INFLUENCE OF NUTRITIONAL METABOLITES AND HORMONAL PROFILES ON REPRODUCTIVE PERFORMANCE OF LACTATING COWS

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A Thesis Submitted to Graduate School in Fulfillment of the Requirements for the Award of the Degree of Doctor of Philosophy in Animal Science of Egerton University

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DECLARATION AND RECOMMENDATION

DECLARATION

I declare that this Thesis is my original work and to my knowledge has not, wholly or in parts, been presented for the award of a Degree in any other University known to me.

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This Thesis has been submitted for examination with our Approval as University Supervisors.

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DEDICATION

I humbly dedicate this work to God the Almighty for enabling me to accomplish this task. To my late son, Jared Indetie, to my parents, Jared and Lydia Indetie for their love, support and encouragement, to my wife Ann Hoka, to my daughters Pauline and Jocyline and my son Jared Indetie (jnr). To my brothers, Livingstone and Daniel and the late George Indetie, to my sisters Winnie Masheti, Joan Okeyo, Sarah Indetie, the late Jocyline Guda and Grace Indetie, and all their families for their love, prayers and support.

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God bless all of them abundantly.

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ABSTRACT

Nutrition influences *postpartum* ovarian activity but the effects of *pre* and *postpartum* feeding on metabolte and hormonal changes and their subsequent influence on reproductive performance remains unclear. This study attempted to explain effects of nutrition on these changes and their ultimate impact on reproductive performance of lactating cows. Forty in-calf cows comprising 20 Friesians and 20 Sahiwals were randomly assigned to 5 dietary groups of 4 cows per breed. Upon calving, cows were fed a basal diet of *Rhodes grass* pasture and supplemented with dairy meal concentrate at the rate of 0, 1, 2, 3, and 4 kgs per day. Body weight and condition, milk yield and composition (% BF, % P, %SNF, density and freezing point), hormonal (progesterone and IGF-1) and metabolites (glucose, urea, NEFA and albumin) levels were measured weekly. The cows were observed for heat, inseminated and monitored up to the next calving. Days to commencement of luteal activity, insemination and calving intervals were also determined. Data was subjected to correlation, general linear models, repeated measures, principal component and regression analysis procedures of SAS. Sahiwals lost less body condition (-0.1 vs -0.4), commenced luteal activity (6 vs 10 weeks) and were inseminated (10 vs 14 weeks) earlier than Friesians. In both breeds, reduced levels of NEFA and threshold levels of 3ng/ml for progesterone coincided with days to commencement of luteal activity and insemination. Only 76% of the cows exhibited heat signs and were served, with 56% of them eventually calving. Friesians attained higher peak milk yield per week (88.9±1.7 vs 68.3±1.6 lts) than Sahiwals. Milk components and reproductive parameters were positively correlated in Friesian but negatively associated in Sahiwals. Services per conception for Friesians were 1.53 compared to Sahiwals 2.42. There were positive correlations (r=0.29) between days to luteal activity and insemination and negative correlations (r=-0.21) with calving intervals. Feeding levels had no effect on days to commencement of luteal activity but influenced metabolites and hormonal levels, particularly glucose and progesterone. Cows that calved expressed heat earlier (70.5±13.4 vs 96.8±21.5 days), had higher ($p<0.05$) mean levels of progesterone (3.78±0.6 vs 3.1±0.42 ng/ml) than those not calving. Cows conceived when IGF-1 levels exceeded 25ng/ml threshold levels. Nutrition had an effect on progesterone, IGF-1, glucose and NEFA that proved suitable predictors of the subsequent reproductive performance of cows after parturition.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACOD	Ascorbate Oxidase
ACS	Acyl-CoA-Synthetase
AFC	Age at First Calving
AI	Artificial Insemination
AIC	Akaike Information Criterion
ANOVA	Analysis of Variance
AR(1)	Autoregressive order one
ATP	Adenosine Triphosphate
BC	Body Condition
BCS	Body Condition Scores
BF	%Butter Fat
BHBA	βHydroxybutyrate
BW	Body Weight
Calfwt	Calf Weight
Cbon	Cow Body Condition Score
CF	Crude Fibre
CI	Calving Interval
CoA	Coenzyme A
CP	Crude Protein
CPM	Counts Per Minute
CS	Compound Symmetry
CV	Coefficient of Variation
D	Density
DIM	Days in Milk
Dins	Days to Insemination
dl	Deciliters
Dlac	Days to Luteal Activity
DM	Dry Matter
DMI	Dry Matter Intake

EB	Energy Balance
ECM	Energy Corrected Milk
EDTA	Ethylenediaminetetra-acetate
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agricultural Organization
Fp	Freezing Point
FSH	Follicular Stimulating Hormone
GH	Growth Hormone
GLM	General Linear Model
GnRH	Gonadotropin Releasing Hormone
HK	Hexose Kinase
IAEA	International Atomic Energy Agency
IGF-1	Insulin-like Growth Factor 1
KARI	Kenya Agricultural Research Institute
KALRO	Kenya Agricultural and Livestock Research Organization
LH	Luteinizing Hormone
MB	Maximum Binding
MEHA	3-Methyl-N-Ethyl-N-(β hydroxyethyl) Aniline
mg/dl	Milligrams per deciliter
ml	Mililiters
mRNA	Messenger Ribonucleic Acid
N	Nitrogen
NADP	Nicotinamide-Adenine Dinucleotide Phosphate
NEB	Negative Energy Balance
NEFA	Non-Esterified Fatty Acids
NE_L	Net Energy of Lactation
ng/ml	Nano grams per milliliter
NSB	Non Specific Binding
OD	Optical Density
P	% Protein
P_4	Progesterone

$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PP	Pyrophosphoric acid
PSPB	Pregnancy Specific Protein β
RDP	Rumen Degradable Protein
RUP	Rumen Undegradable Protein
SM-C	Somatomedin-C
SNF	Solids Not Fat
SRA	Strategy for Revitalizing Agriculture
T	Total Counts per minute
UK	United Kingdom
UN	Unstructured
VFA	Volatile Fatty Acids
Wks	Weeks
Wt	Weight at Calving
Wtins	Weight at Insemination
Wtlac	Weight at Luteal Activity

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Background Information

Lactating cows continuously exposed to inadequate nutrition fail to fully express their genetic potential for production and reproduction performance. This is typical in low input systems where several authors have observed influence of levels of feeding on the low milk yield and delayed age at puberty in heifers, prolonged postpartum intervals to first ovulation, increased incidences of silent heat, and reduced conception rates and birth weights (Topps, 1994; Gong *et a*l., 2002; Lopez *et al*, 2004). The length of calving interval in dairy herds is influenced by number of inseminations per conception and days from calving to conception (Stevenson, 2001). A common feature of feeding regimes in low input systems is characterized by inadequate and variable feeding regimes resulting in large variation in milk yield, ranging from 5 to 15 kg per day (Walshe *et al*., 1991) and long calving intervals, ranging from 308 to 1256 days (Odima *et al*., 1994). For instance, Topps (1994) showed that cows under smallholder production systems in Kenya have a dietary deficit of up to 15 MJ/day of metabolisable energy and effective rumen degradable protein of 235 g per day. Consequently, dairy cows in low input systems are highly sensitive to improved nutrition. This is evidenced in a study by Van der Valk (1992) who obtained with improved nutrition an increase of 140 to 300% over the median milk yield per day in Kenyan smallholder dairy herds.

Low input systems utilize different breeds for milk production of which Friesian and Sahiwal cattle respectively represent the high and low potential milk yielding breeds. In these systems, Friesian breed can produce an average of 72.5 litres of milk per week with a butterfat content of 3.7% in a lactation length of 290 days (Irungu and Mbugua, 1998) while the Sahiwal breed can produce on average 67.2 litres per week in a lactation length of 280 days (Muhuyi and Lokwaleput, 1998). Friesian is a specialized milk breed while Sahiwal is a dual purpose breed and therefore the two breeds will respond differently to the same nutritional regime.

Feeding levels are known to re-initiate *postpartum* ovarian activity in lactating cows but the association with the metabolic and physiological changes during the transition period is unclear.

Some authors have found positive association (Chagas, *et al*., 2006) while others reported negative (Keady *et al*., 2001) or no associated responses (Pushpakumara, *et al*., 2003).Several studies have shown that various nutritional metabolites and hormones circulating in blood and milk can be associated with reproductive performance (Reist *et al*., 2003; Stevenson *et al.,* 2006) and are important in energy homeostasis as well as follicular dynamics (Lucy *et al*., 1992). Therefore, nutritionally induced changes in metabolites and hormonal profiles are candidate indicators that could explain the underlying nutritional effects on the reproductive performance of lactating cows. Potentially useful metabolites and hormones in blood as indicators of body energy balance include Non-Esterified Fatty Acids (NEFA), ß-hydroxybutyrate (BHBA) and insulin-like growth factor-1 (IGF-1) while metabolites in milk include acetone, urea, protein and fat to protein ratio (Reist, *et al*., 2003).

Lactating cows are typically in a state of Negative Energy Balance (NEB) in their postpartum period, because energy expenditure for maintenance and milk production exceeds energy intake (Riest, *et al.,* 2002). In this state, metabolic and endocrine changes allow enhanced mobilization of adipose fat and skeletal muscle breakdown to support mammary glands to provide substrates for milk synthesis (Bauman, *et al*., 1988). The practical indicators of NEB are changes in Body Condition Scores (BCS) and to a lesser extent in live weight changes (Sutter and Beever, 2000). Hormones and metabolites involved in metabolic adaptation, translate the nutritional status to reproductive functioning by interaction with the gonadotropic axis at the hypothalamo-pituitary (central) or gonadal (peripheral) levels (Santos, *et al.,* 2009).

The NEB during the early weeks of lactation and a delayed EB *nadir* (its most negative level) are associated with longer intervals to first ovulation postpartum. Reduced concentration of IGF-1 and estradiol are associated with this ovulation failure (Gong *et al.,* 2002). Milk urea nitrogen provides a rapid and noninvasive means of assessing the dynamics of blood urea nitrogen that can be used to monitor overall protein metabolism in lactating dairy cattle (Gustaffsson and Palmquist, 1993).

1.2 Statement of the Problem

Reproduction is one of the major limiting factors in the production efficiency of nursing or milked cows. Because duration of gestation limits cows to one annual calf crop, losses in potential calves are attributable to either prolonged periods of anestrous, delayed establishment of pregnancy or conception failure that is attributed to various factors of which nutrition is key.

The demand for nutrients during late pregnancy to support foetal growth and lactation after parturition is a major challenge in improving dairy cattle productivity. Low milk yields and poor fertility in tropical cows is a result of nutritional factors due to seasonal variation of forage quantity and quality. Feeding levels have a clear and strong influence on the re-initiation of *postpartum* ovarian activity in lactating cows. But there is conflicting information on the mechanism by which nutrition influences metabolic and physiological changes during the transition period and the subsequent impact on reproductive performance during the *pre* and *postpartum* period.

Supplementary feeding levels induce changes in blood and milk metabolites and hormonal profiles, which have associated influences on milk yield and reproductive performance *postpartum*. The understanding of nutritional influences on metabolite and hormonal mechanisms and their impact on the reproductive processes can be useful in addressing practical problems that improve production and reproductive performance of lactating dairy cows.

1.3 Objective

The overall objective was to asses effects of nutritional metabolites and hormonal profiles associated with reproductive performance of lactating Friesian and Sahiwal cows

1.3.1 Specific Objectives

i. To determine effects of nutrition on milk production and composition, and the subsequent effects on reproductive performance of dairy cows.
ii. To establish nutritional effects on metabolites and hormonal profiles and their subsequent influence on the reproductive performance of lactating cows.
iii. To identify specific nutritional metabolites and hormones and their threshold levels that influence reproduction in dairy cows.
iv. To determine association between conception failure and circulating levels of metabolites and hormones.

1.4 Null Hypothesis

Nutrition, through influence on metabolite and hormonal profiles does not affect reproductive performance of lactating dairy cows

1.5 Justification

Numerous studies have reported that nutrition has major influences on reproductive performance of lactating cows; however, the modalities of the nutritional effects on reproductive performance have been controversial. Some studies show positive influence (Chagas, *et al*., 2007), others show negative influence (Keady, *et al*., 2001) or no response (Pushpakumara, *et al*., 2003). Other studies have shown that nutritional influence on reproductive function is mediated at the ovarian level through the effects of metabolites acting as signals that influence hormonal action on gonads to enhance or impair reproduction in dairy cows (Chagas, *et al*., 2007).

The NEB after parturition impacts negatively on conception due to competing needs for nutrients between reproduction and lactation, resulting in lactational anestrus (Souza, *et al*., 2014). This can be ameliorated by provision of the right quantity and quality of feed that provides the right metabolic status for optimal milk production and conception after parturition.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Nutritional Needs of Lactating Cows

Animal nutrition is the art of balancing feed intake and digestibility, relative to the animals' requirements. Forages are fundamental components for the dairy cow diet in most production systems and profoundly affect protein and carbohydrate intake (Okiely, 1994). The quantity of herbage consumed by grazing animals is typically regulated by grazing time, biting rate and intake per bite (Perez-Ramirez *et al*., 2008; Holmes, 1989).

The most important factor influencing cow productivity is dry matter intake and neutral detergent fiber (NDF) is a good predictor in dairy cattle (Kendall, *et al*., 2008; Waldo, 1986). For high producing dairy cattle the dietary fiber should be more than 20% and 29% for acid detergent fiber (ADF) and NDF respectively to support normal rumen function and maintain a positive body tissue balance. High NDF proportions of the fiber content stimulates chewing activities and hence the rumen buffering effects of saliva and NDF is readily fermented, resulting in rapid production of volatile fatty acids (Russell, *et al*., 1992). Dairy cows consume 28g dry matter per kg live weight in early lactation, which increases to 32g at peak lactation. Animal factors affecting intake include body condition, stage of lactation and metabolic size while herbage factors are, digestibility, size and structure (Forbes, 1986). The nutrients consumed are utilized to meet metabolic needs for maintenance, milk production, growth and reproduction. It is apparent that herbage alone rarely can meet the requirements of an average lactating cow and therefore supplementation with concentrates to mitigate the shortfall in energy and protein is a must (Butler, *et al*., 2003). A high yielding dairy cow requires approximately 7kg of glucose, however, normal absorption from the digestive tract has been shown to be ≤1kg per day suggesting that a dramatic increase in gluconeogenesis is necessary in early lactation to sustain milk production (Bauman and Elliot, 1993). Precursors needed to support increased gluconeogenesis include propionate, lactate amino acids and glycerol and metabolic adjustment is required to avail these substrates.

Lactation and reproduction increases the requirements for nutrients, conversely nutrient supply can influence the lactation and reproductive processes of the cow. Reproduction is an 'all or none' phenomenon and consequences of failure are severe and should be avoided (Butler, *et al.*, 2003). A good plane of nutrition promotes the secretion of insulin which encourages the uptake of glucose in peripheral tissue and synthesis of steroids by the ovary (Garnsworthy, *et al*., 2008).

2.2 Lactation of Dairy Cows

Milk is secreted by the mammary glands and is a complex nutritious product containing more than 100 substances that are either in solution, suspension or emulsion in water (Buttris, 2003). The major component of milk is water with dissolved organic and inorganic elements such as amino acids, urea, water soluble proteins, lactose, minerals, vitamins and milk fat (Auldist, *et al.*, 1998). Lactation is a natural extension of the culminating events of successful reproduction.

It is important to determine the characteristics of the lactation curve of milking cows in order to analyze the milk production potential for improved milk yield and obtain a more desired lactation curve (Keskin and Dag, 2006). Moreover the lactation curve is also useful for assessing the nutritional and health status of milking animals (Dudouet, 1982) and helps to determine the suitable time to end milking (Chang, *et al.*, 2001). In exotic dairy cows, peak milk yield occurs at an average of 50 to 70 days *postpartum* and influences 66-88% of the variation of lactation yield (Roche, *et al*., 2006; Weller, *et al*., 2006). Persistence of lactation is the rate of decline in production after peak milk yield and greatly influences the lactation production levels (Appuhamy, *et al*., 2007). Cole and VanRaden (2006) showed that low producing cows achieve peak production early and at the same time have lower persistence. Stage of lactation influences milk yield and composition in cattle (Ibeawuchi and Dangut, 1996) and goats (Akingbade, *et al*., 2003). Milk composition varies between and within breeds of cattle and is a result of environmental and genetic influences (Legates, 1960). The concentration of protein and fat increases with advancement in lactation in pasture fed dairy cows and reduces when dry matter and energy intakes are low (Auldist, *et al*., 1998; Coulon and Remond, 1991). Dietary protein and energy balance are the major determinants of milk urea concentration (Broderick and Clayton, 1997).

Onset and establishment of lactation and estrus cycles are concomitant, energy-competing processes *postpartum.* This is because the metabolic events essential for milk secretion compete for available nutrients that support the processes leading to the first *postpartum* estrus and subsequent fertility (Butler and Smith, 1989). The onset of heat coincides with significant hormonal changes likely to alter the protein composition. Such alterations could be used to identify estrus-specific proteins by comparing protein profiles of milk at the different stages of the estrus cycle (Morris *et al*, 2001). Understanding the interdependence of lactation and reproduction is essential to optimize reproductive efficiency of the dairy enterprise.

A high yielding dairy cow may produce in a single lactation 5 times as much dry matter in the form of milk as is present in her own body (Riest, *et al*., 2002). Net energy deficits greater than 20 Mcal per day have been reported by Bauman and Elliot (1993) in high yielding dairy cows. The raw material from which milk constituent is derived and the energy for their synthesis in the mammary gland is supplied by the feed consumed. Thus, feed requirements will depend on amount and composition of the milk being produced (Broderick and Clayton, 1997).

About 95% of the nitrogen in milk is in the form of proteins the remainder being urea, ammonia, creatine and glucosamine, which filter from the blood to the milk. In this respect milk serves as an alternative excretory outlet to urine (Morris *et al*, 2001; Plaizier, *et al*., 2005). Milk Fat consists of a mixture of triglycerols comprising saturated and unsaturated fatty acids. Milk fat yield is influenced by the balance of fat synthesis and mobilization and depends on glucogenic substances in the products of digestion. Thus a high proportion of propionate, glucose and amino acids stimulate fat deposition (Blum, *et al*, 2000). Mech, *et al*., (2008) reported that variation in total solids was influenced by variation in milk fat while protein and SNF did not vary across the various lactation stages and is probably not a function of milk yield. The average lactation length was defined as the time where milk yield was less than 30% of the peak. (Chang, *et al*., 2001)

Important characteristics that determine milk quality are density and freezing point and are indicators of the metabolic status of lactating cows (Henno, *et al*., 2008). The specific gravity or density of milk averages 1.032kg per liter, i.e. at 4°C, one liter of milk weighs 1.032 kg. Water without solutes will freeze at 0˚ C and the presence of any solutes will depress freezing point

below 0˚ C (Henno, *et al*., 2008). The freezing point of milk depends upon the concentration of water-soluble components. As milk is diluted, the freezing point will raise closer to zero.

2.3 Body Condition Score as an Indicator of Metabolic Status

Body condition score (BCS) is used as a subjective method to determine body reserves in sheep and cattle (Lowman, *et al*., 1976). The method is based on a visual and tactile appraisal of body fat reserves on the back and pelvic regions and is usually scored on a scale of 1 to 5. Broster and Broster (1998) hypothesized that the ratio of change in body weight relative to change in BCS was estimated at 26kg per unit condition score in cattle.

Energy intake of high yielding cows *postpartum* has been found to be less than half the energy required for production (Van Arendonk, *et al*., 1991), the shortfall must be met through mobilization of body tissue, thus appreciable bodyweight and condition loss is inevitable. Loss of BC at time of AI may negatively influence conception, because cows with BCS of less than 3 at calving were less likely to show heat and BCS loss between calving and 45 days in milk (DIM) was associated with more days open and delayed intervals to 1st insemination (Carvalho, *et al*., 2014; Santos, *et al*., 2009). The BCS of cows at the end of lactation should be 3.25 – 3.75 (Studer, 1998). Energy balance during the dry period and early lactation as monitored by BCS is a better indicator of conception at 1st service than other factors.

The levels and changes of BCS during the lactation period could affect the resumption of estrus cycle and reproductive success and is indicative of the metabolic status necessary to support production and resumption of cyclicity (Pryce, *et al*., 2001; Santos, *et al*., 2009). Regular body condition scoring of cows has been recommended as a means to evaluate the relative degree of energy balance (Heuer, *et al.,* 1999). As genetic merit for production increases, so does mobilization of body reserves creating a larger negative energy balance during early lactation. BCS has a negative genetic correlation with calving intervals of -0.40 and can be used for selection of fertility, with thinner cows, or cows in more negative energy balance, tending to have poorer fertility (Pryc,e *et al.,* 2001). The greatest change in BCS seems to be from calving to week 12 post calving (Pryce, *et al*., 2001). It has been shown that BCS and reproductive measures

have a linear relationship and is negatively correlated to milk yield. High genetic merit cows have lower BCS and lose more body condition than average merit cows (Pryce, *et al*., 2001).

2.4 Physiology of Reproduction

2.4.1 Conception and Pregnancy in Dairy Cows

Conception and establishment of pregnancy are an ordered progression of interrelated events involving various tissues of the reproductive tract and include follicular development resulting in ovulation, fertilization of the oocyte, embryo transport and development, maternal recognition of pregnancy and implantation (Butler, 1998; Cerr, *et al*., 2009).

Reproductive success is a physiological process by which gonadotrophins (FSH and LH) are secreted from the pituitary to the ovary to stimulate follicular development to produce a mature and competent egg (Gong, *et al*., 2002). Nutritional influences on reproduction are mediated by ovarian follicle development in cattle through changes in metabolic hormones such as growth hormone (GH), insulin, insulin like growth factor 1 (IGF-1) and most recently leptin (Boland, *et al*., 2001; Lopez, *et al*, 2004). The interaction between these metabolic hormones and follicular development can be manipulated to improve production and reproductive performance in cattle. These interactions can be on ovarian follicular growth, oocyte maturation and early embryonic development (Santos, *et al*., 2009).To maximize chances of a successful pregnancy, a number of important time-dependent components of the estrus cycle must be well managed. Maximal reproductive efficiency requires management of the calving interval, which consists of 3 main components: the elective waiting period, the active AI breeding period and the gestation period (Stevenson, 2001). The elective waiting period is the rest period *postpartum* and its duration (40-70 days) is based on the need for the reproductive tract of the cow to undergo involution, which is physiologically necessary (Stevenson, 2001; Santos, *et al*. 2009). The AI period begins by the cow exhibiting heat and is subsequently inseminated and the calving interval will depend on the services per conception, while after conception the ability for the cow to hold the pregnancy will further have impact on this interval.

2.4.2 Hormonal and Metabolite Influence on Reproduction

Progesterone is a steroid hormone with key functions in the regulation of female reproduction and plays an important role in the preparation for and maintenance of pregnancy. It is produced during the luteal phase of an estrus cycle by the corpus luteum and depending on the stage of pregnancy and species it is synthesized from cholesterol via pregnenolone (Aufrere and Benson, 1976). The ovary and the placenta are the major production sites, but a small amount is also produced by the adrenal cortex. Circulating progesterone levels, are characteristically low during the follicular phase and increase sharply during the luteal phase of the estrus cycle reaching a peak 5 to 10 days after the mid cycle period (Bridges, *et al*., 2014). Unless pregnancy occurs, a steep decline to follicular levels sets in about 4 days before the next estrus period (Santos, *et al*. 2009).

