The present study showed lower SNF content in both pregnant and non-pregnant cows and lower fat in non-pregnant cows than in reports by Gurmessa and Melaku (2012). Pregnancy results in a decrease in milk fat and protein levels (Loker et al., 2009). However, in the present study, stage of pregnancy did not affect the milk fat, TS and SNF levels in crossbred cows, and milk fat in pregnant cows in both crossbred and HF cows had significantly higher than non-pregnant. The milk fat, protein and TS levels in HF and protein in crossbred cows significantly increased with advancement of pregnancy, with a

non-significant increase in milk fat in crossbred cows, which is in contrast to a previous study that reported a decrease in milk fat, from approximately 90 days in pregnant cows, compared with non-pregnant cows (Bormann et al., 2002).

Milk protein content was significantly affected by reproductive status in crossbreeds, which is in agreement with a previous report (Bormann et al., 2002), while lactation stage affected protein levels in both breeds, but farm management or parity had no effect. Milk protein levels in crossbred cows in the present study was in agreement with reports in local breed cows in Nigeria (Adesina, 2012). However, Adesina (2012) reported higher lactose levels. Farm management did not affect protein, lactose or SNF in both breeds, but significantly affected milk fat in both breeds and TS in HF cows. The higher milk fat level in crossbred cows from smallholder farms than from organised commercial farms could be due to nutritional variation, as cows in organised farms are more exposed to fresh green forage. This is in keeping with a previous report that suggested cows managed under fresh pasture have lower milk fat content compared with cows exposed to silage (Elgersma et al., 2004; Couvreur et al., 2006). However, in contrast to the present findings in crossbred cows whereby cows exposed to green forages (organised commercial) produced lower fat than those not provided, and previous reports (Elgersma et al., 2004; Couvreur et al., 2006), HF cows managed outdoors on grazing had comparatively higher milk fat than those in intensive (indoor) management. Milk fat content was the most affected macronutrient content in both breeds, with comparatively lower overall macronutrient content in crossbred cows. This emphasises the need for proper nutritional and breeding management in crossbred cattle to enhance the nutritional value of milk in the study area.

8.3. GENERAL CONCLUSION

The reproductive performance of crossbred cattle in the study area is not satisfactory which could be due to reproductive diseases and poor breeding and nutritional management. Application of rapid on-farm P4 tests could solve the problem of estrus detection and early pregnancy diagnosis as well as the identification of cycling and non-cycling cattle. P4 and estradiol profiles recorded in this study will form the basis for further studies in the country. However, P4 levels in saliva and urine need to be validated as P4 levels in matched serum did not correlate. The accuracy of both qualitative on-farm and quantitative laboratory P4 assays was similarly high in determining both pregnancy and non-pregnancy, and application of these methods can help to control ovarian function and assist controlled breeding programme. High estrus response and conception rate was obtained using a CIDR-PGF2α-eCG combination estrus synchronisation protocol. Therefore, this protocol is highly recommended to the Ethiopian farming community. Significantly lower milk fat recorded in crossbred than HF cows could be due to breed and nutritional management. These studies offer opportunities for establishing simple field reporting of reproductive status in crossbred dairy cattle, which can have a major impact on breeding management and productivity.

8.4. STUDY LIMITATIONS AND CHALLENGES

In most smallholder farms, there were no proper breeding records. Most questionnaire data including age of heifers/cows, date of insemination, calving date or other related traits were based on (farmers') memory rather than recorded information. This might have influenced the reliability of some of the data, particularly in Chapter 3.

Restraining the study animals during sample collection, particularly for blood and saliva samples, was the hardest challenge of the field study in crossbred dairy cattle. It required more than two strong people to restrain a cow or a heifer. Heifers were more excitable than cows. Some cows that were not approachable due to aggressiveness and attempted butting were not included in the study.

Some farmers would not participate in the questionnaire study (Chapter 3) and some that volunteered for the questionnaire study were not willing to participate in subsequent studies. There were two major reasons given by the farmers for not participating in this project: the first was, farmers complain, for example, that "previous researchers did not come with significant or any solution to our (cattle) problems and that most researchers collect whatever data they want and disappear". The other reason given by some farmers was the fear that their animals would experience pain during blood collection and lose body condition due to stress. However, a few farmers did not volunteer to participate without an explanation. Farmers who did not participate may have been those who had problems on their farm. This might have caused a selection bias to the study, as only those who volunteered were included, which may make it difficult to generalise the findings in this project to a wider population of crossbred cattle in the region (study area), Chapter 3 in particular.