The somatotropic axis consists of growth hormone, growth hormone receptors, insulin like growth factor 1 (IGF-1) and IGF binding proteins. These control nutrient partitioning in early lactation of dairy cows (Etherton, 2004). There is a rapid decrease in blood IGF-1 concentration shortly after calving and this is the process that drives nutrient partitioning to milk production during early lactation (Lucy, 2008) which has a negative influence on reproductive performance (Roche, *et al*., 2007). Nutrition can exert control over the somatotropic axis through its effects on the liver IGF-1 production (Lucy, 2008). Plasma IGF-1 concentrations are an indicator of postpartum energy balance. Better nutrition results in a more positive energy balance leading to liver IGF-1 synthesis and secretion (Butler, *et al*., 2003; Radcliff, *et al.*, 2006). Somatomedin-C (SM-C) or IGF-1 is a basic 70 amino acid single chain polypeptide. The blood concentration of SM-C is more stable due to the binding to carrier proteins. IGF-1 is produced by the liver and other tissues and has endocrine, paracrine and autocrine activities (Lucy, *et al*., 2009). Growth hormone is the most important factor controlling secretion and concentration of IGF-1 (Lucy, *et al*., 2009) while other important factors include age, sex, nutritional status and other hormones like estrogen, thyroxin and prolactin.

Urea is primarily produced in the liver and excreted by the kidneys. It is the major end product of protein catabolism in mammals and primary vehicle for the removal of toxic ammonia from the body. Jung, *et al*. (1975), developed simple and direct procedures for direct measurement of urea concentration in blood or blood urea nitrogen (BUN).

Albumin is the most abundant plasma protein and accounts for about 60% of the total serum protein (Jainudeen and Hafez, 2000). It plays an important physiological role which includes maintenance of colloid osmotic pressure, binding of key substances such as long chain fatty acids, bile acids, calcium and magnesium (Hafez and Hafez, 2000). It has anti-oxidant and anticoagulant effects and acts as a carrier of nutritional factors and is an effective plasma pH buffer. Albumin is a reliable indicator of malnutrition and protein-losing enteropathies (Jainudeen and Hafez, 2000).

The hormonal and metabolic signals that communicate the level of body energy reserves to the reproductive-mammary axis remain undefined in dairy cattle (Chelikani, *et al*., 2009). There is substantial evidence on the effects of nutritional manipulation on the hormonal and metabolic milieu in dairy cows. The interval to first *postpartum* ovulation is related to the period of negative energy balance, as metabolites and metabolic hormones convey information from the cow's metabolic status to her central nervous system (Butler, *et al*., 2003). A high plane of nutrition was associated with increased circulating levels of insulin, glucose and IGF-1, and decreased levels of growth hormone and NEFA and vice versa (Chelikani, *et al*., 2009). Moreover, cows ovulating within 35 days *postpartum* have high levels of IGF-1, insulin and glucose, and lesser concentrations of NEFA and ß-Hydroxybutyrate (Huszenicza, *et al*., 2001).

There is evidence that elevated concentrations of NEFA are associated with compromised reproductive performance in cattle. There is a positive genetic correlation between NEFA concentration with long calving intervals, metritis and reproductive problems while a negative association exists with conception rates to first artificial insemination (Oikonomou, *et al*., 2008). Among blood metabolites, NEFA concentration is assumed to be the best indicator of the cows' energy balance (Reist, *et al*., 2003) because elevated NEFA is the first indication of lipolysis and is a way of adapting to negative energy balance.

A high-energy diet favours activities of the rumen microorganisms leading to the increased production and absorption of volatile fatty acids leading to an increase in insulin and a decrease in fatty acid mobilization from the adipose tissues (Doepel, *et al*., 2002). Among the hormonal signals, a prepubertal increase in circulating concentration of growth hormone and IGF-1 plays a

role in determining the onset of puberty in beef heifers (Yelich, *et al*., 1995), this is because of the role IGF-1 plays in follicular development and maturation (Webb, *et al.*, 2004). In addition to these hormones, leptin, a hormone secreted primarily from adipose tissue is sensitive to dietary manipulation and appears to play an important role in transmitting the metabolic status to the central nervous system to regulate feed intake and reproductive function in ruminants (Zieba, *et al*., 2005). Further, a heifer was considered pubertal and first ovulation occurred when plasma progesterone concentration exceeded 1 ng/ml for the first time (Chelikani, *et al.*, 2003).There is a favorable genetic association between glucose concentration measured in early lactation and reproduction (fertility). Elevated glucose concentration was linked to decreased interval from calving to conception and increased rate of conception (Oikonomou, *et al*., 2008). There is a negative correlation between days open and plasma glucose concentration (Moallem, *et al*, 1997). There is further evidence that glucose promotes steroidogenesis by enhancing cholesterol uptake into the ovarian cells (Rabiee and Lean, 2000) which is a precursor to steroid hormone synthesis and ensures adequate availability.

2.4.3 Fertilization Failure

Fertilization failure is due to factors that fail to facilitate union of a viable egg with a viable sperm. Once fertilization occurs, embryonic death results from failure of normal embryonic development, lack of maternal recognition of pregnancy or normal maintenance of pregnancy (Stevenson, *et al*., 2014). The period from parturition to 1st AI depends on estrus detection and the individual cow‘s fertility status. The level of cow fertility depends on several factors; fertility of the service sire, correct semen handling, AI technique and timing of insemination (Cerri, *et al*, 2009; Stevenson, 2001)

The greatest limiting factor to successful fertilization is detection of estrus. Approximately 59% of estrus periods go undetected on an average dairy farm in the USA (De Vries, *et al*. 2010) and higher producing cows are more difficult to detect estrus (Santos, *et al*., 2009). Mounting activity at estrus which is a behavioral expression of estrus is stimulated by estrogen and inhibited by progesterone (Allrich, 1994; Lopez, *et al.*, 2004). A number of environmental conditions including temperature, nutrition and presence of bulls amongst others, influence whether cows

show estrus or not while cows that are confined or in crowded pens show less estrus (Stevenson, 2001).

Ovulation occurs 24 to 32 hours after the onset of estrus (mean 27.6 hrs.) and is triggered by the hormonal mechanisms that cause the cow to display estrus (Fricke, *et al*., 2014) and once ovulated, the eggs viable life is less than 12 hours unless fertilized. Thawed semen is estimated to have a viable life span of less than 48 hours in the female reproductive duct (Mclaren, 1974). In general, normal sperm need 6 to 10 hours to reach the oviduct after undergoing capacitation. The subsequent 12 to 16 hours represents the period of maximum fertile life of the sperm after which it declines (Fricke, *et al*., 2014). Inseminating pregnant cows can lead to induced embryo death or abortion. Approximately 10% of pregnant dairy cows express estrus (Moore, *et al*., 2005). To avoid abortions of held pregnancies in these cows, semen should be deposited at the mid cervical area (Macmillan, *et al*., 1977).

2.4.4 Embryonic Losses in Cattle

Santos, *et al* (2004) demonstrated that bovine fertilization rates are usually as high as 95% and that embryonic mortality was the main source of reproductive wastage. Causes of embryonic losses could be due to genetic defects of the embryo or uterine factors. Cow factors resulting in such losses were identified as insemination during pregnancy; nutrient and ionic imbalance, rectal palpation, age of cow, heat stress and infection (Stevenson, *et al*., 2014). Nutrition has also been implicated as the most important cause for embryonic loss and is manifest through level of feeding, or imbalance of feed components (McClure, 1994; Santos, *et al*., 2009).

The decreased secretion of interferonτ from day 15 embryos, which is important in sustaining embryonic development and an increased secretion of uterine $PGF_{2\alpha}$ from undernourished ewes (Kim, *et al*., 2011) promotes embryonic loss. This is due to the action of $PGF_{2\alpha}$ regressing the corpus luteum as a result of the reduced action of interferonτ. The early cleavage stages of the embryo is characterized by relatively low levels of biosynthesis, low respiratory rates and a limited capacity to use glucose as an energy source (Khurana and Niemann, 2000). Before the blastocyst formation, metabolic reliance of embryos is on lactate and pyruvate pathways rather than that of glucose and therefore elevated glucose (5-6 mM) during these early stages could be

detrimental to embryo development. Moley, *et al.*, (1996) suggested an elevated concentration of glucose might alter activity of the kreb's cycle, resulting in retarded embryo development.

Cessation of pregnancy in animals occurs at a number of points. Stevenson, *et al*., (2014) defined the bovine conceptus as an embryo from day 14 to 45 after fertilization, and then it becomes a foetus. A number of reasons have been advanced for the increased number of late embryonic and early foetal losses. However, under-nutrition has been identified as the most important (Stevenson, *et al*., 2014). After the egg is fertilized, early embryonic loss may occur at day 3 or if the blastocyst develops by day 7 or 8 (Mclaren, 1974). Thereafter, it enters the uterus by day 5, another critical stage that can result in loss. These losses in addition to fertilization failure are not easily detected because the cow returns to estrus at the regular time of the estrus cycle (Wiltbank, *et al*., 2012).

The next critical stage is around day 15 or 16, when the embryo must be sufficiently developed to overcome the spontaneous uterine secretion of $PGF_{2\alpha}$, which causes the corpus luteum to regress and initiates another period of estrus (Northey and French, 1980). This process is facilitated by foetal trophoblastic cells production of large quantities of interferonτ that is integrated into a multifactorial antiluteolytic function to conserve the established pregnancy (Bazer, *et al*., 2009).

Late embryonic loss may occur from day 25 to 40 after AI due to the failure in attachment of the developing placenta to the uterine wall. This is due to lack of connection of the placental cotyledons to the uterine caruncles of the endometrium that transfer gasses, nutrients and waste products between the uterus and developing calf (Perry, 1981). Sreenan and Diskin, (1986) reported an early embryonic loss of 38% occurring between days 15 to 18 after fertilization while foetal death occurs at a rate of 5 to 8% among dairy cows. However, these later losses are more serious than conception failure and early embryonic death because more time is lost during the period of pregnancy which is subsequently wasted and it takes time to re-establish cyclicity (Santos, *et al*., 2009; Sartori, *et al*., 2006). The simplest method of establishing embryonic loss is the observation of return to estrus or the subsequent drop in progesterone levels more than 25 days after insemination (Stevenson, *et al*., 2014). Progesterone supplementation can improve survival rates of young embryos, especially in sub-fertile animals with lowered plasma

progesterone concentrations; older cows are prone to this problem due to the reduced efficiency of progesterone production by their corpora lutea (Cerr,i *et al*., 2009; Sreenan and Diskin, 1986). Progesterone output from the corpus luteum is connected to energy balance and increased secretion is associated with embryonic survival (Thatcher, *et al.,* 2001)

Pregnancy specific protein B (PSPB) is produced during pregnancy by the binucleate cells of the foetal trophoectoderm and may offer a means of positively identifying the presence of an early conceptus (Humblot, 2001). It was found that blood concentration of PSPB increases gradually during pregnancy, from day 15 to 35 to reach a peak of 2 to 3 ng/L and has a prediction of up to 90% and 99.5% for non-pregnancy. Cows carrying twins had significantly higher concentrations of PSPB, 40 days after ovulation and are a good indicator of foetal wellbeing (Humblot, 2001). Green and Roberts (2006) demonstrated a relationship between foetal growth and plasma PSPB increases.

2.5 Effects of Nutrition on Reproduction

2.5.1 Partitioning of Nutrients to Support Reproduction

Homeorhesis is the coordinated control of metabolism in support of a dominant developmental or physiological process (Bauman and Currie, 1980) while homeostasis is a multiplex compensatory mechanism to maintain physiological equilibrium (controls functions to preserve steady state).

Homeorhetic mechanisms involve the alterations in tissue response to homeostatic controls (Bauman and Curie, 1980). The proposed mechanism include; altered release and /or clearance of a homeostatic signal; changing blood flow to an organ; altered sensitivity of a tissue via receptor numbers and/ or binding affinity and altered tissue responsiveness to a homeostatic signal via changes in the intercellular signal transduction system (2^{nd} messenger, amounts and activation of enzymes) (Bauman and Curie, 1980). These homeorhetic controls cause the direct partitioning of nutrients appropriate for a particular physiological state yet still allows acute regulation to preserve steady state.

Onset of lactation and re-establishment of *postpartum* estrus cycles are energy-competing processes, with lactation having a greater priority for dietary nutrients and body reserves resulting

in prolonged *postpartum* anestrus through homeorhetic controls. Feed intake at this time is insufficient to provide all nutrients necessary for milk synthesis and initiation of cyclicity (Bauman and Curie, 1980). Insulin is a key homeostatic agent involved in the regulation of both glucose utilization (stimulatory) and production (inhibitory). Fat mobilization during late pregnancy is facilitated by the decreased ability of insulin to promote lipogenesis and oppose lipolysis. The suppression of lipogenesis at onset of lactation is associated with low levels of plasma insulin (McNamara, 1988).

NEFA increase just before parturition and at the onset of lactation due to massive adipose tissue mobilization, and decreases as lactation advances and NEB decreases (Danshea, *et al.* 1989; Wathes, *et al*., 2007). This is because of the increased nutrient requirements to support lactation which may not be met by sufficient nutrient intake therefore causing mobilization of stored body fat reserves. NEFA entry rate into the circulatory system is a representation of adipose tissue mobilization, and thus body fat loss as a result of increased adrenergic stimulation of lipolysis. Insulin plays a major role in the regulation of lipid metabolism in adipose tissue, stimulating glucose uptake and activating enzymes associated with lipogenesis. Generally, lipid metabolism shifts from storage of energy during the dry period to a substantial mobilization of energy reserves during early lactation (Bauman and Curie, 1980). Prolactin and somatotropin have been proposed as the homeorhetic controls responsible for these changes.

2.5.2 Effects of Under-nutrition on Reproduction

The period of transition between late pregnancy and early lactation presents an enormous metabolic challenge in dairy cows. There is a dramatic increase in the requirements for nutrients, notably glucose, amino acids and fatty acids that cannot be met by dietary intake (Butler, 2000). Failure to adequately meet these challenges can result in major *postpartum* health and performance problems. Therefore, strategies to facilitate this peri-parturient transition based on quantity and quality of feeds required in supporting late conceptus growth and milk synthesis is very crucial for the dairy enterprise (Leifers, *et al*., 2003).

To ensure sustainability of milk production and regular reproduction to supply herd replacements, Robinson (1990) outlined the effects of underfeeding on puberty through parturition–to–

rebreeding intervals and concluded that, although there was good evidence on the effects of nutrition on blood metabolites, knowledge of the trigger mechanisms, necessary to activate the various stages of the reproductive chain, are not well known. During periods of under nutrition, ruminants should in general be provided with at least a third of their maintenance energy requirement, otherwise lean tissue would excessively be used to provide glucose precursors (Ørskov, 1998). Under nutrition reduces colostrum production and in severe cases delays the onset of lactogenesis. Maternal nutrition therefore, through its effects on foetal metabolism, foetal growth and colostrum production plays a key role in neonatal viability (Robinson, 1990). Nutrition is usually prioritized for maintenance, pregnancy, foetal growth, lactation, cow growth and then body reserves respectively (Fricke, *et al*., 2014). A high rate of gluconeogenesis is ensured in dairy cows during early lactation with increased lipid mobilization and fatty acid oxidations in the liver by regulating metabolite transport across mitochondrial membranes. However, in severely underfed animals, with excessive lipid mobilization and ketogenesis, glucose synthesis is depressed (Danfaer, 1994).

In regions with defined dry and rainy seasons, the ideal time of insemination is determined by the associated fluctuation in nutritional status (Lotthammer, 1991). Underfeeding during growth delays puberty; while in late pregnancy delays the resumption of estrus after parturition; and during the *postpartum* period reduces pregnancy rates (McClure, 1994). It was suggested that energy is the major cause of under nutrition. Adequate levels of feed quantity and quality are rarely available for smallholder dairy cattle in the tropics. Where it is available, they are deficient in some nutrients and have low digestibility, which leads to low intake. This affects body condition at calving and subsequent energy utilization which impacts on the length of the *postpartum* anestrus period (Mukasa-Mugerwa, *et al.,* 1997). Cows in better body condition produce bigger calves, which grow faster, and although they lose weight in the first four months of lactation their anestrus period and calving intervals are shorter than their thinner counterparts (Mukasa-Mugerwa, *et al.,* 1997).

Effects of nutrition can arise as a physiological condition in high-producing dairy cows where physical capacity of the stomach limits the quantity of feed intake and as such, extractable nutrients to meet nutrient demands. Animals subjected to under nutrition have several

physiological mechanisms to cope with this situation. The main evidence of under nutrition is loss in body weight and condition when animals are withdrawing energy from their tissues. In the first place, body fat will be depleted resulting in increases in blood NEFA levels (Grimaud, *et al.* 1998).

2.5.3 Nutrient Balance and Follicular Development

There have been several studies on nutritional influence on body condition and its relationship to fertility. This include 'flushing' before mating to improve fertility and onset of puberty in heifers based on body size rather than age. In cycling heifers, severe restriction in energy intake can induce nutritional anestrus when body weight decreases by more than 15% (Rhodes, *et al*., 1996). This is through the effects on morphological and functional parameters of follicular development such as size, growth rate and steroidogenic activity of the dominant follicles; however, the mechanisms of these effects are not well understood (Rhodes, *et al*., 1996). In *postpartum* dairy cows, extent of negative energy balance and diets with different energy contents has significant impact on population of ovarian follicles and functional competence of the dominant follicle (Gong, *et al*., 2002).

Metabolic hormones, as nutritional signals exert a direct effect at the ovarian level. This is because profiles of hormones such as insulin and IGF-1 are closely associated with nutritionally induced alterations in body energy and protein balance (Pell and Bates, 2000). Ovulation may not occur in animals with low dietary intake due to poor follicular growth which become atretic. Such follicle wave turnover without ovulation has been reported in *postpartum* beef cows in poor body condition (Stagg, *et al.*, 1995). It has been reported that oocyte quality is affected by dietary intake and the degree can be influenced by lactational and physiological state (Wiltbank, *et al*., 2014)

Fat, protein, fat to protein ratio, urea versus kilograms of milk, and milk urea versus protein are important parameters of nutrient balance (Heuer, *et al*., 1999). Milk fat concentration tends to increase and milk protein concentration tends to decrease during *postpartum* negative energy balance. The fat to protein ratio has been suggested as a potential indicator of lack of dietary energy supply, and critical ratios identified to be between 1.35 and 1.5 (Grieve, *et al*., 1986).

Cows with greater ratios than 1.5 were more at risk of getting mastitis, ovarian cysts and had profound increased negative effects on fertility traits (Heuer, *et al*., 1999).

Milk urea nitrogen provides a rapid and non-invasive means of assessing the dynamics of blood urea nitrogen that can be used to monitor overall protein metabolism in lactating dairy cattle (Gustaffsson and Palmquist, 1993; Roseler, *et al*., 1993). Volatile fatty acids (VFA) are produced through the fermentation process in the rumen to produce propionate, butyrate and acetate from concentrate or roughage feed respectively. Roughage feeds prevalently producing acetate also produce less circulating insulin compared to propionate producing concentrates which produce relatively more insulin in plasma (Gong, *et al*., 2002). The high insulin diets advanced the first ovulation *postpartum* and reduced the interval from calving to 1st service and conception and reduced the number of services required per conception and increased conception rates to 1st service (Gong, *et al*., 2002). This diet did not affect FSH and LH secretion suggesting a possible direct effect at the ovarian level. Further, the diet did not affect milk yield, change of body weight and body condition score. Monensin is an ionophore that changes rumen fermentation patterns so that a higher proportion of rumen VFA production is propionate. Feeding monensin has been used to study the effects of energy metabolism on reproduction and associated hormone patterns (Short and Adams, 1988). It was concluded that feeding cows with diets that increase circulating insulin concentrations during early lactation improves reproductive performance.

Proteins in ruminants are mainly supplied by rumen microbes. Dietary protein can be degraded to provide protein for microbes and the protein escaping microbial incorporation ends up as urea in the circulatory system after detoxification of ammonia in the liver (Butler, 1998). Blood urea nitrogen can be determined from plasma or serum and peaks 4 to 6 hours after feed intake (Butler, 1998). A measurement of blood and milk urea nitrogen has provided a useful index for determining the association between metabolism of dietary protein and reproductive efficiency. Conception rates decreased when serum urea nitrogen concentration exceeded 20mg/ per dl on the day of insemination, indicating that excessive degradable protein in the diet contributed to infertility (Butler, 1998). High urea levels are consistent with excess protein intake with concomitant energy shortage and are associated with high levels of ammonia in the circulatory system. High urea nitrogen in plasma and milk (>190 mg/L has been associated with decreased

fertility in dairy cattle due to an altered uterine environment (Butler, *et al.*, 1996). The rumen undegradable protein (RUP) moves into the lower gut for further digestion. Diets that are high in protein (17 to 19% CP) both support and stimulate high milk production in early lactation, however, these levels can also prolong the interval from calving to first ovulation (Souza, *et al*., 2014). This unusual outcome is due to reduced progesterone levels as a result of increased metabolic clearance rates in the liver (Sangstritavong, *et al*., 2002). The ability to produce and maintain optimum levels of progesterone concentrations is important for fertility due to the effects of progesterone in the estrus cycle (Butler, 2000).

Increased milk yield in cows depends on high levels of dietary protein and energy. Depending on the protein quantity and composition, serum concentrations of progesterone may be lowered, uterine environment altered and fertility decreased (Bridges, *et al*., 2014; Butler, 1998). Metabolism and utilization of dietary protein depends on energy availability, the effects of feeding high dietary protein superimposed on the preceding negative EB represents another important interaction of nutrition on reproductive performance of dairy cattle (O'Callaghan and Boland, 1999).

2.5.4 Nutritional Influences on Reproductive Hormones

Energy status is generally considered the most important nutritional factor that influences the reproductive processes, with prolonged low energy intake impairing fertility (Boland, *et al* 2001). In sheep, poor nutrition resulted in lowered ovulation rates which were associated with decreased LH pulse due to inadequate hypothalamic secretion of GnRH (Rhind, *et al*., 1989). These alterations in reproductive parameters were associated with lower plasma insulin and IGF-1 concentrations (Gong, *et al.*, 2000; Block, *et al*., 2003). Long-term restriction to feed intake has been shown to induce anestrus in cattle due to insufficient circulating LH but mode of action is not well established (Rhodes, *et al*., 1996).

A lowered concentration of IGF-1 during negative energy balance reduces steroidogenesis, thus preventing final follicle maturation and the production of LH surge-inducing concentrations of estradiol. It has been established that IGF-1 is a potent stimulator of granulosa cell function, including proliferative steroidogenic activity (Gong, *et al*, 1991). IGF-1 increases LH binding

sites and enhances LH-induced production of progesterone. It can be concluded that negative energy balance during the early weeks of lactation and a delayed EB *nadir* are associated with longer intervals to first ovulation *postpartum*. Reduced concentration of IGF-1 and estradiol are associated with this ovulation failure (Gong, *et al.*, 2000).