In the present study, cows and heifers that naturally came into estrus were either inseminated at the time or they were required for hormonal profile studies (Chapter 4), and/or those with problem of repeat-breeding included in estrus synchronisation study (Chapter 6). Therefore, there was a limitation in the number of animals that naturally came into estrus, available to include as a "control group" to compare their conception rate with

cattle treated with CIDR-PGF2α-eCG combination estrus synchronisation protocol, which therefore requires further controlled study. The farmers were not willing for their animals to be left untreated and used as a control group. Therefore, it is not possible to predict how many of these animals would have come into estrus without intervention. However, in previous studies, where control animals were used, lower estrus response and conception rate were recorded in control group compared with treated animals (Irmã et al., 2008).

Samples collected from crossbred cattle in Ethiopia were heat treated at 56^{o}C for 30 min prior to importing them to the United Kingdom, to avoid the risk of spreading foot and mouth disease (Section 2.7). Consequently, protein hormones, such as FSH and LH could not be measured. FSH and LH profiles have yet to be determined in crossbred cattle in Ethiopia.

8.5. FUTURE DIRECTIONS TO IMPROVE LIVESTOCK REPRODUCTIVE PERFORMANCE AND PRODUCTIVITY

- ✓ Adopt the improved protocol to assess reproductive status and manage productivity.
- ✓ Seek to improve protocols that accurately determine fertility status of both local and crossbred cattle in Ethiopia through application of on-farm ELISAs and portable ultrasound scanners.
- ✓ Longitudinal studies of hormonal profiles of steroid and gonadotrophin hormones at different reproductive stages in both local and cross breeds; however, support is needed to gain more, which could be from government or from external international source (DIFD, Gates, etc.).
- ✓ Assess milk quality and develop strategies to improve the low macronutrient level in milk; resource needed, but also government funding is required for large farms initially.
- ✓ Inform government policy, including salary of AI technicians could be based on per number of AI provided or per successful pregnancy.
- ✓ Training - both farmers and AI technicians. Developing online resources through mobile phone App in local languages to educate and train farmers, veterinary students and AI technicians.

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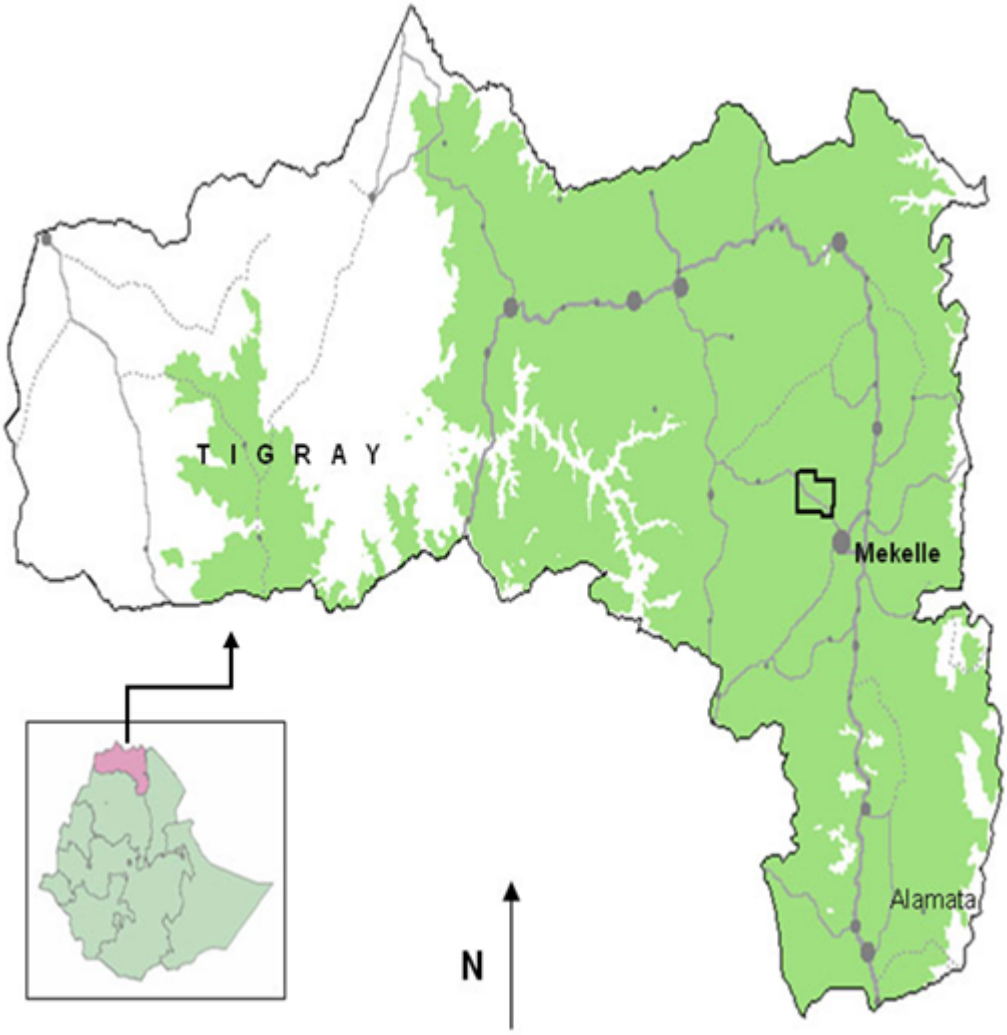
APPENDIXES