The intake of high dietary CP results in elevated concentrations of ammonia and urea. These metabolites are responsible for altering the luminal microenvironment of the uterus by raising the pH which interferes with inductive effects of progesterone (Wiltbank, *et al*., 2006; Butler, 1998), thus decreasing fertility. Progesterone suppresses the production of $PGF_{2\alpha,}$ but the presence of urea significantly increases the secretion of $PGF_{2\alpha}$ which interferes with embryo development and viability (Butler, 1998).

Low progesterone post breeding can reduce fertility (Larson, *et al*., 1997). Progesterone profiles could be used to select for the interval between calving and the start of luteal activity as early establishment of ovarian activity is important for fertility (Darwash, *et al*., 1997). Steroids are selectively stored in fat, and any dietary effect that results in fat mobilization will result in the release of stored progesterone. This may account for the increased progesterone evident in animals on low dietary intakes. Increased concentration of progesterone during the luteal phase before and after breeding have been associated with higher pregnancy rates (Butler, *et al.*, 1996). The rapid improvement of body condition has been associated with an increased ovulation rate and liter size and this alteration is related to the ability of nutrition to increase the cell entry rate of glucose. This effect improves follicle recruitment and growth characteristics (Downing, *et al*., 1995). Leptin, a peptide secreted by white adipocytes, plays a significant role in regulation of body weight and food intake and has recently been implicated in the interaction between nutrition and fertility (Cunningham, *et al*., 1999). Leptin acts as an appetite transducer and as a satiety factor. Thus, it may be speculated that dairy cows overfed during the dry period have reduced feed intake during early lactation, these effects are mediated by increased leptin concentrations (Boland, *et al*., 2001). According to Schwartz *et al*., (1996), increasing leptin in circulation, decreased mRNA for neuropeptide–Y (which stimulates feed intake), and increased mRNA for corticotrophin releasing hormone (an inhibitor of feed intake). Thus, leptin may act as a general

modulator of reproduction by regulating appetite and feed intake and also having direct effects on the reproductive axis, largely by central inhibition of neuropeptide-Y.

2.6 Energy Balance during Early Lactation

The cow must adapt to the increased demand for nutrients by the mammary glands because of onset of lactogenosis (Bauman and Currie, 1980). During late gestation, the foeto-placental unit is a major nutrient consumer and after parturition the mammary glands become the major consumers. Thus, an energy prioritization is manifested that places nutrient use for growth and milk secretion than that of onset of the estrus cycle (Short and Adams, 1989). Cows that consume less dry matter (DM) have delayed 1st ovulation and 1st estrus, produce less milk and are less fertile (Staples, *et al.,* 1990). The severity and duration of negative EB is primarily related to dry mater intake, which in turn, is related to body condition at calving. The energy balance during the first 3 to 4 weeks *postpartum* is highly correlated with days to first ovulation (Butler, 2000; Santos, *et al.,* 2009). The length of the postpartum interval to first ovulation represents an important interaction between energy status and reproductive performance. Components of energy balance include, milk production, composition and dry matter intake (Patton, *et al*., 2007).

Parturition results in an abrupt shift in metabolic demands from nutrient accrual (body reserves and fetal mass) to rapid mobilization of lipid and protein stores in support of increased lactogenesis (Bauman and Currie, 1980). The stimulation of appetite to ensure adequate DM intake in normal healthy cows is essential to provide nutrients for maximizing milk secretion, follicular growth, ovulation, uterine involution and initiation of pregnancy. However, due to the increased energy requirements, lactating dairy cows experience a *postpartum* negative energy balance that reaches its *nadir* during the 1st or 2nd week after calving (Roche, *et al.,* 2000). The timing of the *nadir* has been implicated in the timing of the first ovulation which occurs about 30 days *postpartum* with a range of 17 to 42 days (Butler, 2000). The severity and duration of the negative EB is related to DMI (Staples, 1990). The degree of negative energy balance in the early *postpartum* period and the recovery rate from the negative energy balance are critical for health status and productivity of the cow manifested by the occurrence of ketosis, delayed ovarian cyclicity and low conception rates (Butler and Smith, 1989). The 1st ovulation occurs approximately 10 to15 days after the *nadir* of energy balance (Zurek, *et al.*, 1995). In early

lactation, at peak milk production, metabolic demands are enormous and major amounts of nutrients are required for mammary synthesis of lactose, protein and triglycerides that cannot be met by dietary intake (Bell, 1995). Nevertheless, dairy cows with high DM intake, despite having a negative energy balance, produced more milk, lost less body weight and ovulated earlier *postpartum* than those with low intake (Staples, *et al*., 1990; Zurek, 1995).

Even with good intake, lactating cows remain in negative energy balance until 6 to 10 weeks after calving (Stevenson, *et al*., 1997) which has been associated with prolonged intervals to first ovulation. Despite the negative EB, intake of energy rather than feed capacity may limit dry matter intake. Energy balance has been shown to be positively correlated with insulin and negatively correlated with NEFA during the first 6 weeks *postpartum* (Beam and Butler, 1998). However, the number of days to first ovulation was correlated with energy balance and fat corrected milk production (Santos, *et al*., 2009).

Over conditioned cows at calving will undergo increased mobilization of body fat and accumulate more triacylglycerol in the liver that are associated with longer intervals to first ovulation and reduced fertility (Butler and Smith, 1989; Rukkwamsuk, *et al*., 1999). The negative EB represents a physiological state of under-nutrition which impairs LH secretion and reduces ovarian responsiveness to LH stimulation thereby deterring ovulation (Beam and Butler, 1998). This is further supported by observations that follicles developing after the energy balance *nadir* exhibited greater growth and bigger diameters, enhanced estradiol production, and were more likely to be ovulated than those developing before the *nadir* (Beam and Butler, 1998).

Both plasma concentrations of glucose and insulin are reduced in cows with negative EB and plasma IGF-1 is directly related to energy status and is critical to ovarian follicular development with ovulating cows having 40 to 50% higher levels of circulating IGF-1 than their non-ovulating contemporaries (Beam and Butler, 1998). Furthermore, plasma estradiol concentrations were highly correlated with plasma IGF-1 levels. During the early negative EB period, the ability of follicles to produce sufficient estradiol for ovulation seems to depend on the availability of serum insulin and IGF-1concentrations (Beam and Butler, 1998).

Various metabolic and endocrine blood and milk traits such as NEFA, insulin, ketone bodies, IGF-1(Lucy *et al.*, 1992), milk fat, protein, lactose, fat: protein ratio and fat: lactose ratio (Heuer, *et al*., 2000) have been shown to be related to EB. Therefore, assessment of blood and milk traits may have practical potential in promoting health and productivity of dairy herds by monitoring the nutritional status at the herd level (Reist, *et al* 2003). The EB can be calculated from energy corrected milk yield (ECM), net energy of lactation (NE_L) and bodyweight (BW) using formulae developed by Reist, *et al.*, (2003) and Beam and Butler (1998). It was found that the interval to EB *nadir* was shorter for cows with ovulatory follicles compared to the un-ovulating cows, 7.4 days compared to 16.2 days from calving respectively (Beam and Butler, 1998).

Maintaining adequate body condition and DM intake and balancing diets for cows that are consuming more than 4% of their body weight in daily DM intake are challenges (Chase, 1993). Milk production and DM intake are stimulated by increased dietary protein, but, unfortunately, decreased fertility often is associated with excessive feeding of ruminaly degradable protein (RDP) as assessed by elevated blood or milk concentration of urea (Butler, 1998). Concentration of milk urea N exceeding 19 mg/dl is associated with altered uterine pH and reduced fertility (Butler, 1998).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Site

This study was conducted at Kenya Agriculture and Livestock Research Organization (KALRO), Beef Research Institute, Lanet which is situated in agro- ecological zones 3 and 4 (Pratt and Gwynne, 1977), lying at an altitude of 1600 metres above sea level with a bimodal rainfall pattern averaging 800 mm annually with a relative humidity of 83%. The institute has relatively good quality pastures comprising mainly of planted *Elmba rhodes* grass interspersed with natural *Themeda triandra* and star grass *(Cynodon dactaylon*).

3.2 Experimental Cows

Forty multiparous healthy in-calf cows that were in their third trimester of gestation were selected for this experiment. These cows comprised of 20 Friesians obtained from KALRO, Beef Research Institute, formerly KARI, Lanet Research Centre while 20 Sahiwals were obtained from KALRO, Dairy Research Institute, formerly KARI, Naivasha Research Centre. All the experimental cows were centrally managed at KARI-Lanet Research Centre. The selection was based on relative within breed, weights, parity and pregnancy status.

Upon calving, four cows of each breed were randomly assigned to each of the five dietary treatments of supplementary concentrate dairy meal at the rate of 0, 1, 2, 3, and 4 kgs per cow fed in two portions per day. The cows were allowed a 7 days adaptation period on their respective concentrate diets and during this period, the calves were allowed to suckle colostrum. Data and observations were collected from these cows for 6 months after calving. At the Lanet Centre, experimental cows were grazed together on pasture leys of predominantly *Elmba rhodes* grass for approximately 7 hours per day and supplemented twice daily during the morning and afternoon milking. Mineral licks and water were provided *ad libitum* for all the experimental cows. The cows were dewormed at the start of the experiment and dipped once a week to control ectoparasites, while routine veterinary care was carried out as the need arose. Figure 1 illustrates the hypothesized interactions between nutritional intervention with metabolites, hormonal profiles, production and reproductive parameters observed in this study.

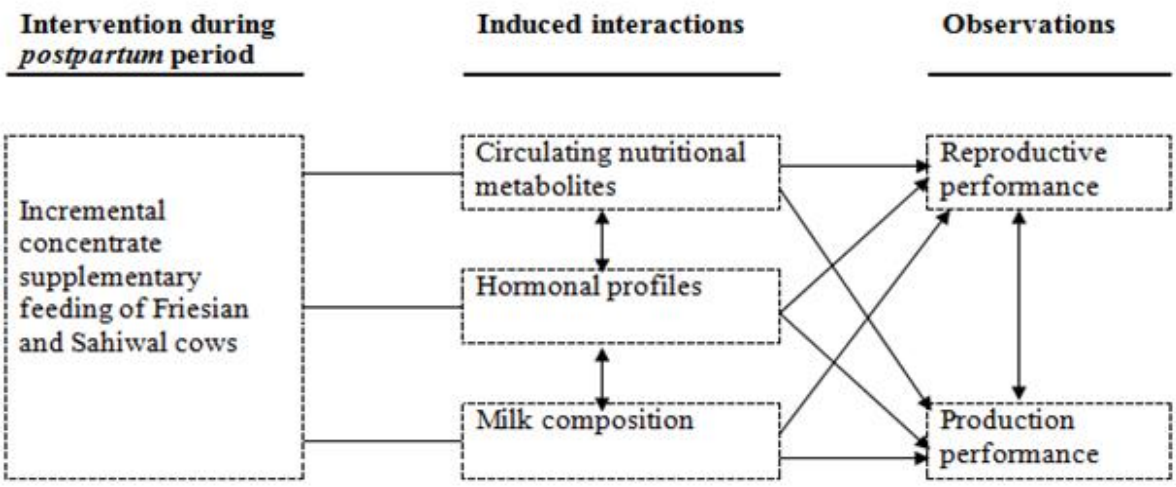

Figure 1. Model of Observations for Experimental Cows

3.3 Determining Feed Nutritional Composition

The pastures were sampled monthly from the grazing fields. This was done by randomly throwing a 1m² quadrant diagonally across the field and the swath within the quadrant was cut using a sickle. After mixing the various quadrant swaths, a sub sample from the mixture was picked in duplicate then prepared for laboratory analysis. The forage and concentrate samples were oven dried at 105˚C for 12 hours after which NDF, ADF and ADL were determined using the Van Soest method while DM and CP were determined by AOAC method (1995). The muffle furnace was used to determine the ash content of the feeds.

3.4 Determination of Body Weight and Scores, Milk Yield and Composition

3.4.1 Body Weight and Body Condition Scores

Upon calving and assignment to their respective dietary groups, the cow and calf were weighed using a weighing scale and the cow body condition scores determined by the same operator using a point scale of 1 to 5 (1= emaciated and 5= extremely fat) as described by Lowman *et al.*, (1976). The cow weights and body condition scores were subsequently measured biweekly for a period of six months *postpartum*. Other cow parameters observed included, date of calving, cow age in months, and parities, which were categorized into 3 classes: 1st and 2nd; 3rd and 4th; and above 5th parities were denoted as A, B, and C respectively.

3.4.2 Milk Yield and Composition

After parturition, the calf was allowed to suck colostrum for one week after which daily milk yield was recorded for the morning and afternoon milking which was summed up for the week for each cow. Milk recording continued daily until the yield was 30% of the peak milk lactation (Mech, *et al.*, 2008) when the cow was considered to be dry. Lactation curves were then derived from the weekly milk production levels using 3rd degree polynomial regression equations for the lactation period.

Homogenized 20 ml milk samples were collected in duplicate into well labeled plastic tubes once a week from each cow for the morning and afternoon milk. The milk samples were then analyzed immediately to determine milk components and characteristics which included percent butter fat (% BF), protein (% P), solids not fat (%SNF), density and freezing point which were measured by infrared spectroscopy (Tietz, 1986) using a machine known as Ecomilk™. Percentage milk fat was divided by % P and % SNF to get two other derived milk parameters for data analysis.

3.5 Blood Sampling

Blood samples were collected weekly (20 ml) from each experimental cow via jugular venipuncture using well labeled tubes containing ethylene diamine tetra-acetate (EDTA) at the rate of 1.8mg/ml as an anticoagulant. The blood samples were then stored in ice until when processed. The blood was centrifuged for 15 minutes at 1600 revolutions per minute to separate the plasma from the solid blood components. The plasma was then pipetted into labeled 2ml plastic vials and stored at -20˚C after which the concentration of nutritional metabolites and hormones were determined. The separate vials containing plasma were assayed in duplicates using commercial kits for Glucose, NEFA, Urea and Albumin using a spectrophotometer calibrated at various wavelengths as specified by the kits for their respective measurements.

Concentrations of progesterone and IGF-1 were measured by radioimmunoassay (RIA) using ^{125}Iodine as the tracer with bench protocols supplied by IAEA (FAO/IAEA, 1999). Progesterone measurements were used to determine commencement of luteal activity *postpartum*, early and late embryonic loss, pregnancy diagnosis and other reproductive disorders.

3.6 Determination of Nutritional Metabolites

3.6.1 Determination of Plasma Urea

The Jung method was used to measure urea concentration utilizing a chromogenic reagent that forms a colored complex specifically with urea (Jung, 1975). The intensity of the colour, measured at an absorbance of wavelength 520nm, is directly proportional to the urea concentration in the sample. Urea in the sample forms a coloured complex that can be measured by a spectrophotometer as described below.

$$\text{Urea} + H_2O \xrightarrow{\text{Urease}} 2NH_4 + CO_2$$

$$NH_4\text{+salicylate} + \text{Naclo} \xrightarrow{\text{Nitroprusside}} \text{Indophénol (chromogen)}$$

The optimized formulation substantially reduces interference by substances in the raw sample.
The procedure involves the addition of a single stable working reagent and incubation for 30 minutes for both cuvette and 96 well plate assays. No pretreatment is required and assays can be performed directly on raw biological samples i.e. in the presence of lipid and protein. The direct assays can be done in serum, plasma urine, and milk and cell tissue cultures.

The kit contains 50ml of reagent A comprising of Sodium salicylate 62mmol/L, sodium nitroprusside 3,4mmol/L, phosphate buffer 20mmol/L and Urease >500 U/mL while reagent B is made up of Sodium hypochlorite 7mmol/L and sodium hydroxide 150mmol/L also supplied with the kit is the urea standard of concentration 50mg/dl. The kits were stored at 2-8˚C while for long term storage they were stored at -20˚C.

Reagents A and B were combined in equal volumes of 50 ml each and equilibrated to room temperature thus making a working solution of 100ml and gently mixed. Plasma samples were assayed directly by transferring 200µL of plasma and 1000µL of working solution (reagent A and B) into assay tubes which were allowed to incubate for 30 minutes at room temperature. The standard of 50mg/dl was diluted to have concentrations of 10, 20, 30, 40 and 50 mg/dl and mixed with 1000µl of working solution and incubated for 30 minutes. The formulated standards and samples were then placed into cuvettes.

The spectrophotometer was calibrated with a blank of distilled water and the absorbance of optical density at wavelength 520nm was read off the LCD scale. The optical densities of the standards were determined and calibration curves for absorbance (optical density) versus urea concentration were developed for the two assays (Figure2). These curves were then used to determine urea concentrations from the plasma samples after their optical densities were read from the spectrophotometer.

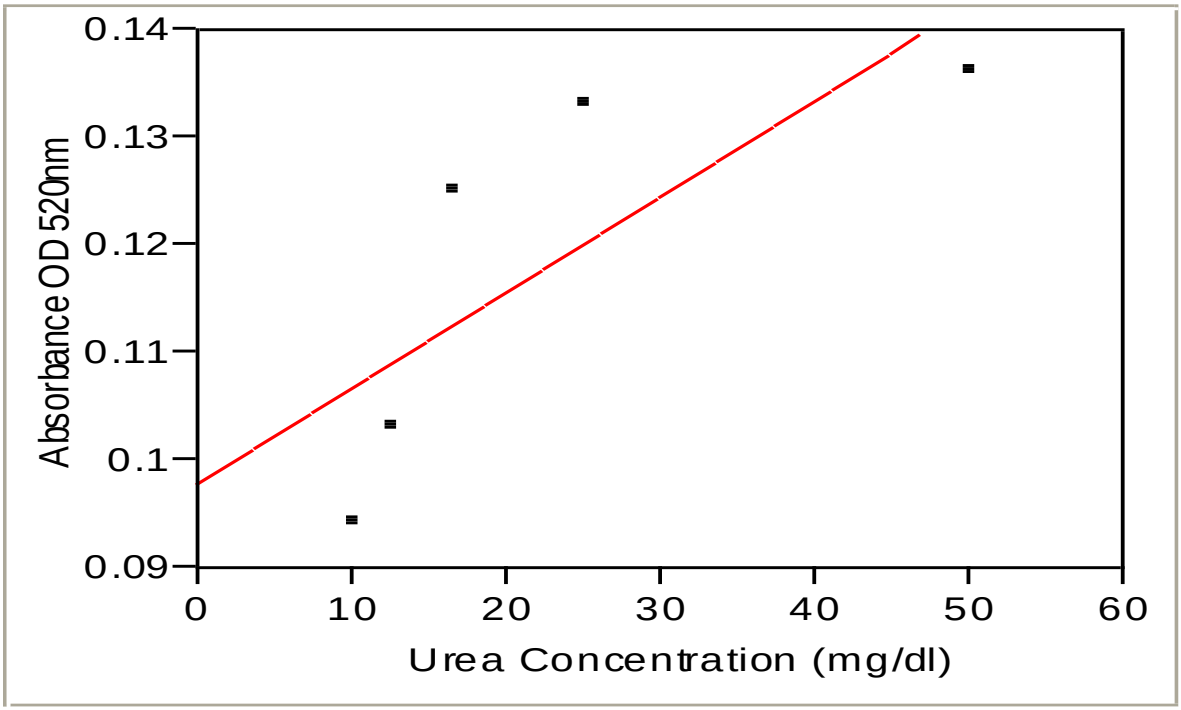

Figure 2. Standard Curve for Urea Concentration

3.6.2 Determination of Plasma Albumin Levels

Direct procedures using kits for measuring albumin in biological fluids without pretreatment have been developed. The method utilizes bromocresol purple that forms a coloured complex specifically with albumin. The intensity of the colour, measured at an optical density of 610nm, is directly proportional to the albumin concentration in the sample. The kit comprises of 50ml of the colour complexing reagent and albumin standard 2 ml of 5g/dl bovine serum albumin stored at -20˚C (Quantichrom™ BCP Albumin Assay Kit, DIAP-250). The standard was diluted using distilled water and 60µL of each dilution point was gently mixed with 1000µl of working reagent

and incubated for 5 minutes at room temperature before transferring contents to cuvettes and reading the respective optical densities at 610nm after which a calibration curve was developed. To determine albumin concentration in the samples, 60µL of sample was transferred into labeled tubes and 1000µL of working reagent added and incubated for 5 minutes. The samples optical density was read at 610nm after which the observed absorbance was read off the standard curve to determine albumin concentration (Figure 3).

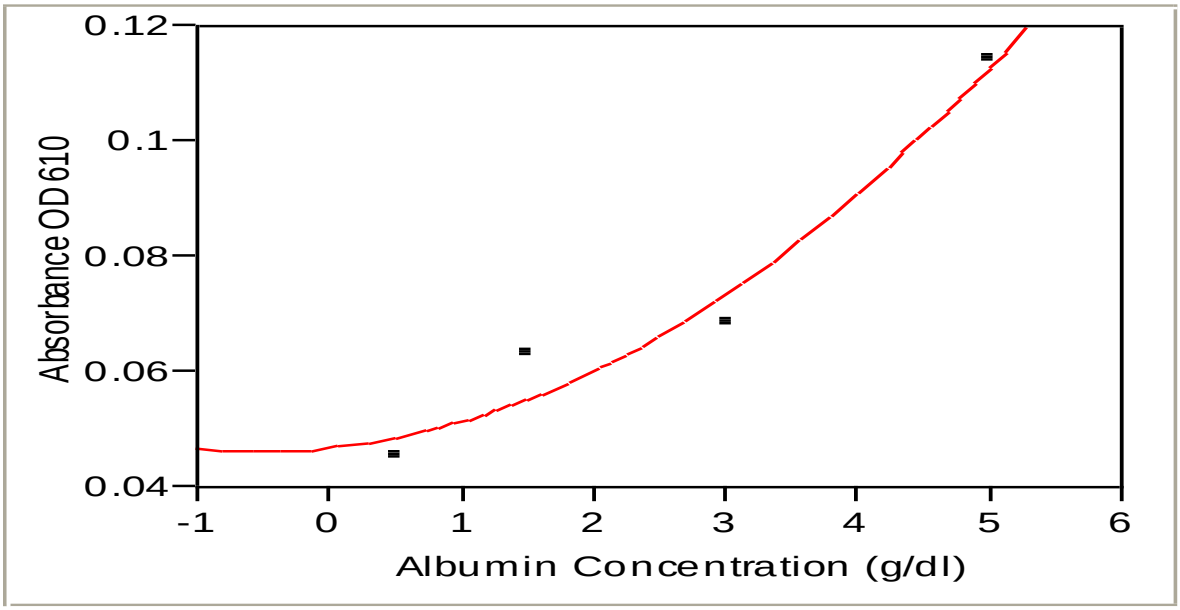

Figure 3. Standard Curve for Albumin Concentration

3.6.3 Determination of Plasma Non-Esterified Fatty Acid

Non-Esterified Fatty Acids (NEFA) levels in plasma are used as an important energy source in peripheral tissues. The amount of NEFA in plasma is dependent on a balance between intake in the liver and peripheral tissues, and its release from adipose tissues. The amount of NEFA decreases due to physical exercise, starvation, cold and fear. The current kit utilizes an in-vitro enzymatic colorimetric method for the quantification of non-esterified (or free) fatty acids in plasma. NEFA in plasma sample is converted to Acyl-CoA, AMP and pyrophosphoric acid (PP) by the actions of Acyl-CoA synthatase (ACS) under coexistence with CoA and ATP. Obtained Acyl-CoA is oxidized and yields Enoyl-CoA and hydrogen peroxide by the action of Acyl-CoA oxidase (ACOD). In the presence of the peroxidase (POD) the hydrogen peroxide formed yields a blue-purple pigment by quantitative oxidation of MEHA. The NEFA concentration was

determined by measuring the absorbance of the purple coloration. The principle of the test using the ACS-ACOD-MEHA Wako method is as follows:-

$$RCOOH(NEFA) + ATP + CoA\text{-}SH \xrightarrow{ACS} Acyl\text{-}CoA + AMP + PPi$$

$$Acyl\text{-}CoA + O_2 \xrightarrow{ACOD} 2,3\text{-}Trans\text{-}Enoyl\text{-}CoA + H_2O_2$$

$$2H_2O_2 + 4\text{-}Aminophenazone + MEHA \longrightarrow Quinoneimine\text{-}colour + 4H_2O$$

ACS: Acyl-CoA-Synthetase
ACOD: Ascorbate Oxidase
CoA: Coenzyme A
ATP: Adenosine Triphosphate
MEHA: 3-Methyl-N-Ethyl-N-(β hydroxyethyl) Aniline
PPi: Pyrophosphoric acid

The intensity of the red pigmentation is proportional to the concentration of free fatty acids in the sample. Ascorbic acid is removed by ascorbate oxidase from the sample.10ml of colour reagent solution A was used to dissolve colour reagent A while 20ml of colour reagent solution B was used to dissolve colour reagent B at room temperature (Table 1).The sample was also prepared and the spectrophotometer wavelength reading set at 550nm and calibrated with distilled water.

Table 1. The Sample and Colour Reagent A Pipetted into Labeled Cuvettes

	Reagent Blank	Standard	Sample
NEFA Standard	-	20µl	-
Sample	-	-	20µl
Color Reagent (A)	0.5ml	0.5ml	0.5ml
Reagent (B)	1.0ml	1.0ml	1.0ml

The cuvettes were thoroughly mixed and incubated for 15 minutes at room temperature (22°C) after which the colour reagent (A) was added and thereafter colour reagent (B) was pipetted into these cuvettes which were vortexed and incubated for another 15 minutes at room temperature. The absorbance of the standard and the samples were read at 550nm against the blank. The sample blank was used to correct for strongly lipaemic or haemolytic samples. The following

formular was used to calculate the concentration of free fatty acids in the plasma samples as indicated below.

$$\text{Free Fatty Acids (mg/dL)} = \text{Absorbance of sample} \times \frac{\text{Concentration of Standard (mg/dL)}}{\text{Absorbance of Standard}}$$

Concentration of the standard provided with the kit was 1mmol/L and the conversion factor to mg/dl is 1mmol/L $_X$28.2 = 1mg/dL

3.6.4 Determination of Plasma Glucose

Carbohydrates supply the body with glucose which is the most important monosaccharide in the blood. Glucose ($C_6H_{12}O_6$) is an indispensable energy supplier which supports cellular function. It is oxidized through a series of enzyme-catalyzed reactions to form carbon dioxide and water, yielding the universal energy molecule Adenosine Triphosphate (ATP), through a process known as glycolysis. Due to its importance in metabolism, glucose level is a diagnostic parameter for many metabolic disorders. The plasma glucose concentration was assayed calorimetrically using commercial kits (Glucose Hexokinase method, mti diagnostics GmbH™)

Sample and colouring solution were mixed and incubated for 5 minutes at room temperature (20-25°C) and the absorbance was read on the spectrophotometer.

$$\text{Glucose + ATP} \xrightarrow{\text{HK}} \text{Glucose-6-Phosphate + ADP}$$

Hexose Kinase (HK) catalyses the phosphorylation of glucose to glucose-6-phosphate (G-6-P) by ATP.

$$\text{Glucose-6-phosphate + NADP} \xrightarrow{\text{G-6-PDH}} \text{Gluconate-6-P+ NADPH+ } H^+$$

Glucose-6-phosphate dehydrogenase (G-6-PDH) oxidizes glucose-6-phosphate in the presence of nicotinamide-adenine dinucleotide phosphate (NADP) to gluconate-6-phosphate. No other carbohydrate is oxidized. The rate of NADPH formation during the reaction is directly proportional to the glucose concentration and can be measured photometrically.

The glucose hexokinase fluid kits have been designed to measure glucose directly in plasma without pre-treatment and are a quantitative colorimetric glucose determinant. The improved o-toluidine method utilizes a specific colour reaction with glucose. The absorbance at 340nm is directly proportional to glucose concentration in the sample when the spectrophotometer is placed at factor 1 reading of 284.

The procedure involved the addition of 1000µl of coloring regent to 10µl of sample which were incubated at room temperature (22°C) for 5 minutes. The glucose concentration was then read directly in mg/dl from the spectrophotometer.

3.7 Determination of Hormonal Profiles

3.7.1 Determination of Plasma Progesterone

Plasma progesterone (P_4) profiling is a simple and reliable method for detection of ovulation (Fricke, *et al*., 2014) and tracking of reproductive events in dairy cows (Lamming and Bulman, 1976). Progesterone, which is present in most body fluids, can be measured more effectively in plasma or milk using the "self-coating" or the Coat-A-Count® progesterone procedure which is a solid phase radioimmunoassay, whereby ^{125}Iodine labeled progesterone competes for a fixed time with progesterone in the sample for antibody sites. Because the antibody is immobilized to the wall of the polypropylene tube, simply decanting the supernatant suffices to terminate the competition and to isolate the antibody-bound fraction of the radio-labeled progesterone. Counting the tube in a gamma counter yields a number, which converts by way of a calibration curve to a measure of progesterone present in the sample. The advantage of this procedure is that there is no extraction or pre dilution as required by the other methods.

The kit for the Coat-A-Count® procedure consists of progesterone antibody coated polypropylene tubes coated with rabbit antibody to progesterone, ^{125}I iodinated progesterone packed in 105ml which are both stored at 2-8˚C and plain polypropylene tubes (12x75mm). Seven progesterone calibration vials representing 0, 0.1, 0.5, 2, 10, 20 and 40 nanograms per milliliter (ng/ml) which is equivalent to 0, 0.3, 1.6, 6.4, 31.8, 63.6 and 127.2 nanomoles per liter (nm/L). Conversion factor is ng/ml X 3.18 = nmol/L. Other requirements are foam decanting racks, logit-log graph paper and a serum-based immunoassay control.

For each assay the frozen plasma samples were allowed to attain room temperature before assaying and mixed by a gentle swirl to allow homogeneity. Four plain tubes were labeled in duplicate as T (total count) and NSB (non-specific binding). Fourteen progesterone antibody coated tubes were labeled A through to G in duplicate for the calibrators or standards which were used to draw the standard curve from which the unknown samples were extrapolated to determine progesterone concentration.

One hundred µL of the zero calibrator A were pipetted into the NSB and A tubes (maximum binding), and 100µL of the calibrators B through to G into correspondingly labeled tubes. Another 100µL were pipetted into the control and well labeled plasma sample tubes. One ml of ^{125}I progesterone was added to every tube and vortexed for 5 seconds and allowed to incubate for 1 hour in a water bath at 37˚C. These tubes were then decanted thoroughly allowing all liquid to drain completely after which the tubes were subjected to γ radiation count for 1 minute in a single well gamma counter.

The resulting counts per minute (CPM) for each tube were used to obtain the progesterone concentration from the logit-log representation of the calibration curve. First the NSB counts per minute were determined to allow for the calculation of net counts for each pair of tubes: Net counts = CPM –NSB counts. The binding of each pair of tubes as a percentage of maximum binding was then determined; the % Bound = Net Counts/Max binding Counts * 100. Using a 3-cycle semi-logarithmic graph paper, the progesterone calibrators percentage bound was plotted on the vertical (probability) axis against concentration on the horizontal (logarithmic) axis for each of the non-zero calibrators and a straight line was drawn approximating the path of these points (Figure 4). The results for the unknown samples were then read from the line by interpolation.

T = (Total Counts per minute) for the tracer
%NSB = (Average NSB counts/Total Counts)*100
%MB = (Net Counts/Total Counts)*100

For quality control the intrassay (within assay) coefficient of variation (CV) was determined to assess precision and was done by analyzing a number of samples multiple times and calculating

the CV for each of the samples. The interassay or between assay variability is also important to reflect the consistency of various assays giving similar results from the same animal between the different assays.

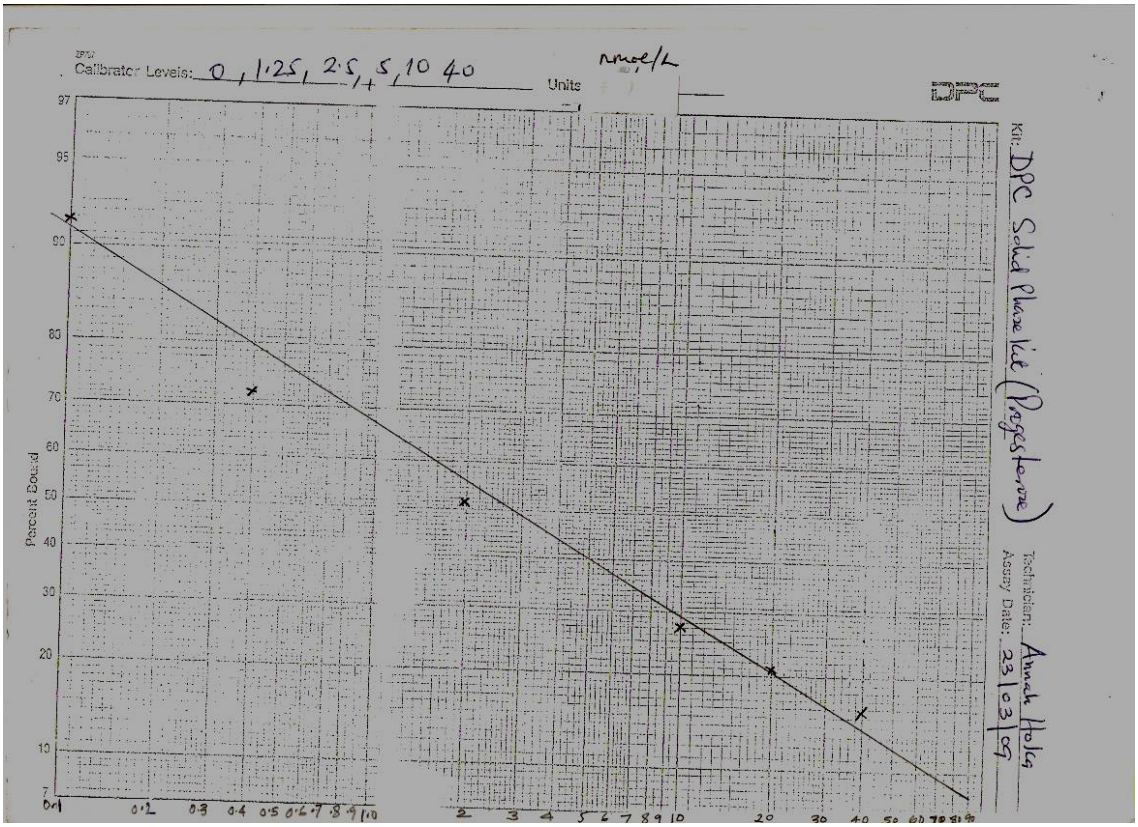

Figure 4. Logarithmic Graph for Progesterone Standard Curve

3.7.2 Determination of Plasma IGF-1

The kit provided for IGF-1 has a pre-treatment step to improve the clinical performance of the assay which is an acid-ethanol procedure (Daughaday and Rotwein, 1989). A fixed amount of labeled ^{125}Ioidine somatomedin-C (IGF-1) competes with the IGF-1 in the sample or in the calibrator for a fixed amount of antibody sites being immobilized to the wall of a polystyrene tube. After an overnight incubation at 2-8°C, an aspiration step stops the competition reaction. The tubes were then washed with 3ml of wash solution. A calibration curve was plotted for known concentrations of IGF-1 supplied with the kit and concentrations of IGF-1 in the samples were then determined by dose interpolation from the calibrated curve.

In the pre-treatment step the sample and standard plastic tubes were labeled in duplicate and 100µl dispensed for each sample and standard after which 400µl of pre-treatment solution was added to each tube. The tubes were then shaken at 1200rpm for 30 minutes and thereafter centrifuged for 10 minutes at 1500g and 100µl of supernatant was transferred to fresh labeled tubes in which 600µl of neutralization solution was added and vortexed. Coated tubes were labeled in duplicate for each calibrator (Standard), sample and controls while for the determination of Total Counts, two un-coated tubes were also labeled. In each tube, 100µl of controls, sample and standard were dispensed into the respective tubes after which 500µl of ^{125}Iodine was dispensed into all the tubes including the uncoated ones for total counts. The tubes were shaken in their racks and incubated overnight at 2-8°C. The contents of the tubes were aspirated (decanted) and washed with 3ml of working wash solution and then the gamma radiations from the tubes were counted in a gamma counter for 60 seconds.

The mean of duplicate count determinations were calculated and the bound radioactivity as a percentage of the binding determined at the zero calibrator point according to the following formula.

$$B/B_0\ (\%) = \frac{\text{Counts (calibrator or Sample)}}{\text{Counts (Zero Calibrator)}} \times 100$$

Using a 3cycle semi-logarithmic graph paper B/B_0 (%) values were plotted for each calibrator point as a function of the IGF-1 concentrations (Figure 5). By interpolation of the sample B/B_0 (%) values, the concentration of IGF-1 in the sample were determined from the calibration curve (Figure 5). The concentrations read on the calibration curve were multiplied by 35 because of the dilution factor during the pre-treatment phase to get the actual concentration of IGF-1 in the plasma samples.

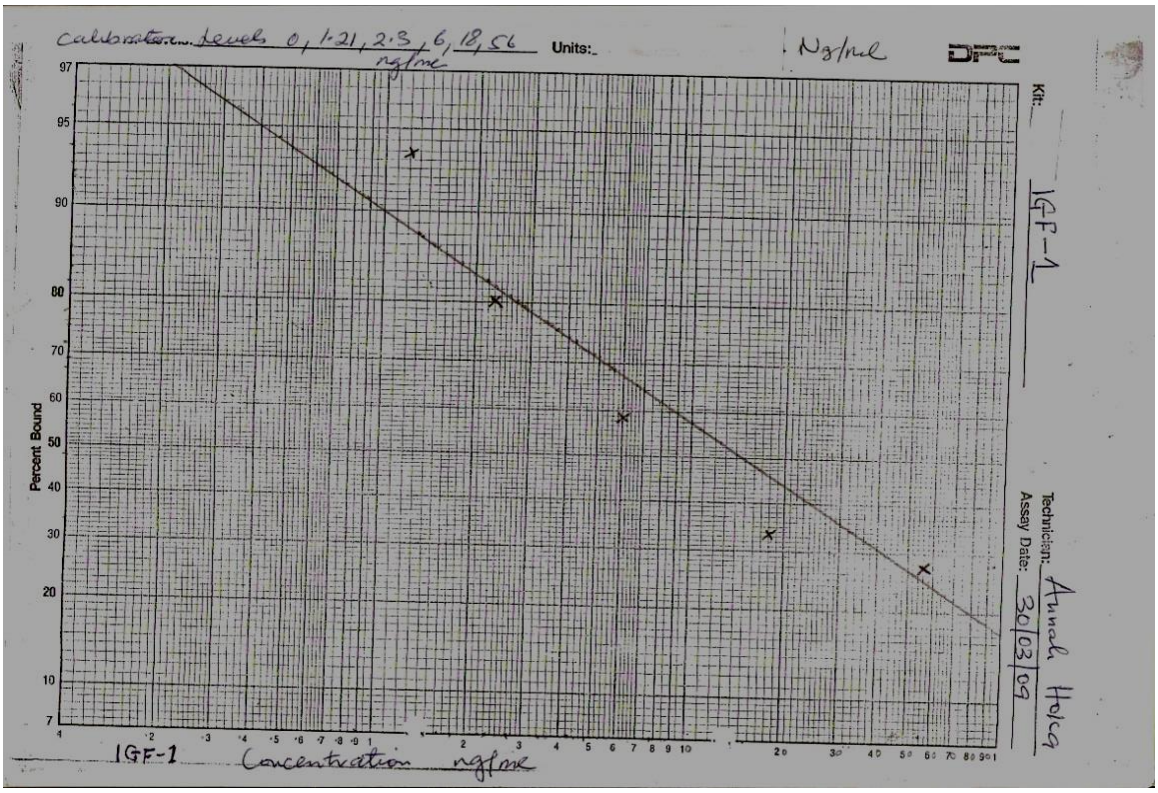

Figure 5. Logarithmic Graph for IGF-1 Standard Curve

3.8 Measuring Reproductive Performance

After parturition, the cows were visually observed for behavioral signs of estrus, with observations carried out by the day livestock handlers and the milkers during the morning and afternoon milking. The cows were served using artificial insemination upon exhibiting appropriate standing heat and clear mucous discharge from the vulva. Cows observed on heat in the morning were served in the afternoon while those exhibiting heat in the later part of the afternoon were served the following morning. The cows were inseminated up to the fourth time, after which they were considered open, but this was to be confirmed with the progesterone profiles to determine the actual reproductive status of each cow.

The recorded reproductive performance variables included, Days from calving to Commencement of Luteal Activity (Dlac) as determined from the progesterone profiles (≥ 3ng/ml); Days to first Insemination (Dins) *postpartum* and calving intervals (CI). After insemination the cows were monitored for return to heat and those returning were re-inseminated. Pregnancy diagnosis was done by rectal palpation, 90 days after insemination (Ott, *et al*., 2014). Conception rates were

determined by the number of cows bred that became pregnant during the study period divided by the number of cows exposed to AI. Other reproductive parameters recorded included foetal loss for inseminated cows that were confirmed having conceived but returned to heat later on or had observed diminished progesterone levels after a sustained rise beyond 3ng/ml. After insemination the cows were further divided into two categories comprising those that conceived and eventually calved, and those that didn't calve and their metabolic and hormonal profiles were examined to determine associations between these profiles and the calving status.

3.9 Data Analysis

The original dataset was divided into two with variables being classified as single observation point's data and repeated time series data. This was to distinguish between observations taken once per animal during the study phase and variables observed severally on the same animal overtime in a longitudinal observation.

3.9.1 Single Data Measurement Statistical Analysis

The first analysis involved the assessment of effects of various factors on milk parameters of yield, peak milk production and days to peak milk production and the reproductive performance of experimental cows as detailed in the model below. The data was subjected to Analysis of Variance (ANOVA) performed using the General Linear Model (GLM) procedure of SAS program, release 8.2 (SAS, 2001). Simple mean separations were performed using the t-test with p-values pegged at 0.05 significant levels. The models fitted for the single measures were:-

$$Y_{ijkl} = \mu + bi + t_j + (bt)_{ij} + p_k + d_m + \varepsilon_{ijklm}$$

Where;

Y_{ijkl} were the dependent observations of milk parameters (yield, peak milk production and days to peak) and reproductive performance (Dlac, Dins and CI); while μ, b, t, p and d represent the overall mean, the fixed effects of breed, treatment (feeding levels), parity and BCS respectively, while ε was the residual error.

3.9.2 Repeated Measures Variables Analysis

These were measurements made on the same animal over a period of time and are more likely to be more correlated than those on different animals. For this analysis to be valid, the covariances among repeated measures must be modeled using the proper covariance structure. The three most commonly used covariance structures are, Compound Symmetry (CS), Unstructured (UN) and Autoregressive order one [AR(1)] (Littell, *et al*., 1998).

The variables measured in this study included milk production, composition, metabolic and hormonal parameters. The milk parameters included weekly milk production, % Butterfat (BF), % Protein (P), % Solid Not Fat (SNF), Density (D) and Freezing Point (Fp) while metabolic parameters were glucose, non-esterified fatty acids (NEFA), urea and albumin. The hormonal observations were progesterone and insulin-like growth factor 1 (IGF-1).

ANOVA was carried out using the mixed model procedures of SAS program package release 8.2 (SAS, 2001) with repeated measures as described by Plaizer, *et al*., (2005). Milk production and composition, metabolite and hormonal levels measured every week were the repeated factors while the cow was the subject. The fixed effects of breed, feed, time (week) and their respective interactions on milk parameters, nutritional metabolites and hormones were then determined using the following model:-

$$Y_{ijkl}=\mu+b_i+t_j+w_k+bt_{ij}+bw_{ik}+tw_{jk}+b(t)_j+\varepsilon_{ijkl}$$

Where;

Y_{ijkl} =Milk parameters, metabolic and hormonal variables, μ=overall mean, b_i=breed, t_j=feed levels, w_k=week (time), bt and bw are interactions, $b(t)_j$=breed effects nested within feed, ε_{ijkl}=residual error. The [AR(1)] covariance structure was used for urea, albumin NEFA, progesterone and IGF-1while (US) was used for glucose. These were the structures which gave the lowest Akaike's Information Criterion (AIC) (Littell, *et al*., 1998) used in selecting the most suitable structure for longitudinal data analysis.

3.9.3 Principal Component Analysis

Principal component analysis is a statistical approach (Cattell, 1978) for removing redundant information from correlated variables to represent the original variables with a smaller set of derived variables called principal components. It is appropriate when a number of observed variables are associated with one another, possibly because they are measuring the same construct and which are used to identify groups of observed variables that tend to hang together empirically. The variables that are closely correlated form the principal components, which may be used as predictor variables in further analysis, thus forming a smaller number of artificial variables (called principal components) that account for meaningful amount of variance for the response variables. Empirically the number of components extracted in a principal component analysis is equal to the number of observed variables analyzed. However, in most analyses, only the first few components account for meaningful amounts of variance, and are retained for interpretation and subsequent analyses, the remaining components account for trivial amounts of variance and hence not included for further analysis.

Principal component analysis were performed on milk composition variables, metabolite and hormonal variables to determine those closely correlated and therefore measuring similar constructs that account for most of the variance in the observed variables.

3.9.4 Multiple Regression Analysis

Multiple linear regression models were developed for the hormones of progesterone and IGF-1 as response variables while the metabolites of glucose, NEFA, urea, and albumin were the explanatory variables. These were to determine the influence of metabolites on the hormonal profiles.

$$Y_i=\alpha+\Sigma b_i X_i+\varepsilon_i$$

Where;

Y_i were hormonal variables, α=intercept, b_i=regression coefficients, X_i=metabolites (glucose, NEFA, urea, albumin) and ε_i=residual error

3.9.5 Logistic Regression Analysis

A stepwise logistic regression was fitted with the response variable being progesterone concentration of cows categorized as those having mean P_4 levels above 3ng/ml while the other category comprised of cows whose level of P_4 were below 3ng/ml. Breed and feeding levels were included in the model to determine their effects on the P_4 category, while metabolites were nested within feeding levels. The stepwise regression retained breed and feeding levels nested within NEFA and albumin in the model respectively. The rest of the variables were eliminated because their inclusion did not significantly improve the model. This model was to predict the probability of metabolite concentration influence given the various feeding levels on plasma progesterone concentration, which may have a bearing on reproductive performance. The model developed was as indicated.

$$Y_i = \alpha + bi + NEFA\ (t)_{ij} + Albumin\ (t)_{ik} + \varepsilon_{ijk}$$

Where;

Y_i were the two categories of progesterone levels to luteal activity; with α, b representing the intercept and effects of breed while NEFA and albumin nested within feed (t) and ε the residual error.