Appendix 1: Map of Tigray and Ethiopia (in the square) (**Source:** Water, 2011).



Appendix 2: Questionnaire for surveying reproductive performance and problems of crossbred cows/heifers prepared in English.

1. Name of the farm or farm owner_____.

2. Address of the farm: _____; phone no. _____

3. When was the farm established? ☐ 1-5 years ☐ 6-10 years ☐ > 10 years

4. Type of farm: ☐ Intensive ☐ Semi-intensive ☐ Extensive

5. Number of cattle in the farm (owned) _____.

a. No. of cows_____ c. No. of bulls_____

b. No. of heifers_____ d. No. of calves_____

6. Types of feed cows or heifers are fed:

☐ Hay ☐ Straw ☐ Concentrate ☐ Concentrate and hay or straw

7. How many times do animals get feed per day? _____

8. Do animals get water regularly? _____; how many times a day?_____

9. Age of cows and/or heifers and parity (years):

Cow 1. _____ (parity_____); heifer 1. _____

Cow 2. _____ (parity_____); heifer 2. _____

Cow 3. _____ (parity_____); heifer 3. _____

Cow 4. _____ (parity_____); heifer 4. _____

Cow 5. _____ (parity_____); heifer 5. _____

Cow 6. _____ (parity_____); heifer 6. _____

Cow 7. _____ (parity_____); heifer 7. _____

Cow 8. _____ (parity_____); heifer 8. _____

Cow 9. _____ (parity_____); heifer 9. _____

Cow 10. _____ (parity_____); heifer 10 _____

10. What is the number of pregnant cows/ heifers in the farm?

A) Cows: _____ (trimester:_____ 1st; _____2nd; ____3rd)

B) Heifers: _____ (trimester: _____1st; _____2nd; _____3rd)

11. Method of insemination? Natural AI Both

12. Number of animals in estrous now? A) Cows: _____; B) Heifers: _____

13. How many lactating cows do you have? _____

14. What is the number of anestrous animals in the farm? A) Cows? _____; B) Heifers:

15. Have any of your cattle encountered reproductive diseases or problems in the past?

☐ Yes ☐ No

16. If yes, what reproductive diseases or problems were observed?

_____

17. Did any of your cows suffer from abortion? ☐ Yes ☐ No

18. If yes, what month of the pregnancy did abortion occur?

a. before 3 months c. over 6 months

b. 3 to 6 months d. a, b and c

19. Did any of your cows encounter pregnancy overdue in the past? ☐ Yes ☐ No

If yes, how many months was the cow pregnant before it was given veterinary assistance?

_____? No. of cows diagnosed positive? _____

20. Did any of your cows or heifers give birth to a dead calf at a normal gestation length (9 months) in the past? ☐ Yes ☐ No

If yes, how many cows? _____

21. Did any of your cows suffer from dystocia in the past? ☐ Yes ☐ No

If yes, how many of your cows suffered from dystocia? _____

22. Did any of your cows suffer from metritis in the past? ☐ Yes ☐ No

If yes, number of cows or heifers suffered from metritis? _____

23. Did any of your cows or heifers suffer from vaginal, cervical or uterine prolapse in the past?

☐ Yes ☐ No

If yes, number of cows or heifers with vaginal prolapse? _____; Cervical prolapse?_____ or uterine prolapse?_____

24. Did any of your cows encounter RFM in the past? _____

How did you solve the problem?