Pearsons linear correlation coefficients were used to determine the associations between milk parameters, metabolites, hormones and reproductive performance.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Nutritive Value of Feeds

The nutritional composition of forage and concentrates fed to experimental cows are summarized in Table 2. These values were similar to those reported by Smith, *et al.* (2006) in Ethiopia and Irungu and Mbugua (1998) in Kenya. They reported a mean crude protein of 15.8% and 8.3%, while crude fibre was 9.6% and 32.3% for dairy meal and pasture respectively. High values for NDF as indicated below reflect the good pastures used, and were suitable in enhancing rumen function (Russel, *et al.* 1992).

Table 2. Mean±SD of Nutritional Values for Forage and Concentrate Fed to Cows

Nutritional Component	Forage (%)	Concentrate (%)
Dry Matter	36.8±2.5	89.4±3.7
Crude Protein	4.7±2.2	16.8±1.1
Neutral Detergent Fiber	41.7±6.7	45.7±9.5
Acid Detergent Fiber	42.6±2.5	22.4±4.9
Acid Detergent Lignin	4.7±1.6	3.1±0.6
Ash	8.9±1.3	9.42±2.1

4.2 Changes in Body Weight and Condition on Reproductive Performance

The mean age and weights of cows and their calves at commencement of the experiment are presented in Table 3. The Sahiwals were much older ($p<0.05$) while Friesians were heavier ($p<0.05$) at commencement of the experiment. The mean calf weights for the two breeds were significantly different ($p<0.05$) and as a proportion of cow weight at calving were higher in Friesian (6%) than in Sahiwals (5%) respectively. The Friesian calf weights in this study were lower than the mean of 32.7kgs reported by Irungu and Mbugua, (1998). The mean age at first calving for cows in this study showed that Sahiwals averaged 48 months (Table 3) which occurred much later than 44 months reported by Ilatsia, *et al*., (2007), while that of Friesians was similar to 39.2±7.5 months reported by Tadesse, *et al*., (2010) in the highlands of Ethiopia.

Table 3. Mean ± SD of Age and Weight of Cows

	N	Age at First Calving (Months)	*Age of Cows (Months)	Weight at Calving (kg)	Calf Birth Weight (Kg)
Friesian	16	37.3±5.6[a]	75.4±22.8[a]	442.2±47.9[a]	26.6±4.2[a]
Sahiwal	17	48.5±5.5[b]	102.4±20.9[b]	385.7±23.6[b]	19.2±3.1[b]
All Cows	33	43.1±7.9	89.3±25.2	413.2±46.6	22.8±5.2

[ab] *Different superscripts within column are significant P<0.05*
**Age of cows at commencement of the experiment*

Table 4 presents body weight and condition scores at commencement of luteal activity and at insemination for Sahiwal and Friesian cows. Though both breeds had comparable body weight loss from calving to commencement of luteal activity, (27 vs. 26 kg equivalent to 6% and 7% of weight at calving), Sahiwal had a higher ($p<0.05$) average daily weight loss (0.64 kg vs. 0.38 kg/day). This difference is due to the shorter number of days to get to luteal activity by the Sahiwals compared to the Friesians. The mean body weight loss for both breeds from calving to commencement of luteal activity averaged 0.53kg which is similar to the findings of Gutierrez, *et al*., (2006) who reported a weight change of 0.56kg per day after parturition in Friesian cows. However, at insemination the weight loss for Friesians reduced to 1.8% while for the Sahiwals increased to 8% relative to weight at calving. This is an indication of differences in adaptation and recovery rates for the two breeds associated with parturition due to the demand for nutrients for milk production. These findings are much lower than the 10% weight loss, 20 days after parturition of Holstein Friesians fed on pasture in New Zealand as reported by Clark, *et al*., (2005).

Table 4. Changes in Body Weight and Condition Scores at Commencement of Luteal Activity and Insemination

Breed	**Mean±SE Live Weight (kg)**			**Mean±SE Body Condition Scores**		
	Calving	Luteal Activity	At Insemination	Calving	Luteal Activity	At Insemination
Friesian	442±48[a]	416±47[a]	434±27[a]	4.0±0.06[a]	3.6±0.08$_a$	3.5±0.05[a]
Sahiwal	385±24[b]	358±29[b]	351±21[b]	3.4±0.05[b]	3.3±0.04[b]	3.2±0.04[b]
All	413±47	384±47	377±46	3.6±0.06	3.4±0.06	3.3±0.05

Superscript within column show significant differences p<0.05

Figure 6 shows that Friesians lost weight rapidly and quickly recovered at time of insemination while the weight loss for the Sahiwals was gradual and took longer to recover and is a reflection of how the two breeds adapt to energy balance after parturition. The *nadir* for weight depression occurred between weeks 5 and 7 *postpartum* and coincides with peak milk production. This may be due to competition for nutrients between milk production and the accumulation of body reserves in preparation for the next reproductive cycle. These findings are similar to those reported by Banos, *et al*., (2006) who showed a cumulative energy balance decrease up to week 7 *postpartum* and increased thereafter during the recovery phase.

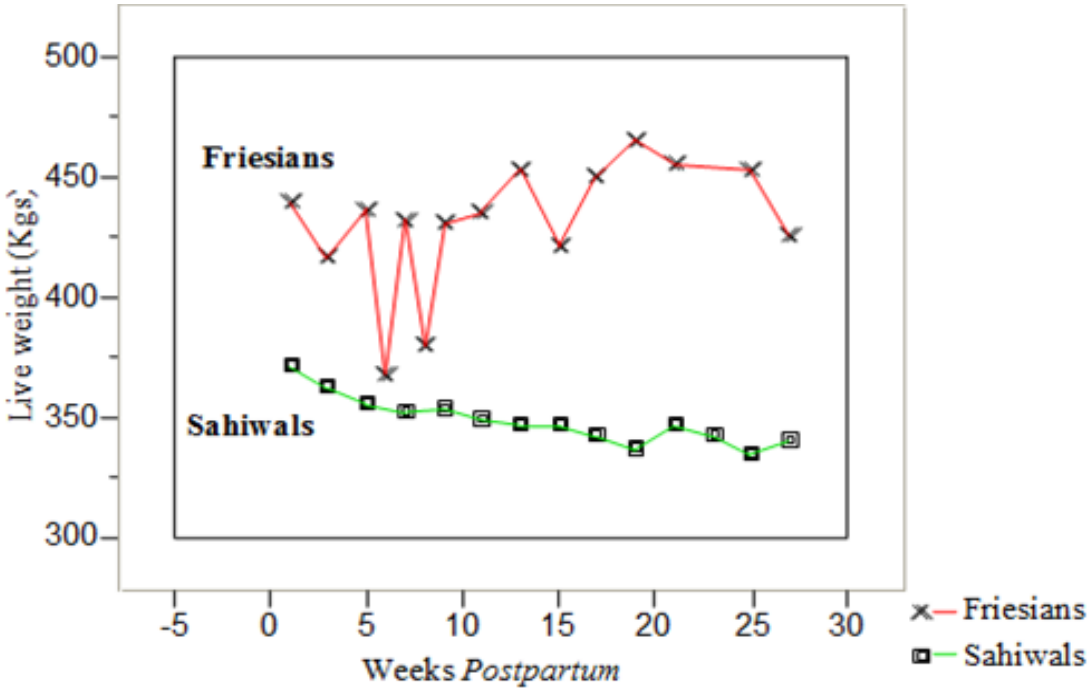

Figure 6. Changes in Body Weight for Cows after Parturition

Body condition scores for both breeds were depressed up to week 6 *postpartum* (Figure 7) before starting to recover. The mean loss in body condition for the two breeds shows that Sahiwals lost less body condition at the two points than the Friesians (Table 4). This could be the reason Sahiwals got into cyclicity and were inseminated earlier than the Friesians. Thus, body condition scoring could be a better indicator of the cumulative energy balance of the cows *postpartum* than body weight and agrees with the findings of Banos, *et al*., (2006). The *nadir* for body condition loss in Sahiwals occurred during week 7 and recovered from this depression after week 16

postpartum (Figure 7). Compared to Friesians, the BCS patterns are similar with *nadir* occurring at week 6 and full recovery realized at week 15 *postpartum* (Figure 7). This is similar to the findings of Chagas, *et al*., (2007) who reported a *nadir* for BCS at day 50 *postpartum* in Friesian cows. The recovery phase continues after the *nadir* and luteal activity commences week 10, while insemination occurs week 14 *postpartum* in Friesians. As for the Sahiwals, luteal activity commenced at week 6 and insemination occurred week 10 *postpartum*. This shows that the threshold for initiating luteal activity despite negative energy balance in Sahiwals is higher than for Friesian and can be attributed to adaptation.

It was noted that insemination in both breeds occurred 4 weeks after commencement of luteal activity and it can be concluded that body condition scores are a better indicator of the metabolic energy balance for the two breeds than body weight changes *postpartum* under tropical production systems. This agrees with the findings of Okantah, *et al*., (2005) who found that body condition losses in Sanga cattle during early lactation led to extended anestrus and is a reflection of negative energy balance (Roche, *et al*., 2007).

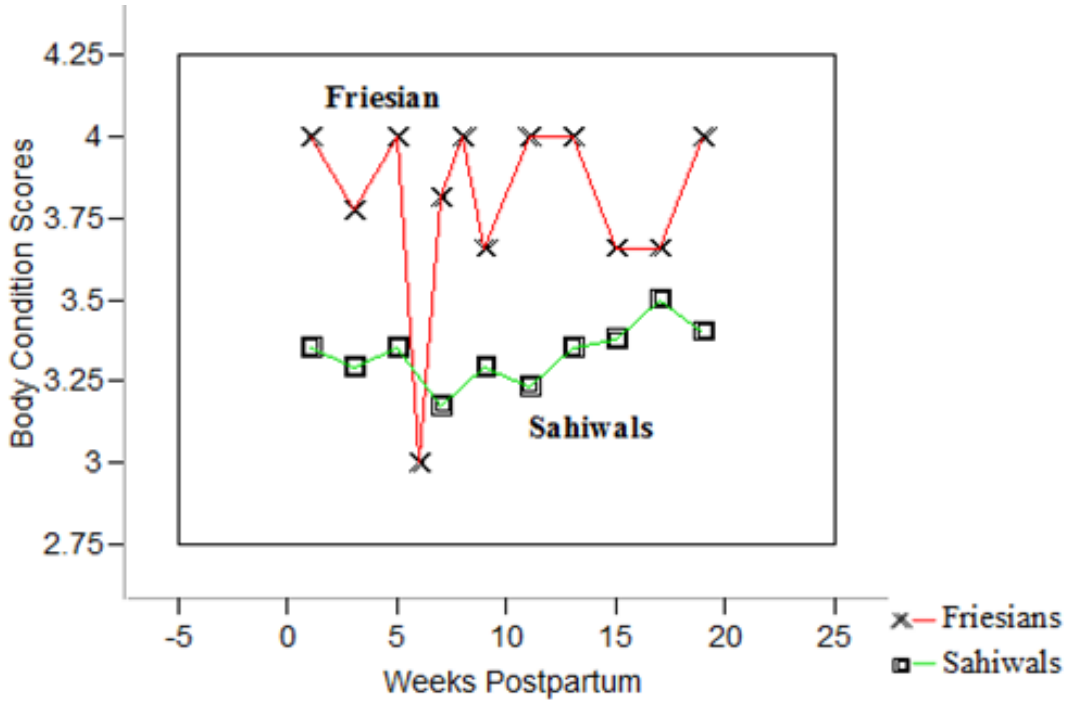

Figure 7. Change in Body Condition Scores for Cows after *Parturition*

First ovulation *postpartum* in dairy cows occurs some days after the *nadir* in energy balance (Beam and Butler, 1998). Therefore, the longer it takes to reach the nadir in energy balance the longer the delay to first ovulation *postpartum.* The main factor causing delayed ovulation postpartum is low LH pulsatility (Butler, 2000). Moreover, altered LH pulsatility is linked to nutritional changes. Wu, *et al*., (2013) showed that the direction of body weight changes affected the pattern of LH secretion in heifers, with heifers gaining weight and condition having higher LH pulse frequency than those losing weight, and thus inducing ovulation.

Heavier cows at calving take longer ($P<0.05$) to commence luteal activity (Table 5), but also have shorter calving intervals. This could be due to increased feed intake resulting in increased blood flow to the liver causing enhanced metabolism of progesterone due to increased metabolic clearance rate of steroids (Sangstritavong, *et al.,* 2002). The increased metabolic clearance rate of steroids results in depressed levels of progesterone and estrogen that are useful hormones for expression of estrus (Vasconcelos, *et al.,* 2003). Older cows have reduced days to commencement of luteal activity, this could be due to well-developed body reserves which do not therefore compete for nutrients for lactation, and allowing for cyclicity to proceed early unlike in younger cows where demands for nutrients for growth mitigate against reproduction.

Larger calves at calving result in delayed commencement of ovulation (Table 5). This could be because of higher nutrient demands for foetal growth and preparation for lactation during the third trimester leaving the dams in more NEB, thus impacting negatively on reproduction (Sangstritavong, *et al.,* 2002). Heavier cows also had shorter calving intervals and heavier calves at birth, this is a reflection of condition and metabolic status of the cows implicating a favorable energy status resulting in quick resumption of cyclicity and the foetus having adequate nutrients to enhance growth in the uterus. The cow's weight at commencement of luteal activity is closely associated with the body condition score and this mirrors the metabolic status of the cow, this agrees with the findings of Garnsworthy, (2007) who found a strong association of $r^2=0.82$ and is influenced by genetic merit.

Higher calf weights and better cow body condition at calving were negatively associated with days to insemination, which indicated that cows with heavier calves and in better body condition

were inseminated earlier than their counterparts (Table 5). Again, this is a reflection of a favorable metabolic status of the cow to support foetal growth, and upon calving, recover quickly from lactational nutrient demands. Good body condition at calving is essential for better calf weights and subsequent reproductive performance. Calving interval (CI) was negatively associated with weight of cow at calving indicating that lower than normal weight at calving led to prolonged calving intervals due to delayed commencement of cyclicity and more repeats before conception, these findings agree with those reported by Chagas, *et al*, (2007).

Table 5. Linear Correlations of Reproductive Traits with Cow Parameters

	Dlac	**Dins**	**CI**	**Wt**	**Wtlac**	**Wtins**	**AFC**	**Age**	**Calfwt**	**Cbon**
Dlac	1	0.29	-0.21	0.37*	0.02	0.18	-0.14	-0.26	0.31*	0.14
Dins		1	-0.14	-0.07	-0.12	-0.05	-0.12	-0.13	-0.12	-0.5*
CI			1	-0.47*	-0.27	-0.25	-0.05	-0.02	-0.15	0.22
Wt				1	0.57*	-0.54*	-0.32	-0.34*	0.43*	0.24
Wtlac					1	0.72*	-0.53*	-0.48*	0.56*	0.55*
Wtins						1	-0.54	-0.49*	0.61*	0.51*
AFC							1	0.49*	-0.49*	-0.46*
Age								1	-0.35	-0.46*
Calfwt									1	0.58*
Cbon										1

**significant at p<0.05*

There is a positive linear correlation (r=0.29) between days to commencement of luteal activity and days to insemination (Table 5) which means that cows taking longer to commence luteal activity also delay in showing heat signs for insemination. This means that phenotypically, the interval to first service increases as the interval to commencement of luteal activity increases (Royal, *et al*., 2002). Thus, it has been suggested that the interval from calving to commencement of luteal activity be incorporated in a selection index as an indicator of reproductive efficiency (Darwash, *et al*., 1997; Royal, *et al*., 2002)

However, there is a negative correlation (r=-0.21) between days to commencement of luteal activity (Dlac) and calving interval (CI) which indicates that animals taking longer to get into luteal activity have sufficient time to regain their metabolic status and therefore, would require

less repeats of inseminations before conception leading to reduced calving intervals. Older cows take a relatively longer time (r=-0.26) to get into cyclicity than younger cows (Table 5) and could be a reflection of reducing reproductive efficiency with age. This agrees with the findings of Cavestany, *et al.,* (2009), who explained this difference to be due to reduced concentration levels of IGF-1 in multiparous cows relative to primiparous cows.

4.3 Milk Production and Reproduction

An ANOVA for milk parameters comprising milk yield, peak milk production and days to peak for the experimental cows indicated that breed, feeding levels and body condition influenced all the milk parameters as observed in Table 6, while parity only had an effect on peak milk production ($p<0.05$). There were also significant interactions between feeding levels and breed (Table 6) for all the milk parameters. Age and weight when included as covariates influenced milk production and days to peak milk production (Table 6).

Table 6. Analysis of Variance of Milk Parameters Measured

		F Ratio		
Source	**DF**	**Milk Prod**	**Peak Milk**	**Days to Peak**
Breed	1	4.72*	78.25*	9.72*
Feeding Levels	4	11.13*	14.36*	23.11*
Breed*Feeding Levels	4	13.77*	43.60*	54.29*
Parity	2	1.36	3.52	36.77*
BCS	2	36.30*	58.15*	9.72*
Age	1	11.97*	4.69	30.89*
Weight	1	5.50*	0.58	53.72*

**Significant at p<0.05*

Friesians produced significantly ($p<0.05$) more milk than the Sahiwals (66.3 vs 58.9 lts) respectively (Table 7) which is similar to 72.5 liters per week reported by Irungu and Mbugua (1998) for Friesians while Sahiwals produced more milk per week than that reported by Ilatsia, *et al*., (2007) of 34.3 liters. This is to be expected as Friesians having been selected for milk production are expected to produce more milk than the Sahiwal which is a dual purpose animal.

The two breeds also exhibited different milk peaks, with Friesians and Sahiwals attaining peaks of 88.9±1.7and 68.3±1.6 liters per week after 52 and 42days *postpartum* respectively (Table 7). The Friesian peak is similar to that attained at 50 to 70 days reported by Roche, *et al*., (2006).

Commencement of luteal activity and first insemination in Friesians occurred 2 and 6 weeks after peak milk production respectively. For Sahiwals, peak milk occurred at the same time as commencement of luteal activity which was 6 weeks *postpartum* and insemination occurred 4 weeks after the peak milk production. The two breeds took similar periods of 4 weeks from commencement of luteal activity to insemination despite them having different periods to commencement of luteal activity from calving. Banos, *et al.,* (2006) found that net energy balance of lactating Friesian cows decreased until week 10 of lactation that coincided with peak milk production and this balance improved thereafter, thus having a direct implication on when the cows begin cycling.

Body condition scores and parity also had an effect ($p<0.05$) on the milk parameters (Table 7). Cows with higher body condition scores produced more milk progressively and had higher peaks compared to those with lower condition (Table 7), however, this group of cows also took longer to reach peak milk production. This is a reflection of the positive net energy balance resulting in more nutrients being availed for milk production for cows in better body condition. Cows of parity 3 and 4 (B) produced higher average milk production ($p<0.05$) and peak milk (Table 7) while those of parities greater than 5 (C) reached peak milk production much earlier.

Table 7. Least Square Means±SE of Milk Production Parameters

	Factor	N	Milk Production (Lts/week)	Peak Milk Production (Lts/week)	Days to Peak Milk Production
Breed	Friesian	16	66.3±2.4[a]	88.9±1.7[a]	52.1±2.1[a]
	Sahiwal	17	58.9±2.3[b]	68.3±1.6[b]	42.9±2.0[b]
Parity	A	16	62.4±3.6[a]	74.3±2.5[a]	65.3±3.1[a]
	B	9	64.7±1.7[a]	80.2±1.2[b]	43.3±1.5[b]
	C	8	60.7±2.2[b]	81.4±1.5[b]	33.9±1.9[c]
Body Condition	3	6	45.9±1.8[a]	64.7±1.3[a]	40.7±1.6[a]
	4	23	57.8±2.0[b]	71.1±1.4[b]	49.9±1.8[b]
	5	4	84.1±4.4[c]	100.1±3.1[c]	51.9±3.8[b]

[a,b] *Indicates values with different superscripts within column differ significantly ($p<0.05$)*

The lactation curves for both breeds during the experimental period are represented in Figure 8 with R^2 of 0.96 and 0.98 for Friesians and Sahiwals respectively after fitting a third order degree polynomial. The Friesians weekly milk levels declined to less than 30% of peak lactation of 26.7 liters after 29 weeks while Sahiwals declined to 20.5 liters after 32 weeks of lactation as described by Mech, *et al.*, (2008) who reported that a cow should be considered dry when milk production declines to less than 30% of the peak milk yield. Thus, the mean lactation lengths for Friesians and Sahiwals were 203 and 224 days *postpartum* respectively as indicated in Figure 8. This is much less than the conventional 305 days for most dairy cows (Appuhamy, *et al*., 2007) and 282 days reported by Ilatsia, *et al*., (2007) for Sahiwals. Both breeds of cows dried about 3 months earlier than expected.

The characteristics of the lactation curve are important as they determine the production potential and allow for assessment of the nutritional and health status of a milking cow (Dudouet, 1982; Chang, *et al.*, 2001). A number of studies have shown that excess mobilization of body reserves or negative energy balance resulting from the energetic demands for milk production during early lactation is associated with a decline in reproductive performance (Lucy, 2001; Butler, 2000). This is because of selection for milk production thus compromising reproductive performance (Pryce, *et al*., 2002).

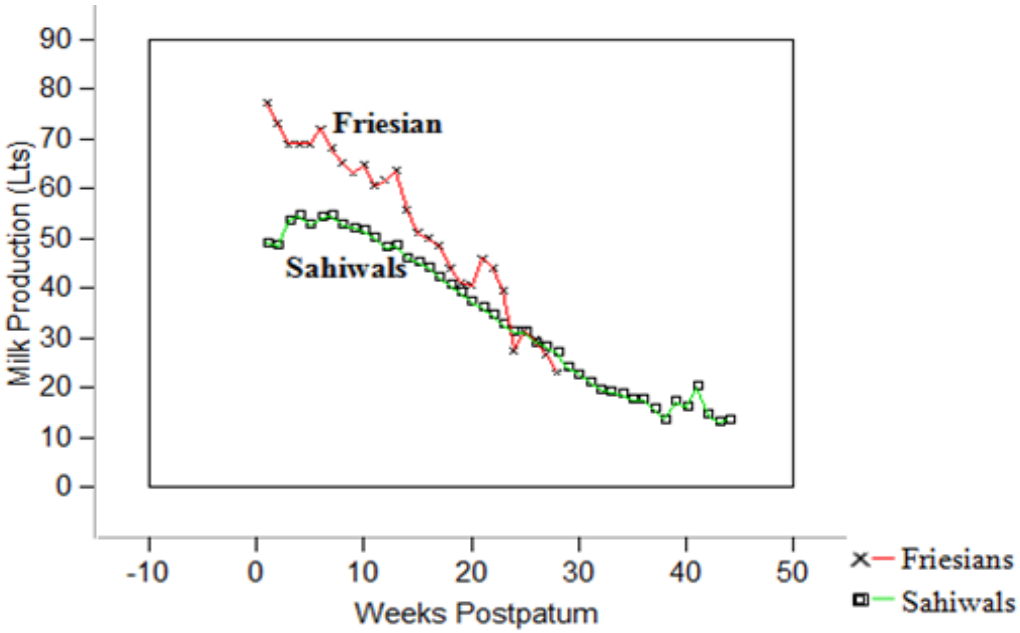

Figure 8. Lactation Curves for Friesian and Sahiwal Cows Postpartum

4.4 Milk Composition

Milk composition varies depending on the species, breed, feed, and the stage of lactation (Mech, *et al.*, 2008). Charles (1998) reported that Holsteins tend to have the lowest percentage milk composition as compared to Jersey, Ayrshire and Guernsey breeds. These breed differences are due to efficiencies in energy conversions to specific feed types (Rodriguez, *et al.*, 1997). The differences in milk composition between the Jerseys and Holsteins showed that Jerseys had greater efficiency of energy conversion than Holsteins.