_____

25. Have any of your cows or heifers been repeater (repeat breeder)?

If yes, what do you think the cause/s for the animal to come back in to estrus again?

What measure did/do you take to get the animal pregnant?

_____

26. Did any of your cows suffer from metritis in the past? ☐ Yes ☐ No

If yes, what treatment did the cow get?_____

What was the effect of the treatment in the reproductive status of the cow after that?_____

27. Did any of your animals come to estrous, but missed insemination or mating in the past?

☐ Yes ☐ No

If yes, why was the animal not inseminated or mated?

a. Lack of bull

b. Lack of AI technician

c. Lack of means of communication to AI technician

d. Called AI technician, but did not come to inseminate or did come very late

e. Reproductive tract problem

f. Size of the animal was very small to breed

g. Lack of nutrition

h. All or some of the above causes

28. Did any of your animals suffer from any health problem other than reproductive health?

If yes, please describe the disease and signs the animals showed.

_____

29. What is your future plan to improve the reproductive performance of your animals?

_____

30. What do you think the government (agriculture, universities, etc.) and private sectors should do to support the improvement of fertility of your animals? _____

31. Do you have any comment? _____

Thanks!!

Appendix 3: English to Tigrigna (Ethiopian language) translated Questionnaire.

ኣብ መቐለን ከባቢኣን ዝተኣታተዋ ድቓላ ፀባ ከፍቲ ዘጋጥመን ዘሎ ኩነታት ሕማም ስነ ተዋልዶ ንምፅናዕ ስለዝተሓሰበ እዚ ቅጥዒ እዚ ካብ ሓረስቶትን ማነጀራትን ክእከብ ዝተደለየ መረዳእታ ንምእካብ ዝተዳለወ እዩ

1. ሽም ወናኒ ትካል /ሕርሻ ____

2. ትካል ዝርከበሉ ቦታ/ኣድራሻ:________________; ስልኪ ቁፅሪ ____________

3. እዚ ትካል ካብ ዝጣየሽ ክንደይ ዓመት ገይሩ? ካብዞም ዝስዕቡ ምረፁ

a) ካብ 1-5 ዓመት

b) ካብ 6-10 ዓመት

c) ልዕሊ 10 ዓመታት

4. ናይዚ ትካል ዓይነት/ናይ አረባብሓ ሜላ:

a) ሙሉእ ብሙሉእ አሲርካ ምቅላብ

b) ብክፋል አሲርካ ምቅላብ

c) ስዲ ጋህፂ

5. ክንደይ ከፍቲ አለዋኩም? (በዝሒ ዘለዋኹም ከፍቲ በዚ ዝስዕብ መሰረት ብቑፅሪ ኣቐምጡ)

a) ምውላድ ዝጀመራ አላሕም__________

b) በዝሒ ኣዕሩሕ/ምውላድ ዘይጀመራ_________

c) በዝሒ አብዑር__________

d) በዝሒ አምራኩት_________

6. ንእንስሳኩም እትቐርብሉን ቀለብ ዓይነት ግለፁ:

a) ጸርቋ

b) ሓሰር/ቃንጫ

c) ዝተመጣጠነ መግቢ

d) ዝተመጣጠነ መግብን ሓሰርን/ጸርቅን ሓዊስካ

7. እንስሳትኩም ንመዓልቲ ክንደይ ግዜ ቀለብ ተቕርቡለን? ___________________

8. እንስሳትኩም ኩሉ መዓልቲ ማይ ይረኽባዶ? _________; ክንደይ ግዜ ኣብ መዓልቲ?___________

9. ኣብ ትካልኩም ዘለዋ እንስሳ ብዕድመ ብዝወለዳኦ ብዝሒ ኣፍራያተን ኣቀምጡ

ላሕሚ ቁፅሪ 1. __________ (ኣብ ሂወታ ዝወለደተን በዝሒ) ዓርሒ ቁፅሪ 1.__________

ላሕሚ ቁፅሪ 2. __________(ኣብ ሂወታ ዝወለደተን በዝሒ); ዓርሒ ቁፅሪ 2.__________

ላሕሚ ቁፅሪ 3. __________ (ኣብ ሂወታ ዝወለደተን በዝሒ); ዓርሒ ቁፅሪ 3.__________

ላሕሚ ቁፅሪ 4. __________ (ኣብ ሂወታ ዝወለደተን በዝሒ); ዓርሒ ቁፅሪ 4.__________

ላሕሚ ቁፅሪ 5. __________ (ኣብ ሂወታ ዝወለደተን በዝሒ)ዓርሒ ቁፅሪ 5.__________

ላሕሚ ቁፅሪ 6. __________ (ኣብ ሂወታ ዝወለደተን በዝሒ)ዓርሒ ቁፅሪ 6.__________

ላሕሚ ቁፅሪ 7. __________ (ኣብ ሂወታ ዝወለደተን በዝሒ)ዓርሒ ቁፅሪ 7.__________

ላሕሚ ቁፅሪ 8. __________ (ኣብ ሂወታ ዝወለደተን በዝሒ)ዓርሒ ቁፅሪ 8.__________

ላሕሚ ቁፅሪ 9. __________ (ኣብ ሂወታ ዝወለደተን በዝሒ)ዓርሒ ቁፅሪ 9.__________

ላሕሚ ቁፅሪ 10. __________ (ኣብ ሂወታ ዝወለደተን በዝሒ)ዓርሒ ቁፅሪ 10.__________

10. ምልኦ ዘለወን ኣላሕምን ኣዕሩሕን ክንደይ እየን?

A) ኣላሕም:

- ናይ 3 ወርሒ ምልኦ ዘለወን በዝሒ:_______

- ናይ 6 ወርሒ ምልኦ ዘለወን በዝሒ:_____
- ናይ 9 ወርሒ ምልኦ ዘለወን በዝሒ:_____

B) ኣዕሩሕ:

- ናይ 1-3 ወርሒ ምልኦ ዘለወን በዝሒ:_____
- ናይ 4-6 ወርሒ ምልኦ ዘለወን በዝሒ:_____
- ናይ 7-9 ወርሒ ምልኦ ዘለወን በዝሒ:_____

11. እንስሳኹም ብኽመይ ተዳቕሉወን?

a) ብኣብዑር ነስርረን

b) ሰብ ሰራሕ ምድቃል

c) ክልቲኡ

12. ኦሚ መዓልቲ ስራ ዝደለያ (ዘላዓላ) ከፍቲ ክንደይ ኣለዋ?

a) በዝሒ ኣላሕም: _____

b) በዝሒ ኣዕሩሕ: _____

13. ክንደይ ዝሕለባ ኣላሕም ኣለዋ? _____

14. ኦሚ መዓልቲ ስራ ዘየልዓላ ከፍቲ ክንደይ ኣለዋ?

a) በዝሒ ኣላሕም: _____

b) በዝሒ ኣዕሩሕ: _____

15. ኣብ ዝሐለፈ እዋናት ኣላሕምኩም ናይ ሕርሲ ሕማም ገጢሙወን ይፈልጥ ዶ? እወ ወይ ኣይገጠመንን ብምባል መልሱ

a) እወ

b) ኣይገጠመንን

16. መልስኩም እወ እንተኮይኑ እንታይ ዓይነት ሕማም ወይ ናይ ወሊድ ፀገም ከም ዝገጠመን ብዝርዝር ፀሓፉ

_____

17. ኣብ ዝሓለፈ እዋናት ዘወሃያ/ዘጨንገፉ ኣላሕም ኣለዋኩም ዶ? እወ ወይ አየወሃያን ብምብል መልሱt?

a) እወ

b) ኣያወሃያን

18. መልስኩም እወ እንተኮይኑ ምልኦ ካብ ዝሕዛ ኣብ መበል ክንደይ ወርሒ ምኳኑ ይግለፁ

a) ቅድሚ 3 ወርሒ

b) ኣብ ሞንጎ 3 ን 6 ወርሒ

c) ድሕሪ 6 ወርሒ

19. ካብ ክወልዳሉ ዝግባእ መዓልቲ ኣሕሊፈን ዝወለዳ ኣለዋኩም ዶ?

a) እወ

b) ኣይፉሉን

መልስኩም እወ እንተኮይኑ ሕክምና ከይተገበረለን ክንደይ ጊዜ ፀኒሐን?_____?

እዋነን ዘሕለፉ መላዓት ኣላሕም ክንደይ ይኾና? _____

20. ትሽዓ ወርሐን መሊአን ግን ድማ ምዉት ዝወለዳ ኣዕሩሕ ወይ ኣላሕም ኣለዋኩም ዶ?

a) እወ

b) ኣይፉሉን

መልስኩም እወ እንተኮይኑ ክንደይ ኣላሕም ምውት ወሊዱን? _____

21. ኣብ ምውላድ ዝተፀገማ ኣላሕም ኣጋጢሙኩም ይፈልጥ ዶ?

a) እወ

b) ኣይፋሉን

መልስኩም እወ እንተኮይኑ ክንደይ ኣላሕ ፀገም ምውላድ ገጢሙወን? _____

22. ናይ ማህፀን ቁስሊ/ረክሲ/ ዘጋጠመን ኣላሕም ኣለዋኩም ዶ?

a) እወ

b) ኣይፋሉን

መልስኩም እወ እንተኮይኑ ክንደይ ኣላሕ ፀገም ምቁሳል ማህፀን ገጢሙወን?

_____

23. ኣብ እዋን ወሊድ ናይ ብልዕቲ ወይ ናይ ኣፍ ደገ ማህፀን ወይ ናይ ማህፀን ምግልባጥ ዝገጠመን ኣላሕም ኣለዋኩም ዶ?

a) እወ

b) ኣይፋሉን

መልስኩም እወ እንተኮይኑ ክንደይ ኣላሕ ፀገም ገጢሙወን?

a) ናይ ብልዕቲ ምግልባጥ ዝገጠመን በዝሒ_____ወይ

b) ናይ ኣፍደገ ማህፀን ምግልባጥ ዝገጠመን በዝሒ_____ ወይ

c) ናይ ማህፀን ምግልባጥ ዝገጠመን በዝሒ_____

24. ኣብ ዝሐለፈ እዋናት ሓከብ ምውዳቅ ዝኣበየን ኣላሕም ኣለዋኩም ዶ?

a) እወ

b) ኣይፋሉን

ኣጋጢሙኩም እንተኮይኑ እቲ ፀገም ከመይ ፈቲሕኩምዎ?

---

25. ንተከታታሊ ክልተ ግዜን ልዕሊኡን ተሰሪራ ወይ ተዳቂለን ምልኦ ዘይሓዛ ኣላሕም ነይረናኩም ወይ ኣለዋኩም ድየን?

a) እወ

b) ኣይፋሉን

መልስኩም እወ እንተኾይኑ ምክንያቱ እንታይ ይኸውን?

ምልኦ ንክሕዛ ዝገበርኩምዎ ፃዕሪ እንታይ ኣሎ?

---

26. ናይ ማህፀን ቁስሊ/ረክሲ/ ዘጋጠመን ኣላሕም ኣለዋኩም ዶ?

a) እወ

b) ኣይፋሉን

መልስኩም እወ እንተኮይኑ እንታይ ዓይነት ሕክምና ተገይሩለን?

እቲ ዝወሰድኦ ሕክምና እንታይ ምምሕያሽ ኣምፂኡለን?

a) ፅቡቅ ምምሕያሽ ኣምፂኡለን

b) ምንም ለውጢ የብሉን

27. ካብ ኣላሕምኩም ስራ ኣርእያ ግን ድማ ከይተዳቀላ ዝሓለፈን ኣለዋ ዶ

a) እወ

b) ኣይፋሉን

መልስኩም እወ እንተኮይኑ ንምንታይ ከይተዳቀላ ወይ ከይተሰረራ ሓሊፉወን?

a. ዝሰርር ብዕራይ ስለዘየለ

b. ሰብ ሰራሕ መዳቀሊ ስለዘየለ

c. ነቶም ዘዳቅሉ ንምርካብ ሕፅረት መራኸቢ ስለዘሎ

d. ዘዳቅሉ ሰብ ሞያ ፀዊዕና ከይመፀ ብምትራፉ ወይ ብምድንጓዩ

e. ናይታ ላሕሚ ማህፀን ፀገም ስለዝነበሮ ኣያዳቀልናያን

f. እታ እንስሳ ብጣዕሚ ደቃቅ ሰለዝነበረት

g. ናይ ቀለብ ሕፅረት ስለዝነበረ

h. ካብቶም ኣብ ላዕሊ ዝተጠቀሱ ኩሎም ወይ ፍርቆም ምክንያት እዮም

28. እንስሳኩም ካብ ስነ ተዋልዶ ወፃኢ ካልእ ሕማም ይሓማ ዶ? _____

መልስኩም እው እንተኾይኑ ዓይነት ሕማምን ምልክታትን ይግለፁ.