Feeding levels did not affect ($p>0.05$) milk components apart from percent fat (Table 8). These findings are similar to those reported by Alzahal, *et al*., (2009). In the current study, breed, had a significant ($p<0.05$) influence on percent fat, SNF, Protein and freezing point of milk. Parity had an effect on SNF, protein, density and freezing point while there were significant differences for body condition for all the milk components apart from density (Table 8). Body weight of cows at calving affected fat and protein composition of milk. There were also significant interactions of breed and feed for all components apart from milk density (Table 8).

Table 8. Analysis of Variance for Milk Composition of Experimental Cows

		F-ratios					
Source	DF	Fat	SNF	Protein	Density	Freezing Point	Fat/ Protein Ratio
Breed	1	3.12*	1.24**	0.17**	6.32	139.72**	0.09
Feed	4	14.41**	0.53	0.09	13.55	17.05	1.37*
Parity	2	2.94	2.46**	0.33**	40.96**	148.56**	0.38
Body Condition	2	22.39**	1.37*	0.21**	8.02	49.03*	1.82**
Breed*Feed	4	36.80**	3.15**	0.46**	34.72	158.91**	3.2**
Weight	1	5.19*	0.68	0.10*	1.77	20.61	0.40
AFC	1	7.16**	0.49	0.03	22.55*	19.3	0.51*

**Significant at p<0.05 ** Significant at p<0.01*

There were significant ($p<0.05$) breed differences for most of the milk components with Sahiwals having higher values than Friesians (Table 9). This was expected as Zebus, normally have higher values for milk components relative to the exotics (Charles, 1998; Walsh, *et al.*, 2008). The Sahiwals with higher amounts of solids in milk had relatively higher milk densities and lower

freezing point depressions (Table 9) than the Friesian cows and this was expected (Henno, *et al*., 2008). Body condition and breed by feed interaction influenced ($p<0.05$) milk composition apart from density which means that different breeds when supplemented, partition nutrients differently at parturition due to the efficiencies of energy conversions (Rodriguez, *et al.*, 1997; Walsh, *et al*., 2008).

Table 9. Means of Milk Components for the two Breeds

Breed	N	%Fat	%SNF	% Protein	Density	Freezing Point ºC	Fat/Protein Ratio
Friesian	16	3.47 ± 0.18^{a}	8.15 ± 0.06^{a}	3.07 ± 0.02^{a}	1.026±.029	-0.535 ± 0.004^{a}	1.13 ± 0.06^{a}
Sahiwal	17	5.19 ± 0.07^{b}	8.71 ± 0.04^{b}	3.30 ± 0.01^{b}	1.027±.017	-0.565 ± 0.002^{b}	1.58 ± 0.02^{b}

^{ab} *Different subscripts within column are significant ($p<0.05$)*

Duffield, *et al*., (1997); de Vries and Veerkamp (2000) and Heuer, *et al*., (2001) consistently found that in cases of negative energy balance during early lactation, there was an increase in percentage butter fat due to lipid mobilization which find their way in the milk, while there is a decrease in the percentage of milk protein, thus increasing the fat to protein ratio. It was therefore, suggested that this ratio is a potential indicator of lack of energy supply through feed. Heuer, *et al*.,(1999) indicated that cows with fat to protein ratios greater than 1.5 were more at risk of getting mastitis, ovarian cysts and had profound increased negative effects on fertility traits.

In the current study, the fat to protein ratio indicated that Sahiwals were in more negative energy balance than the Friesians; however, this is contrary to the reproductive performance, because Sahiwals came into cyclicity and were inseminated much earlier than the Friesians, which would indicate that they were in a more favourable metabolic state than the Friesians. However, this can be explained by the fact that percentage fat values for Sahiwals were consistently higher than those of the Friesians while the values for protein were quite similar in both breeds (Table 9). Thus, this ratio can only be meaningful when compared within breed and not across breeds because of the inherent differences of milk composition and ability to mobilize fat among the different breeds.

The link between higher milk production and poorer reproduction is well established. Madouasse, *et al*., (2010) suggested that after milk yield, percentage of protein followed by percentage of lactose were the most important variables associated with the probability of conception.

For milk fat and protein components, it was observed that despite the supplementation, Sahiwals maintained higher ($p<0.05$) values consistently (Figures 9 and 10). For % fat and protein, supplementation levels in Sahiwals increased up to 3kg and gave the best levels for the two components while Friesians showed best levels at 2kg supplementation after which there was depression (Figures 9 and 10). These can be regarded as the optimal supplementation levels for enhanced fat and protein levels in milk for the two breeds. This agrees with the findings of Walker, *et al*., (2004) who demonstrated that breed and feeding regime affected milk composition on pasture fed cows in Australia.

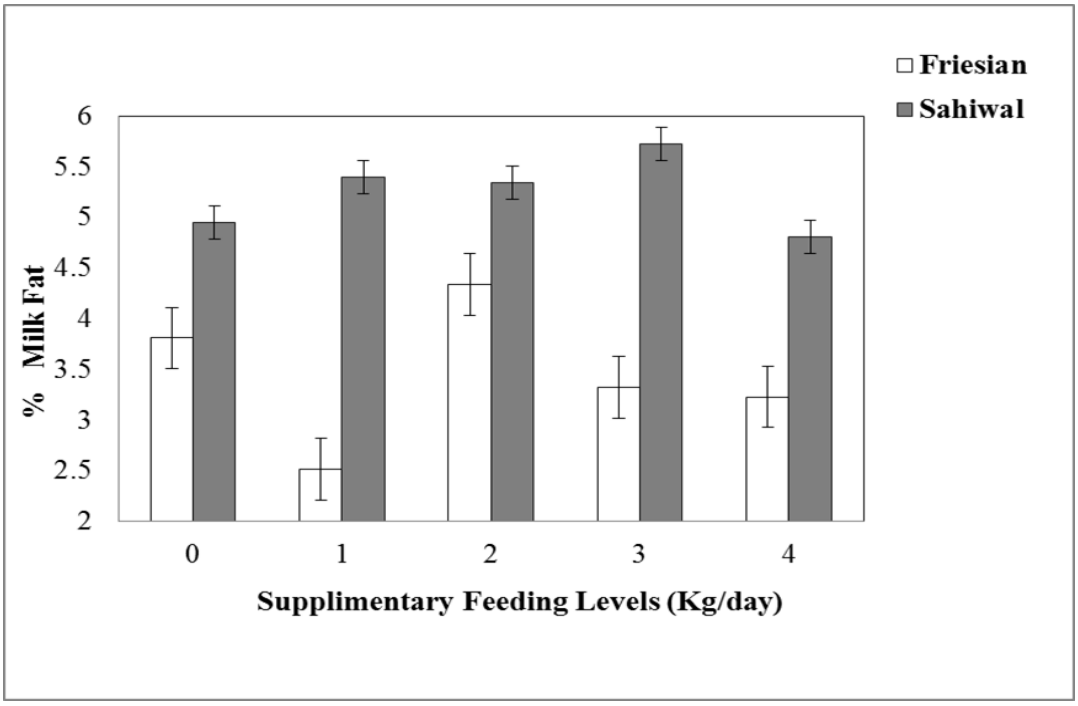

Figure 9. Percent Milk Fat by Feed Supplementation

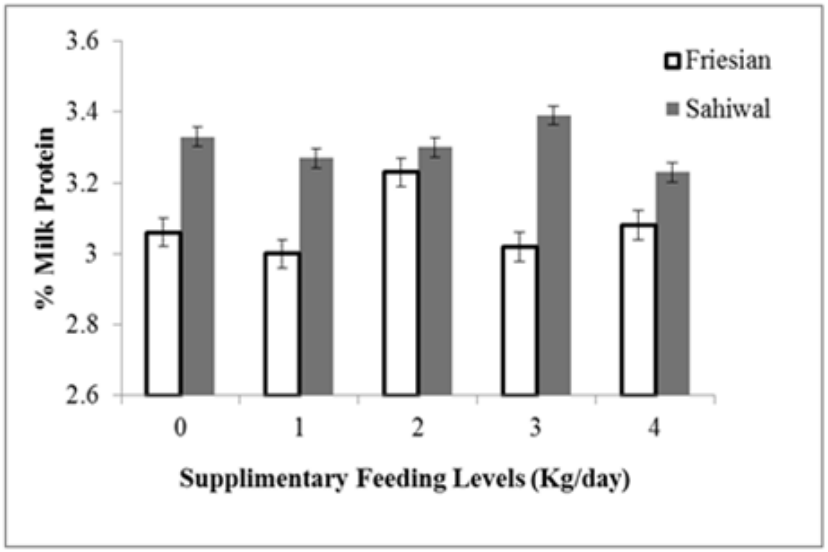

Figure 10. Percent Milk Protein by Feed Supplementation

There was significant positive association ($p<0.05$) between all milk components and reproductive parameters of days to luteal activity (Dlac) and insemination (Dins) for Friesian cows but little association with calving intervals (Table 10). This was bhjo particularly so for SNF and protein which indicates that the higher the values of the components the longer it takes to initiate luteal activity and insemination and can be explained by the fact that if milk components are high, then these have been derived from the blood thus depriving the reproductive system of the necessary nutrients to initiate reproduction for Friesians *postpartum*. This is in agreement with the findings of Clark, *et al.,* (2005) who reported that milk components have a relationship with energy balance after parturition that affect reproduction on grass fed Friesian cows.

Table 10. Correlation of Milk Components with Reproductive Parameters for Friesians

	% Fat	% SNF	Density	% Protein	Dlac	Dins	CI
%Fat	1.00	0.04	-0.43**	0.10	0.28*	0.27*	-0.03
%SNF		1.00	0.89**	1.00**	0.61**	0.64**	0.06
Density			1.00	0.86**	0.43**	0.46**	0.06
% Protein				1.00	0.63**	0.66**	0.05
Dlac					1.00	0.94**	-0.20*
Dins						1.00	-0.07
CI							1.00

**Significant at p<0.05 ** Significant at p<0.01*

Sahiwals showed a positive association between SNF, % protein and density with days to commencement of luteal activity (Table 11), which is an indication that higher values for these components results in prolonged resumption of cyclicity. This is expected because as more nutrients are derived from the system for milk production; the cows are exposed to negative energy balance resulting in prolonged anoestrus. However, the relationship with days to insemination was negative (Table 11) implying that cows with higher values get into insemination earlier, which is an indication that higher values for milk components in Sahiwals result in reduction in the period between commencement of luteal activity and time of insemination. This could be an inherent characteristic for the Sahiwals and by extension the Zebu which normally have higher values for milk components than their Friesian counterparts.

However %fat exhibited a strong positive association with calving interval (Table 11) an indication that higher milk fat results in longer calving intervals and is a reflection of increased fat mobilization due to negative energy balance resulting in increased repeat inseminations causing prolonged calving intervals and is in agreement with the findings of Madouasse *et al*., (2010). This implies that the relationship of milk components with reproduction for the two breeds is mediated differently after parturition.

Table 11. Correlations of Milk Components with Reproductive Parameters for Sahiwals

	% Fat	**% SNF**	**Density**	**% Protein**	**Dlac**	**Dins**	**CI**
% Fat	1.00	-0.13*	-0.54**	-0.07	-0.19*	0.00	0.43**
% SNF		1.00	0.83**	0.99**	0.14*	-0.32**	0.16*
Density			1.00	0.80**	0.23*	-0.27**	-0.13*
% Protein				1.00	0.13*	-0.31**	0.17*
Dlac					1.00	-0.15*	-0.52**
Dins					1.00	-0.15*	0.23
CI							1.00

**Significant at p<0.05 ** Significant at p<0.01*

There were no significant associations between milk components and the hormones apart from percent milk fat that had a significant correlation with progesterone of 0.22 in both breeds. This is expected because progesterone is a fat derived hormone. The association between milk

components and nutritional metabolites were observed as being low and insignificant in both breeds.

There was a negative correlation ($p<0.05$) between days to peak milk production with peak milk production and calving intervals in Friesian cows (Table 12). This indicates that Friesian cows with high milk peaks take shorter time to reach peak production from calving and is a characteristic of the lactation curve. It was also observed that there was a positive association ($p<0.05$) between peak milk production with mean milk yield and calving interval which indicates that Friesian cows with high peaks also tend to have higher milk yields and longer calving intervals. This is a reflection that increased milk production impacts negatively on fertility as a result of the increased nutrient demands for lactation and agrees with the findings of Pryce, *et al*.,(2002) who found a negative association between milk production and cow fertility.

Table 12. Correlation of Milk Attributes with Reproductive Parameters for Friesians

	Days to Peak	**Milk Prod**	**Peak Milk**	**Dlac**	**Dins**	**CI**
Days to Peak	1	-0.09	-0.43**	0.75**	0.47**	-0.41**
Milk Prod		1	0.73**	-0.35*	-0.09	0.66**
Peak Milk			1	-0.79**	-0.83**	0.65**
Dlac				1	0.93**	-0.28*
Dins					1	-0.16*
CI						1

**Significant at p<0.05 ** Significant at p<0.01*

Average milk production and peak milk production were negatively associated with start of luteal activity and days to insemination in Sahiwals (Table 13). These seems unusual because higher peaks and milk production means that more nutrients are directed to milk production and should impair reproduction leading to longer intervals to initiation of luteal activity and insemination as demonstrated by Madouasse, *et al*., (2010). However, these two milk attributes were positively associated with calving intervals as expected implying that the more milk produced the more likelihood of reproductive impairment.

Table 13. Correlations of Milk Attributes with Reproductive Parameters for Sahiwals

	Days to Peak	Milk Prod	Peak Milk	Dlac	Dins	CI
Days to Peak	1	-0.60**	-0.69**	0.66**	0.28*	-0.87**
Milk Prod		1	0.80**	-0.35*	-0.45**	0.38*
Peak Milk			1	-0.39*	-0.63**	0.41*
Dlac				1	-0.08	-0.51**
Dins					1	-0.15
CI						1

**Significant at p<0.05 ** Significant at p<0.01*

4.5 Reproductive Parameters Observed after Calving

After initial selection of in-calf cows, the cows calved and were closely monitored to establish the beginning of luteal activity using progesterone profiles followed by observation of heat expression and subsequent insemination. Of the initial 40 cows selected and identified as pregnant, 18% did not calve due to late loss of foetus while 82% calved (Table 14). The seven cows that did not calve were censored and not included in the subsequent analysis. They comprised of 4 Friesians and 3 Sahiwals, thus making 20% and 15% of their respective breeds of the initially 20 selected cows from each breed respectively. This is higher than the findings of Sreenan and Diskin (1986) who reported late foetal loss of 5 to 8% among dairy cows, however, findings from this study were similar to those reported by Falkenberg, *et al*., (2008) who found that of all the cows inseminated, 20.7% did not conceive after 200 days *postpartum*. Another observation after calving showed that 12% of the cows did not show any luteal activity (Table 14) as observed from progesterone levels being below 3ng/ml, 20 weeks post calving, this denotes a temporary or permanent state of ovarian inactivity with no manifestation of estrus and could be due to environmental, nutritional or pathological conditions (Jainudeen and Hafez, 2000; Tadasse, *et al*., 2010). While another 12% showed luteal activity through observation of hormones but with no behavioral heat signs and could be termed as silent heats. Only 76% of the initial in-calf cows that calved, exhibited heat, were served and 56% of them eventually calved for the second time after commencement of the experiment (Table 14). This outcome is comparable to that reported by Masama, *et al*., (2006) in Zimbabwe.

Table 14. Summary of Reproductive Parameters of Experimental Cows

Breed	In-calf	Lost foetus	1st Calving	No Luteal Activity	Heat and Inseminated	Conceived	2nd Calving
Friesian	20	4 (20)*	16 (80)	3 (19)	11 (69)	6 (55)	6 (55)
Sahiwal	20	3 (15)	17 (85)	1 (6)	14 (82)	13 (93)	8 (57)
All	40	7 (18)	33 (83)	4 (12)	25 (76)	19 (76)	14 (56)

**Numbers in parenthesis are percentages.*

It was observed that Sahiwals exhibited better reproductive performance than their Friesian counterparts (Table 14) as determined by the reproductive parameters discussed above and agrees with the findings of Blache, *et al*. (2007) who reported that genotype affects the ability to resume estrus after parturition.

There were breed differences ($p<0.05$) from calving to commencement of luteal activities with Sahiwals getting into cyclicity earlier than the Friesians (Table 15), this was expected because the demand for nutrients on the Friesians for milk production rendered them to be in more negative energy balance after parturition than the Sahiwals, which produced significantly less milk (Blache, *et al*., 2007). These findings were also similar for number of days to insemination but the calving intervals for Sahiwals are longer because of the higher number of inseminations per conception. This could be that Sahiwals being shy breeders are more difficult to detect heat than the Friesians and so the high number of insemination per conception, thus elongating the calving intervals. It was also observed that the two breeds took 4 weeks from commencement of luteal activities to insemination (Table 15).The interval from calving to initiation of luteal activity and eventual insemination is important for reproductive efficiency because it influences the calving interval and determines number of calves born over the lifetime of the cow (Cavestany, *et al*., 2009).

Table 15. Effects of Breed on Reproductive Parameters

Breed	Reproductive Parameters		
	Days to Luteal Activity	Days to Insemination	Calving Intervals (Days)
Friesian	68.6±7.7 (10 weeks)[a]	98.6±13.7 (14 weeks)[a]	442.7±22.8 (14.7 Months)
Sahiwal	42.5±5.4 (6 weeks)[b]	73.1±11.2 (10 weeks)[b]	533.1±12.5 (17.8 Months)

[ab] Different superscripts within column are significant

It was observed that cows that conceived after insemination and eventually calved a second time had relatively shorter intervals from calving to commencement of luteal activity and insemination compared to those that did not calve (Table 16) and is in agreement with the findings of Falkenberg, *et al*., (2008) who found that cows with favorable pregnancy rates had shorter calving to first insemination periods and is an indication of cow fertility. The mean days to insemination of 70.5±13.4 for cows that calved and 96.8±21.5 days for those that didn't calve (Table 16) are lower than 116 ± 6.6 days reported by Obese, *et al*., (2009) for Sanga cattle in West Africa and 107 ± 9.2 days reported by Swai, *et al*., (2007) for Friesian cows in Tanzania. However, these findings are in agreement with those of Reist, *et al.,* (2003) who reported 71 days to first service *postpartum* for cows that calved. The long calving intervals exhibited by these cows could be because of the repeated inseminations particularly in Sahiwals which was attributed to several factors despite the shorter anestrus period *postpartum* (Table 16) and agrees with findings of Tadasse, *et al*., (2010).

There were significant breed differences between intervals from calving to commencement of luteal activities (Dlac) with Friesian taking longer ($p<0.05$) to exhibit luteal activity compared to Sahiwals in both categories of cows that calved or not (Table 16). The number of days from calving to first insemination (Dins) were different ($p<0.05$) for the two breeds for cows that did not calve but similar for cows that were served and calved, the average days to first insemination for both breeds were 70 days *postpartum* (Table 16). This is similar to that reported by Royal, *et al.,* (2002) who found intervals to first insemination of 74.0±0.4 days *postpartum.*

Table 16. Reproductive Parameters on Cows Calving Status in Days

Calving Status	Breed	N	Dlac	Dins	Calving Intervals
No	Friesian	10	74.8±10.6[a]	121.5±40.9[a]	
No	Sahiwal	9	42.0±6.6[b]	75.7±18.8[b]	
All		19	58.4±7.2	96.8±21.5	
Yes	Friesian	6	57.4±8.8[a]	70.6±28.2[a]	442.7±91.3[a]
Yes	Sahiwal	8	43.1±9.6[b]	70.4±13.5[a]	533.1±51.7[b]
All		14	49.1±6.8	70.5±13.4	490.9±50.2

[ab] *Different superscripts within column differ significantly (P<0.05)*

Early re-establishment of ovarian activity has been associated with fertility (Darwash, *et al.*, 1997). These authors reported that cows starting to cycle early had reduced interval to conception by having higher chance of early insemination *postpartum*, higher pregnancy rates and fewer services per pregnancy than those with long anovulatory periods. Findings from the current study are in agreement with these observations which showed, cows that calved successfully after insemination commenced luteal activity after 49.1±6.8 days relative to their counterparts that didn't conceive which required 58.4±7.2 days (Table 16). This could be because, non-calving cows had a problem with cyclicity due to low average levels of circulating progesterone which averaged 3.10±0.42 ng/ml for non-calving cows, compared to 3.78±0.60 ng/ml for those cows calving and this would appear as the threshold level for progesterone in calving cows under our production system. These findings agree with those reported by Lucy, (2001) and Washburn, *et al*., (2002) who found that low levels of circulating progesterone affect reproductive efficiency. This is because elevated progesterone levels play a critical role in supporting uterine function and numerous studies have linked low progesterone secretion to reduced embryo development and early embryonic loss (Souza, *et al*., 2014; Mann and Lamming, 2001).

Calving interval is an important index for cow reproductive performance that is defined as the average interval between the two most recent consecutive calvings for each cow, an interval of 365 days is desirable for efficient production (Esslemont, 1993). Kanuya, (2000) defined a standard recommended interval of 430 days under tropical conditions, while Irungu and Mbugua, (1998) reported intervals averaging 429 days for Friesians in Naivasha. In the current study, Friesians recorded a mean calving interval of 442 days (Table 16) which is higher than those reported above, but is lower than the findings of Swai, *et al*., (2007) who reported intervals of 476 days for Friesians in Tanzania. Joshi *et al*., (2001) reported 420 days intervals for Sahiwal cows in Pakistan while Ilatsia, *et al.*, (2007) reported 468 days for the National Sahiwal stud at Naivasha, which is much lower than the current findings of 533 days for the Sahiwals in this study (Table 16). A probable reason could have been due to the age of the cows used in the experiment that averaged 102 months. This agrees with the findings of Tadasse, *et al*., (2010) who reported increased calving intervals with age and parity for dairy cows in the highlands of Ethiopia.

It was observed that Friesians averaged 98 days to first insemination, which is similar to 90 days reported by Ott, *et al*., (2014) and the second insemination averaged 134 days *postpartum* while

Sahiwals averaged 73 days to first insemination and the second insemination occurred after 122 days. These findings are more favorable than 115±1.7 days to first insemination reported by Tadasse, *et al*., (2010) but similar to the ideal range of 60-90 days recommended by Berry, *et al.*,(2003).Thus, the long calving intervals observed for the Sahiwals despite favorable timing to first and second insemination can be attributed to the number of services per conception which averaged 1.53 and 2.4 for the Friesians and Sahiwals respectively, this agrees with the findings of Ilatsia, *et al*. (2007) who reported services per conception for Sahiwals of 2.2. These finding for Friesians are similar to a mean of 1.8 reported by Tadasse, *et al*., (2010). Successful insemination depends on many factors such as quality of semen, skills of the inseminator, proper timing, cow related factors and management attributes.

Early resumption of ovarian activity is important to allow for more luteal phases before first service, which has been shown to increase fertility of lactating dairy cows (Townson, *et al.*, 2002). In this study, 52% of the cows showed ovarian activity below 50 days after parturition and contrasts with the findings of Cavestany, *et al*. (2009) who reported 66% of lactating cows resuming ovarian activity before 49 days *postpartum.*

In the current study feeding levels did not significantly influence resumption of luteal activity, suggesting that levels of supplementation were not sufficient enough to change body energy reserves *postpartum* so as to influence resumption of ovarian activity and concurs with the conclusion of Meikle, *et al.,* (2004) and Cavestany, *et al.,* (2009). However, there were significant ($p<0.05$) interactions between breed and feeding levels (Figure 11) which is an indication that supplementation affects the two breeds differently by altering their metabolic status and by extension, its influence on reproductive performance. It was observed that cows on pasture alone as in treatment one, the Sahiwals took relatively longer to commence luteal activity (Figure 11) an indication that pasture alone was not providing adequate nutrients to counter the effects of negative energy balance for Sahiwals relative to Friesians which is contrary to expectation as Friesians with higher milk output would have registered extended days to commencement of luteal activity. However, upon supplementation Sahiwals took relatively shorter days to get into cyclicity *postpartum* at all levels relative to the Friesians (Figure 11). Sahiwals respond more rapidly to supplementation than the Friesians, meaning that the relative supplementation thresholds for Sahiwals are lower than those of the Friesians for cylicity. The longer periods to get

into cyclicity for Friesians could be due to the increased milk production of the Friesians relative to the Sahiwals with more nutrients being channeled to milk production and therefore compromising resumption of reproduction. These findings are similar to those reported by Wu, *et al.*, (2013) who linked nutrition to commencement of luteal activity in various breeds.

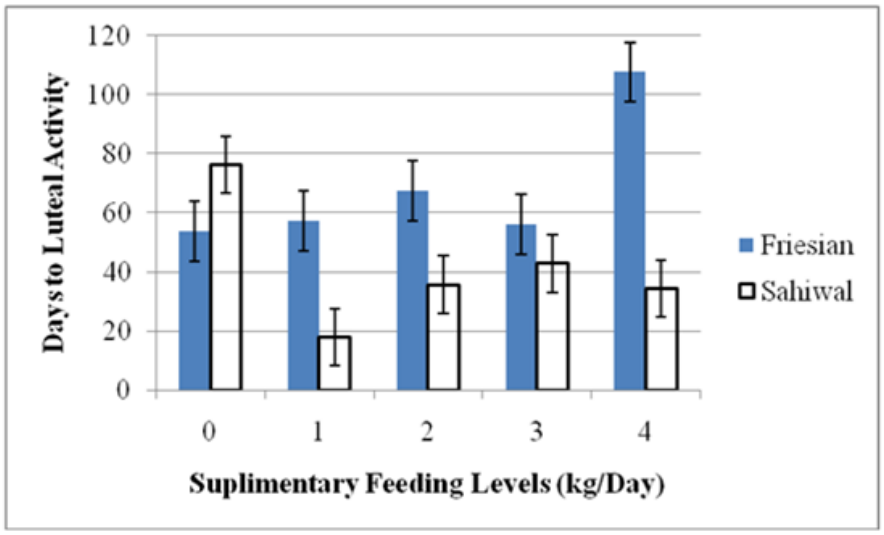

Figure 11. Interaction of Breed and Diet on Commencement of Luteal Activity

The general trend was for Friesians to gradually have increasing duration for days to commencement of luteal activity with incremental supplementation up to 3kgwhen it decreased before rapidly increasing with further supplementation (Figure 11). This could be that prior to 3kg supplementation the cows are in negative metabolic status as a result of nutrients being channeled for milk production, which eventually reaches an equilibrium at 3kgs supplementation and further increases leads to the cow being over conditioned resulting in negative effects on reproduction causing an increase in number of days to resumption of cyclicity at 4 kg of supplementation. This agrees with the findings of Lucy, *et al.*, (2009) who found that a high plane of nutrition negatively affects the reproductive tract leading to infertility. Sangsritavong, *et al*., (2002) demonstrated that increased feed intake leads to increased metabolic clearance of rate of steroids in the liver and affects the circulating levels of progesterone, therefore influencing reproduction.

4.6 Metabolite and Hormonal Profiles of Cows after Calving

The repeated measures analysis for variables measured over time showed that there were breed differences ($p<0.05$) over time for all the metabolites and hormones except glucose and albumin, which meant that the two metabolite concentrations (glucose and albumin) did not vary significantly in a given pattern for the two breeds (Table 17). Feeding levels influenced ($p<0.05$) all metabolites and hormones except for NEFA as shown in Table 17 which is an indication that supplementation levels in this study had an effect on the metabolic and hormonal profiles over time and by extension may affect reproductive parameters. In general this agrees with the findings of Chelikani, *et al*., (2009) who reported that feeding different diets to *postpartum* dairy cows resulted in an increase of glucose and reduced NEFA levels as the diet energy density increased. However, the influence of increasing supplementation on NEFA was surprising considering that increased supplementation would ameliorate against NEB thus reducing lipolysis and therefore reduced levels of circulating NEFA in *postpartum* cows.

It was also observed that only the hormones had definitive patterns overtime while the rest of the metabolites varied randomly with time (Table 17), this agrees with the findings of Alzahal, *et al*., (2009) who reported that there was no effect of time on the variation of metabolites in Holstein cows fed varying dietary levels.

Table 17. ANOVA for Repeated Measures of Metabolites and Hormones

		Metabolites				Hormones	
		Glucose	NEFA	Urea	Albumin	Progesterone	IGF-1
Factor	Covariance Structure	US	AR(1)	AR(1)	AR(1)	AR(1)	AR(1)
Breed		0.74	90.81*	10.28*	4.9	16.64*	8.73*
Feed		4.36*	2.06	10.36*	2.89*	5.55*	19.83*
Week		0.99	1.39	1.5	1.22	2.60*	2.1*
Breed*Feed		4.68*	3.07*	16.09*	1.8	3.05	12.09*
Breed*Week		1.71*	1.15	1.97	1.41	0.99	6.7*
Feed*Week		1.24	1.79	1.42	1.23	1.22	2.35*

**Significant at p<0.05*

The two breeds also responded differently for the metabolites and hormones when given incremental feed levels apart from albumin and progesterone (Table 17) by secreting varying levels of metabolites and hormones over time.

It was observed that Sahiwals had higher ($p<0.05$) mean concentrations of NEFA, Urea, Progesterone and IGF-1 than Friesians (Table 18). It has been reported by Reist, *et al*., (2003); Wathes, *et al*., (2007) and Wiltbank,*et al*., (2012) that higher plasma concentrations of IGF-1 and progesterone results in shorter calving to conception intervals as demonstrated by the Sahiwals. It would appear that the two hormonal influences on reproduction supersede the negative attributes of the high levels of NEFA and urea as demonstrated by the Sahiwals (Table 18).

Table 18. Means±SD of Metabolite and Hormone Concentrations

		Metabolites				Hormones	
Breed	N	Glucose (mg/dl)	NEFA (mg/dl)	Urea (mg/dl)	Albumin (g/dl)	Progesterone (ng/ml)	IGF-1 (ng/ml)
Friesian	16	65.1±3.8[a]	28.8±3.1[a]	8.3±5.2[a]	4.3±0.02[a]	2.6±1.9[a]	30.1±27.2[a]
Sahiwal	17	64.2±5.9[a]	35.4±2.7[b]	19.1±13.9[b]	4.1±0.01[a]	4.1±1.9[b]	51.7±41.7[b]

[ab]*Different superscripts within column are significantly different ($p<0.05$)*

Basic observations of trends for plasma concentration of NEFA for Friesians, were high initially and started to reduce immediately after parturition up to 10 weeks, and this is when luteal activity commenced, it then rose again and reduced to low concentration during week 15 (Figure 12) *postpartum* and this is when insemination occurred. As for Sahiwals, NEFA rose from parturition but began to decrease after week 5 *postpartum*, which coincided with commencement of luteal activities; this continued to further reduce up to week 10 when insemination occurred (Figure 12). These patterns of NEFA profiles over time coincided with cow body condition scores and reproductive status of the cows in early lactation. When NEFA levels were reducing, cows were in the recovery phase of the negative metabolic status occasioning initiation of luteal activity and insemination for both Sahiwals and Friesians. These profiles of NEFA patterns *postpartum* are in agreement with those reported by Nielsen, *et al*., (2007).

NEFA is a proxy for negative energy balance *postpartum* and high levels impact negatively on reproductive parameters. NEFA is produced due to mobilization of body energy reserves to

support milk production and resumption of cyclicity after parturition. Pryce, *et al*., (2001) and Patton, *et al*., (2007), demonstrated that poor body condition had adverse effects on cow reproductive performance, while Walsh, *et al*., (2007) and Wathes, *et al*., (2007) reported that high blood concentration of NEFA was negatively associated with reproductive performance and is a reflection of the metabolic status of the cow. In this study, it was observed that Sahiwals had significantly higher values for NEFA than the Friesians (Figure 12), however, the Sahiwals came into reproduction much earlier than their Friesian counterparts, who had relatively lower concentrations of plasma NEFA, however, the Sahiwals required more inseminations per conception. This could be that the threshold levels at which NEFA starts influencing reproduction in Sahiwals is higher than Friesians, particularly for days to commencement of luteal activity and insemination. Reist, *et al*., (2003) identified NEFA as the best indicator of a cows energy balance because it is the first indication for lipolysis. When cows fail to adapt to negative energy balance, NEFA is converted to ketone bodies in the liver and predisposes the cows to reproductive failure (Patton, *et al*., 2007).

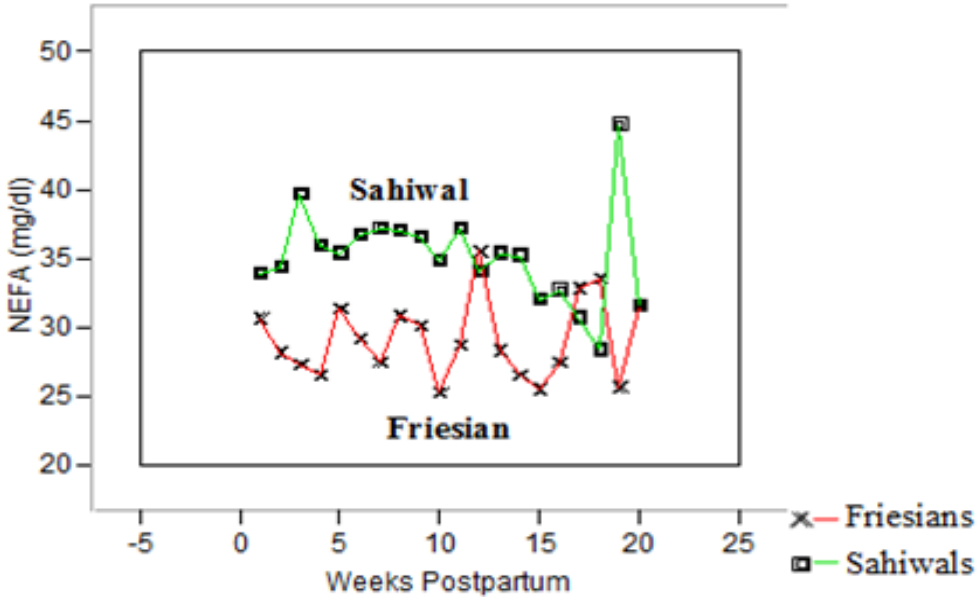

Figure 12. Plasma NEFA Concentration overtime for both Breeds

Breed differences were also significant ($p<0.05$) for urea (Figures13) while albumin showed mixed patterns for the two breeds as observed in Figure14. Sahiwals exhibited higher plasma levels for urea while Friesians had higher average concentrations of plasma albumin than the

Sahiwals (Figure 13) and a pattern almost like urea though not significant. There seems to be two peaks for urea in both breeds, with one occurring during week 5 *postpartum* and a second appearing during week 15. In early lactation, cows are in negative nitrogen balance which has been reported at -560g/day and is compensated for by mobilizing amino acids from skeletal muscle as evidenced by 25% reduction in muscle fibre diameter in dairy cows (Reid, *et al*., 1980). Urea and albumin concentration in plasma due to varying diets does not sufficiently influence metabolic hormones as to adversely affect reproductive function and this is in agreement with the findings of Garnsworthy, *et al*., (2008).

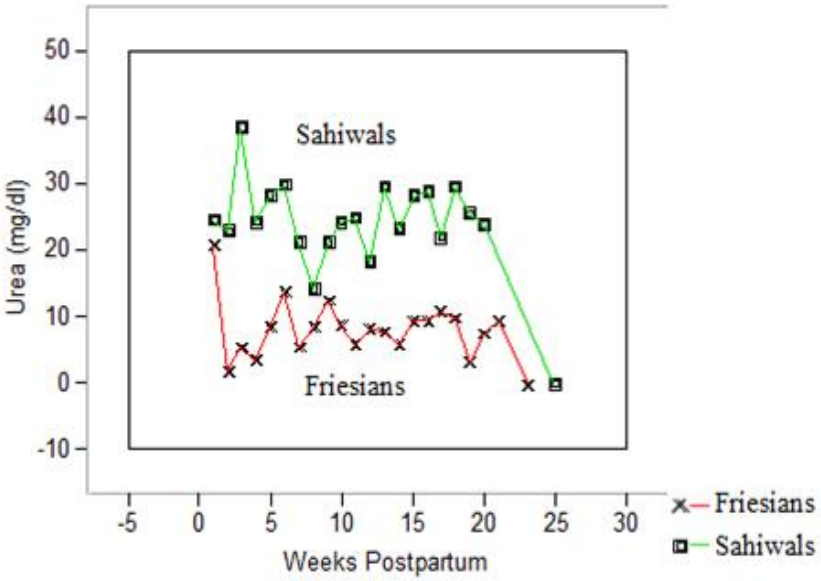

Figure 13. Plasma Urea Concentration overtime for both Breeds

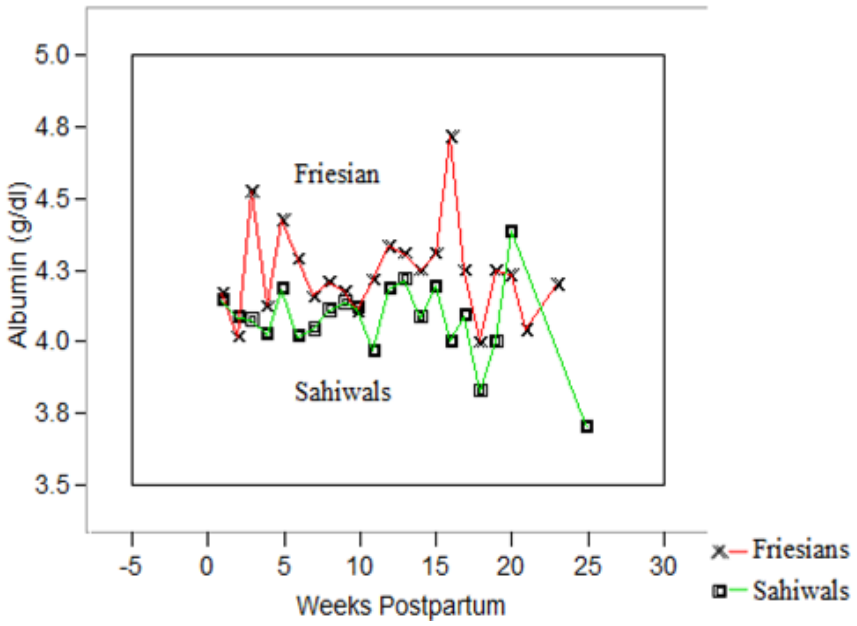

Figure 14. Plasma Albumin Concentration overtime for both Breeds

As for the hormones, mean progesterone levels rose steadily in both breeds and peaked at week 18 *postpartum* before they began to reduce. The Sahiwals had higher levels on average than the Friesians over time (Figure 15). It was observed that progesterone levels sufficient for ovulation, which is 3ng/ml was reached at 5 weeks *postpartum* for the Sahiwals while Friesian attained these levels at week 10 which coincided with commencement of luteal activity in both breeds. Insemination in Sahiwals occurred 10 weeks *postpartum*, when the progesterone levels were 6 ng/ml while Friesians registered more than 4ng/ml at 14 weeks *postpartum* (Figure 15). This could be the reason why Sahiwals exhibited better reproductive performance than the Friesians and agrees with the findings of McNeill, *et al*., (2006) and Santos, *et al*., (2009) who reported that reduced circulating concentrations of progesterone are associated with reduced pregnancy rates. Progesterone has been reported to favour conceptus development (Garret, *et al*., 1988) and synthesis of interferon-tau (Ott, *et al*., 2014; Thatcher, *et al.*, 1997), which inhibits the biosynthesis of $PGF_{2\alpha}$ in the endometrium to block luteolysis (i.e. maternal recognition of pregnancy).

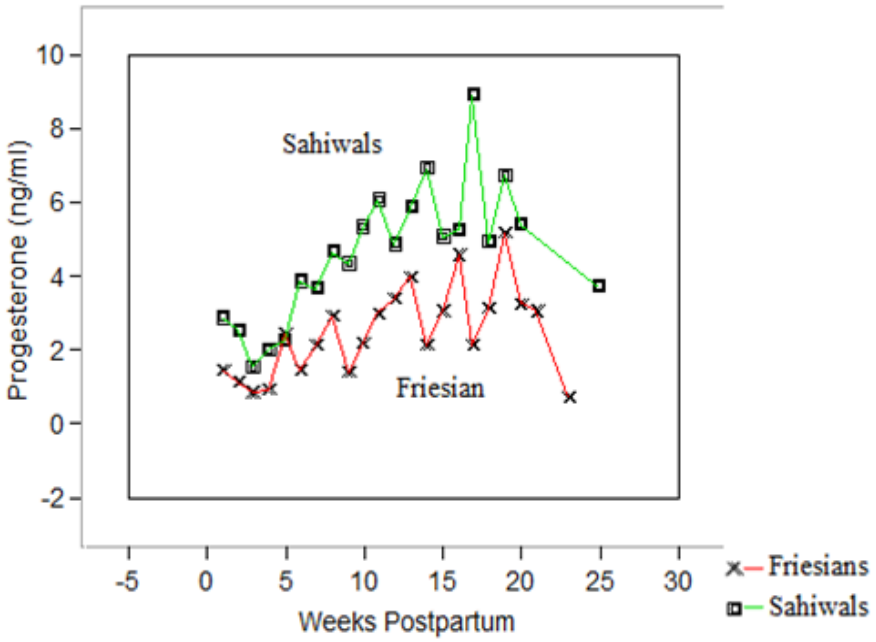

Figure 15. Plasma Progesterone Concentration for Friesian and Sahiwal Cows

The pattern of IGF-1 plasma concentration in Sahiwal reduced gradually and rose to peak at week 5 *postpartum* and reduced again before a small peak occurred at week 10 before finally showing a large peak at week 16. While for Friesians the concentration reduced to a *nadir* at week 10 and then rose to peak 15 weeks *postpartum* (Figure 16). There appeared to be three distinct peaks in both breeds, which occurred at 5, 10 and 16 weeks *postpartum* (Figure 16). At week 16 *postpartum* is when the progesterone and IGF-1 levels in both breeds were highest and compared to body condition scores, this is when the cows recover fully from the metabolic depression caused by the NEB after parturition. These findings are similar to those reported by Patton, *et al*., (2007) who expressed that conception rates were enhanced when insemination occurred at peak levels of IGF-1 concentration in blood plasma and cows were less likely to conceive when their plasma IGF-1 levels were lower than 25ng/ml *postpartum* (Taylor, *et al*., 2004; Santos, *et al*., 2009) resulting in longer intervals from calving to resumption of cylicity (Beam and Butler, 1998; Wu, *et al*., 2013;). This is because of the role IGF-1 plays in follicular growth, maturation and development of the embryo (Diskin, *et al*., 2003; Lucy, *et al*., 2014).

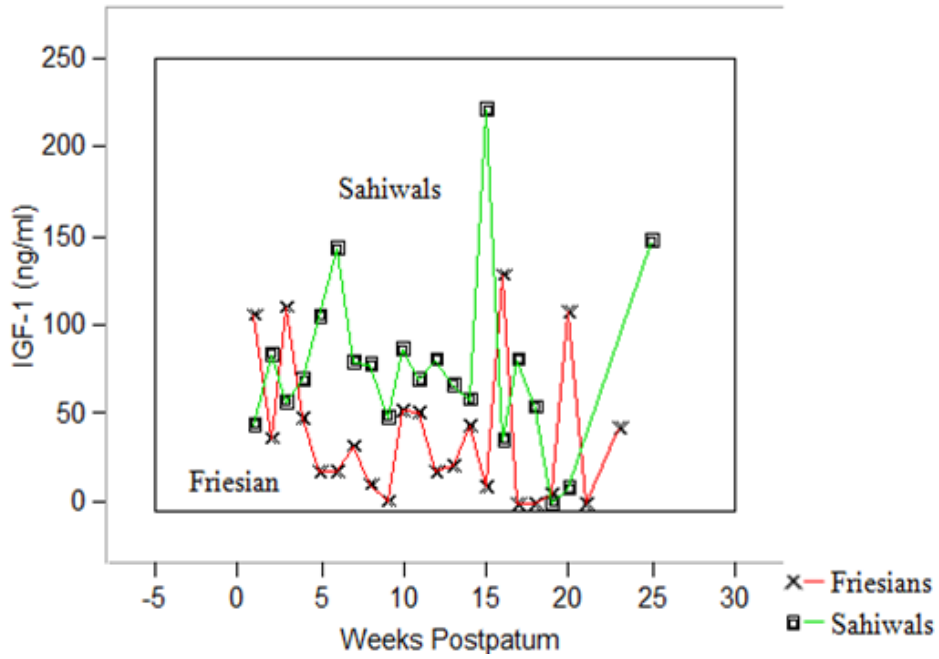

Figure 16. Plasma IGF-1 Concentration for Friesian and Sahiwal Cows

4.7 Metabolite and Hormonal Profiles for the two Calving Categories

Changes in metabolic hormones are pertinent for dairy cow fertility, because they interact with the reproductive hormones that control ovarian function (Garnsworthy, *et al*., 2008). The mean values of metabolites and hormones for the two breeds of cows that calved and those that failed to calve during the observation period are represented in Table 19. It was observed that there were no significant ($p>0.05$) breed differences for glucose levels within calving category. NEFA showed breed differences ($p<0.05$) within calving category, with Sahiwals having higher values in both categories. This was the same for urea while albumin did not show breed differences for non-calving cows but Friesians had higher ($p<0.05$) concentrations for cows that calved.