_____

29. ናይ እንስሳኹም ናይ መፍረያይነት ኩነታት ንምዕባይ ታይ ትልሚ ኣለኩም?

_____

30. ናይ ከባቢ ኣካላት ማለት ቤት ፅሕፈት ሕርሻ፣ ዩኒቨርሲቲ፣ ሕርሻ ምርምር፣ ወዘተ መፍረያይነት ንምምሕያሽ እንታይ ክሕግዙ ኣለዎም ትብሉ?

_____

31.ካልእ ርኢቶ ኣለዎም ዶ?

_____

የቐንየለይ

Appendix 4: Cross-reactivity of estradiol assay (company report: Demeditec Diagnostics GmbH).

Steroid	Cross Reactivity (%)	Steroid	Cross Reactivity (%)
Estradiol	100	11-Desoxycorticosterone	0
Estrone	0.2	21-Desoxycortisol	0
Estriol	0.05	DHT	0
Androstenedione	0	Dihydroepiandrosterone	0
Androsterone	0	20-Dihydroprogesterone	0
Corticosterone	0	11-Hydroxyprogesterone	0
Epiandrosterone	0	17a-Hydroxyprogesterone	0
16-Epiestriol	0	17a-Pregnenolone	0
Estradiol-3-sulfate	0	17a-Progesterone	0
Estradiol-3-glucuronide	0	Pregnanediol	0
Estradiol-17α	0	Pregnantriol	0
Estriol-16-glucuronide	0	Pregnenolone	0
Estrone-3-sulfate	0	Progesterone	0
DHEA	0	Testosterone	0
11-Desoxycortisol	0		

Appendix 5: Assessment and follow up format for the on-farm P4 assays to assess reproductive status in crossbred heifers and cows.

1. Owners or farm name_____

2. Address: _____, Phone No. _____

3. Cow's/heifer's name_____; Problem reported_____

4. Age of the cow/heifer _____

5. Body condition score (1-5)? _____

6. Number of calving? _____

7. Date of last calving? _____

8. Date the animal come to estrus after calving? _____

9. When did the animal show signs of estrus?

☐ Morning ☐ Afternoon ☐ Evening

10. When was the animal mated or inseminated?

☐ Morning ☐ Afternoon ☐ Evening

11. Date of last mating or insemination? _____

12. Has it come back to estrus again? _____; if yes, how many times did it come back to estrus after insemination? _____; when did it come back to estrus, last? _____

13. What method was used to conceive the animal? ☐ Natural mating ☐ AI

14. How many times was the animal mated or inseminated when it came to estrus?

☐ Once ☐ Twice ☐ More

15. Did you get the animal checked for pregnancy? Yes ☐ No ☐

16. When was the animal last checked for presence of pregnancy? _____

17. Examination of ovary by rectal palpation (tick the findings):

a. Presence of follicular structure on the ovary

b. Presence of CL

c. Presence of fetal membrane

d. Presence of fetus

e. Presence of placentomes

f. Other findings (like pyometra, fetal maceration or mummification, follicular/luteal cyst) _____

g. Diagnosis: the cow /heifer is ☐ Pregnant ☐ Not pregnant, but cycling

☐ Neither pregnant nor cycling (no follicle or CL on the ovary)

16. Dipstick (P4 Rapid/RPEDT)

i. First test: Date_____; P4 level: ☐ high ☐ low ☐ very low

ii. Second test: Day_____; P4 level: ☐ high ☐ low ☐ very low

17. On-farm P4 ELISA: First test result_____

Second test result_____

18. Reproductive status of the animal:

☐ cyclic ☐ pregnant ☐ anestrus (cystic ovary luteal) ☐ Follicular cyst

Other finding_____.

Appendix 6: Representative pictures of some of the procedures in milk composition analysis.