The hormones showed that progesterone and IGF-1 had different ($p<0.05$) blood concentrations for the two breeds within calving category, with Sahiwals again exhibiting much higher values than Friesians (Table 19). However, cows that calved had relatively higher levels of progesterone for Friesians than those not calving and this is in agreement with the findings of McNiell, *et al*., (2006) and Santos, *et al*. (2009) who reported that reduced circulating concentration of progesterone was associated with reduced pregnancy rates. The Sahiwals exhibited similar

progesterone and IGF-1 concentrations for both categories (Table 19). However, those cows that calved had higher mean IGF-1 concentrations above the threshold required for conception of 25ng/ml as reported by Santos, *et al*., (2009). Fulkenberg, *et al*., (2008) reported that cows with higher IGF-1 at calving are more likely to conceive and calve relative to those with lower levels of IGF-1 at parturition.

Table 19. Means±SE of Metabolite and Hormone Concentrations of Cows Calving Status

			Metabolites				Hormones	
Calving status	Breed	N	Glucose	NEFA	Urea	Albumin	Progesterone	IGF-1
No	F	10	65.2±1.3[a]	27.8±0.8[a]	7.9±1.7[a]	4.2±0.1[a]	2.2±0.4[a]	32.8±9.2[a]
	S	9	66.2±1.2[a]	34.7±0.9[b]	18.6±4.7[b]	4.1±0.03[a]	4.1±0.6[b]	52.0±14.7[b]
Yes	F	6	64.8±1.6[a]	30.5±1.4[a]	8.9±2.2[a]	4.4±0.1[a]	3.4±1.1[a]	25.6±10.4[a]
	S	8	61.9±2.6[a]	36.1±0.8[b]	19.6±5.2[b]	4.1±1.0[b]	4.1±0.7[b]	51.3±14.8[b]

[ab] *Different subscripts within calving category indicate significance ($p<0.05$)*

4.8 Feed Supplementation on Plasma Concentration of Metabolites and Hormones

It was observed that the breeds responded differently ($p<0.05$) when supplemented with varying levels of concentrate feed for mean plasma metabolites and hormonal concentrations except albumin and progesterone. There was a general gradual increase of glucose concentrations when supplementation levels were increased from 0 through to 4 kg in both breeds (Figure 17). The 4kg supplementation seemed most appropriate in enhancing glucose levels which is a reflection of the energy balance status *postpartum*, with cows having higher values giving better energy balance and being more probable of conceiving than their counterparts with lower glucose concentrations (Huszenicza, *et al*., 2001).

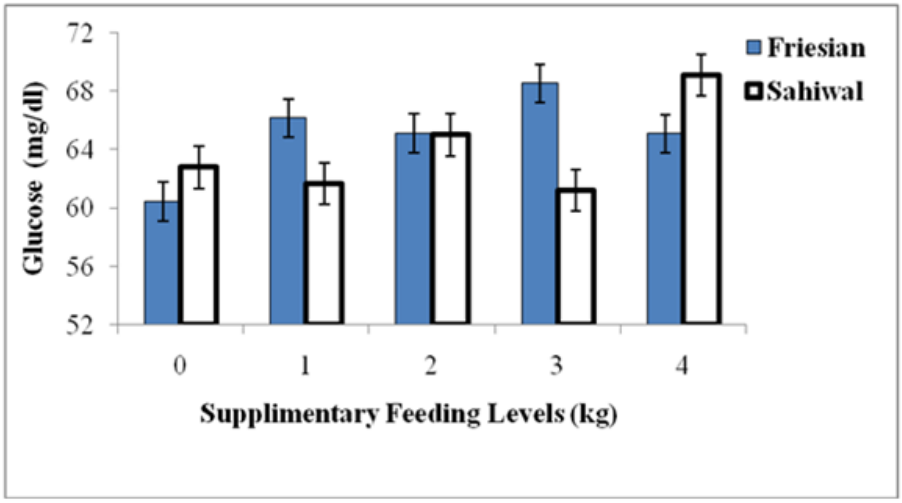

Figure 17. Glucose Concentration by Breed and Feeding Levels (Kg/day)

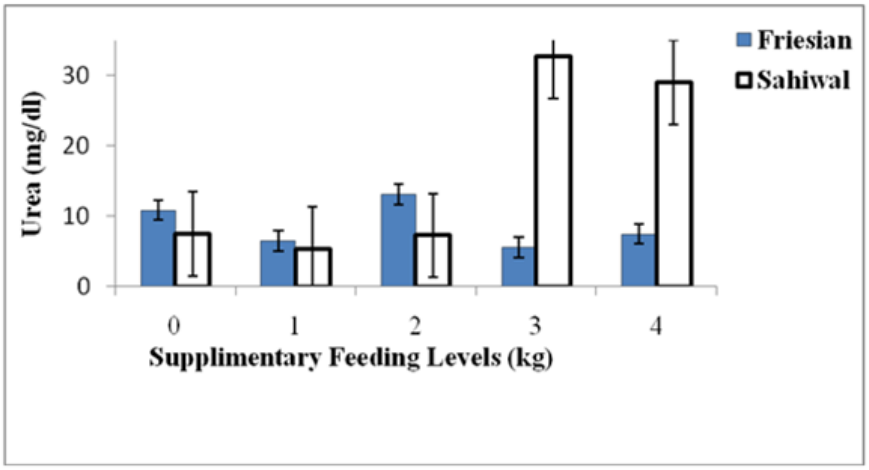

Figure 18. Urea Concentration by Breed and Feeding Levels (kg/day)

Friesians had relatively higher values for urea compared to the Sahiwals during early supplimentation, however, as supplimentation increased, urea plasma levels for Sahiwals rose astronomicaly as shown in Figure 18. This could be that as supplimentation increased the protein levels for Sahiwals could have been in excess for the body to absorb and therefore incresed levels of deamination in the liver resulting in high urea levels being channeled to body fluids for excretion (Heuer, *et al*., 1999). This was also observed for glucose with high levels for Sahiwals at 4kg supplimentation (Figure 17). Friesians had higher plasma concentration for albumin than

the Sahiwals across all supplimetation levels. This is an indication that the two breeds metabolise protein-based nutrients differently.

Friesians had relatively ($p<0.05$) low levels of progesterone compared to Sahiwals and there was a general trend for progesterone levels gradually increased as levels of supplementation increased (Figure 19). This is necessary for improved conception rates (Santos, *et al*, 2009), however, at the higher levels the concentrations tended to reduce in both breeds and Friesians had more reduction in progesterone at higher supplementation levels than Sahiwals. The optimum levels of supplementation in both breeds should be between 2 and 3kg supplementation (Figure 19). This is in agreement with the findings of Sangsritavong, *et al*., (2002) who reported that increased concentrate intake promotes progesterone metabolism by increasing the metabolic clearance rate in the liver resulting in reduced plasma progesterone levels. The clearance rates for Friesians at higher levels of supplementation is more than that observed for the Sahiwals, thus explaining the better reproductive outcomes exhibited by the Sahiwals.

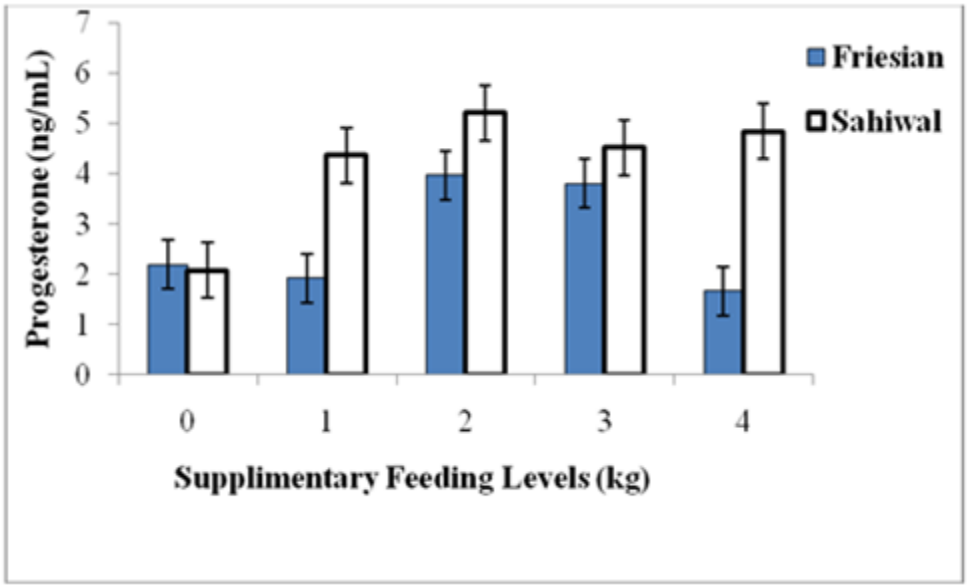

Figure 19. Progesterone Concentration by Breed and Feeding Levels (Kg/day)

In un-supplemented cows, IGF-1 levels were relatively low for Friesians while it was relatively high for Sahiwals. However upon supplementation the plasma levels of IGF-1 were highest when the cows were supplemented at the rate of 3 kg per day and Sahiwals had relatively higher values

than Friesians (Figure 20) but both were beyond the threshold of 25ng/ml as reported by Santos, *et al.*, (2009).

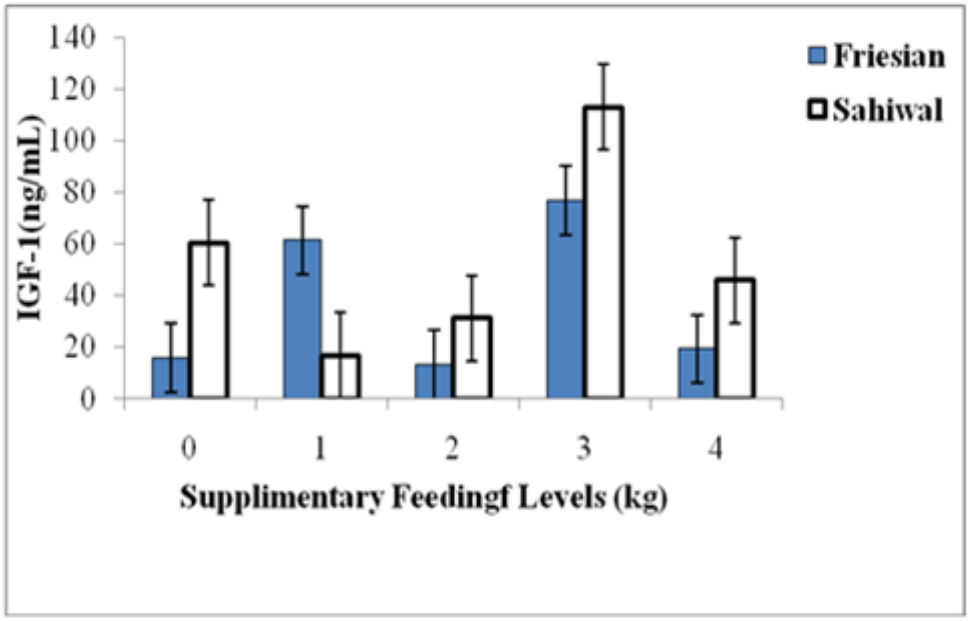

Figure 20. IGF-1 Concentration by Breed and Feeding Levels (kg/day)

4.9 Principal Component Analysis for Milk, Metabolite and Hormonal Variables

Measurements made on the same subject are likely to be more similar than measurements made on different individuals, thus eliciting multicolinearity among measurements. Thus, Principal Component Analysis (PCA) was performed on milk, metabolites and hormonal variables. The method was relevant for this analysis because some of the variables of interest were highly correlated. The extracted Principal Components (PCs) derived are uncorrelated and account for most of the total variation contained in the fitted models. The PCA results for the milk, metabolites and hormonal variables were based on a factor loading of 0.4 and a principal component selection criterion of more than one (1) Eigen value per component also known as the Kaiser criterion (Kaiser, 1960).

4.9.1 Principal Component Analyis for Milk Constituents

When the milk variables were subjected to the principal component analysis, it was observed that they clusterd into two Priciple Components (PC), with the first one comprising of SNF, density, freezing point and protein which are density related molecules and accounted for 62.6% of the total variance while the second PC comprised of fat and its derivatives of fat to protein ratio accounting for 36.8% of the variance (Table 20). This implies that percentage fat is the distinguishing milk parameter.

Table 20. Rotated Correlation Coefficients Factor Patterns of Milk Components

	Principal Components	
Milk Parameters	1	2
% Fat		0.97
Fat to Protein ratios		0.99
% SNF	0.99	
Density	0.89	
Freezing Point	0.99	
Protein	0.98	
Variance Explained (99.4%)	62.6%	36.8%

4.9.2 Principal Component Analysis for Metabolites and Hormones

Two PCs were also extracted for the hormones and metabolites and accounted for 43.7% of the total variance (Table 21). PC1 comprised of protein-based metabolites of urea and albumin and the hormone IGF-1 and this explained 24% of total variance while the second component accounted for 19.8% of the variance and comprised of glucose and NEFA. The loadings indicate that IGF-1 influences metabolic status during early lactation through the protein based metabolites (Ward, *et al.,* 1995), while glucose and NEFA are closely associated in regulating energy metabolism (Weber, *et al*., 2013; Garnsworthy, *et al*., 2008). The two components can be labeled as protein and energy related constructs respectively that influence reproduction in lactating dairy cattle. However, their specific actions need to be explored further.

Table 21. Rotated Correlation Coefficients Factor Patterns of Metabolites and Hormones

Metabolites and Hormones	Principal Components	
	1	2
Urea	0.38	
Albumin	0.32	
IGF-1	0.49	
Glucose		0.64
NEFA		0.49
%Variance Explained (43.7%)	23.9	19.8

4.10 Regression Analysis

Multiple linear regression models were developed, with hormones comprising, progesterone and IGF-1 as response variables while the metabolites were the explanatory variables, it was observed that for progesterone, there were a significant ($p<0.05$) regressions with glucose, NEFA and albumin (Table 22). This agrees with the findings of Riest *et al*, (2003) who showed a close association between energy related attributes of glucose *nadir* and peak of NEFA with progesterone levels. While the regressions with IGF-1 showed that only NEFA was significant. This is an indication that glucose, NEFA and albumin had an influence on progesterone concentrations while only NEFA had an influence on IGF-1 concentrations during early lacataion (Table 22). Thus, any circumstance that alters significantly the concentrations of these two metabolites will affect the concentrations of progesterone and IGF-1 hormones which have an influence on reproductive performance in *postpartum* cows. NEFA seems to have the greater significant influence on the two hormones compared to the rest (Table 22). This agrees with the findings of Weber, *et al*., 2013 and Clark, *et al*., (2005) who concluded that NEFA as an indicator of NEB also affects reproductive and metabolic hormones. When milk components were included in the multiple regression models for progesterone and IGF-1, % fat was the only component associated with reproductive attributes of the lactating cows.

Table 22. F-Values for Multiple Regressions of Hormones in Relation to Metabolites

	Metabolites			
Hormones	Glucose	NEFA	Urea	Albumin
Progesterone	2.43*	3.66*	0.64	2.9*
IGF-1	1.19	2.52*	1.13	1.43

**Significant at p<0.05*

A stepwise logistic regression model was developed to predicting the probability of nutrition being able to influence metabolites and hence the circulating levels of progesterone on the commencement of luteal activity in lactating cows. The response variable of progesterone was categorised as cows with progesterone ≤ 3ng/ml forming one category, while those with progesterone ≥ 3ng/ml formed the other category. The explanatory variables were the metabolites nested within feeding levels. The stepwise model retained the effects of breed, NEFA and Albumin nested within feed as significant parameters, the rest were discarded as not contributing significant variance on the response variable (Table 23). Sahiwals and Friesians responded differently with nutritionally induced changes in metabolites leading to varying threshold levels of progesterone that influence conception. Feeding levels influenced albumin and NEFA levels differently ($p<0.05$) in predicting the probability of when luteal activity commences in *postpartum* cows as determined by the rise of progesterone levels beyond 3ng/l (Table 23). This agrees with the findings of Clark, *et al*., (2005) who indicated that NEFA is a suitable indicator of NEB and is closely associated with prolonged days to luteal activity (Bisinotto, *et al*., 2012). However, when cows are fed concentrates during this state of negative energy balance, the effects of NEFA on reproductive performance are normaly reduced.

Milk components did not significantly associate with progesterone to be able to predict reproductive function using the logistic regression when progesterone levels were categorized above 3ng/ml as the threshold of commencing luteal activity.

Table 23. Stepwise Logistic Regression of Progesterone

Effect	DF	Chi Square
Breed	1	7.66*
NEFA(Feed)	5	24.78*
Albumin(Feed)	5	22.96*

**Significant at p<0.05*

CHAPTER 5

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

From this study, Sahiwals lost more average daily weight (0.64 kg vs. 0.38 kg), but lost less body condition (-0.1 vs -0.4) from calving to commencement of luteal activity compared to Friesians. They also commenced luteal activity (42 vs. 68 days) and were inseminated earlier (73 vs. 98 days) than the Friesians respectively. Thus, body condition scores were better associated with energy balance of cows *postpartum* than body weight and therefore an indicator of reproductive potential. Despite the two breeds having varying periods from calving to commencement of luteal activity, the period from commencement of luteal activity to insemination was 4 weeks in both breeds.

Cows with better body condition scores at calving produced more milk progressively and had higher milk peaks relative to those with lower condition but also took longer to attain peak milk production. The mean lactation lengths for Friesians and Sahiwals were 203 and 224 days respectively. The fat to protein ratio of 1.58 in Sahiwals indicated that they were in more negative energy balance than the Friesians; however, they came into cyclicity and were inseminated much earlier than the Friesians. This factor should be used for comparisons within breed because of the inherent breed differences in milk composition. Higher values for milk components, particularly SNF and protein were associated with longer periods to luteal activity and insemination for Friesians. It was also observed that the higher the peak of milk production, the longer the calving interval and was a reflection of increased milk production impacting negatively on fertility as a result of the increased nutrient demands for lactation. Shorter intervals from calving to commencement of luteal activity and higher concentration of progesterone were suitable indicators of cow fertility. The number of services per conception for Friesians were 1.5 while those of the Sahiwals were 2.4.

Feeding levels did not significantly affect resumption of luteal activity, suggesting that supplementation was not sufficiently high to influence body energy reserves to initiate estrus. Sahiwals responded to supplementation more rapidly than Friesians, meaning that the relative

threshold for nutrients in Sahiwals was lower than that of Friesians because of the relatively smaller metabolic size of the Sahiwal. Feeding levels influenced all metabolites and hormones except for NEFA, particular effects on glucose and progesterone had an effect on reproduction. The threshold levels at which NEFA starts to affect reproduction in Sahiwals is higher than that of Friesians, particularly for days to commencement of luteal activity and insemination. It was observed that the progesterone threshold for ovulation, which occurs at 3ng/ml, was reached at 5 weeks for Sahiwals while that of Friesian was attained at week 10 *postpartum* that coincides with commencement of luteal activity in both breeds. However, cows attaining a plasma progesterone concentration of 3.78ng/ml *postpartum* were more likely to get into cyclicity and conceive earlier than those with lower levels

The pattern of IGF-1 plasma concentration in both breeds peaks at week 16 *postpartum* this is when the progesterone levels in both breeds are highest and compared to body condition scores, this is when the cows recover fully from the metabolic depression caused by the NEB after parturition. The two breeds responded differently when supplemented with varying levels of concentrate for mean plasma metabolites and hormonal concentrations except albumin and progesterone. Friesians had relatively low levels of progesterone than the Sahiwals and there was a general trend for progesterone and glucose levels to increase as supplementation increased, however, at the higher feeding levels the concentrations tended to reduce in both breeds with Friesians having more reduction in progesterone than Sahiwals. The optimum levels of supplementation in both breeds were found to be 3kg per day. IGF-1 influenced metabolic status during early lactation through the protein based metabolites of urea and albumin while glucose and NEFA were closely associated with progesterone. There is an indication that glucose, NEFA and albumin had an influence on progesterone concentrations while only NEFA had an influence on IGF-1 concentrations during early lacataion. Thus, any feeding strategy that alters significantly the concentration of these three metabolites will affect the circulating plasma levels of progesterone and IGF-1 hormones and thus influence reproductive performance in *postpartum* cows. Feeding levels influenced albumin and NEFA levels differently in predicting the probability of when luteal activity commences in *postpartum* cows as determined by the rise of progesterone levels beyond 3ng/ml.

It has been demonstrated in this study that nutrition has an effect on albumin, glucose and NEFA which influences circulating levels of progesterone and IGF-1 and can be suitable predictors of the subsequent reproductive performance of cows after parturition. They mimic the metabolic status of the cow for sustaining increased milk production and subsequent resumption of cyclicity after calving in dairy cows.

5.2 Recommendations

Feed supplementation levels of 3 kgs of concentrate per day for dairy cows after calving is recommended to maintain a body condition score of 3 and above which supports high milk production and early resumption of cyclicity *postpartum* for dairy cows. Feed ingredients that enhance gluconeogenesis and secretion of progesterone and IGF-1 should be recommended to enhance milk production and improved reproductive performance. These aspects are mitigated by nutritional metabolites of glucose and NEFA which have an effect on the hormonal concentrations of IGF-1 and progesterone that enhance reproductive performance. The threshold levels should be maintained at above 3 and 25ng/ml for progesterone and IGF-1 respectively for optimal reproduction in the dairy enterprise. These can be referred to as metabolic markers that give an indication of the status of energy balance of cows in early lactation that could be used to predict milk production potential and subsequent reproductive performance.

There is need for further work to determine the specific actions of NEFA and glucose on the gonadotropic axis in influencing progesterone and IGF-1 levels in *postpartum* cows and particularly how different breeds of cattle respond to supplementation and the subsequent influence on reproduction.

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APPENDIX

Submitted Titles for Publication

Submitted to Bulletin of Animal Health and Production in Africa (July 2015)

1) Effects of Nutritional Supplementation and Genotype on Milk Production and Fertility of Lactating Dairy Cattle under Tropical Conditions

Indetie, D[1]., Musalia, L[2]., Bebe, B[2]., Wathuta, E[2]., Indetie A[1]., Kinywa[1], J. & Lukibisi, F[1].
[1]Kenya Agricultural Research Institute, P.O. Box 3840, Nakuru, Kenya;[2]Egerton University, P.O. Box 536, Egerton, Kenya.

2) Effects of Body Weight and Body Condition Changes after Parturition on the Reproductive Performance of Sahiwal and Friesian Cattle

D. Indetie[1], L. Musalia[2], B. Bebe[2], E. Wathuta[2], A. Indetie[1], J. Kinywa[1], and F. Lukibisi[1]
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Submitted to Livestock Research for Rural Development (August 2015)

1) The Role of Nutrition on Metabolic and Hormonal Profiles and their Influence on Reproductive Performance of lactating Dairy cattle

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