

**THE EFFICIENCY OF ULTRASONOGRAPHY IN MONITORING OVARIAN STRUCTURES
AND FOETAL DEVELOPMENT IN GOATS, SHEEP AND CATTLE AS VERIFIED THROUGH
LAPAROSCOPY AND LAPAROTOMY**

By

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DECLARATION

I, Mbonalo Steven Siphugu, the undersigned, hereby declare that this dissertation submitted for the Master of Science in Agriculture (Animal Science) degree at the University of Venda is my original work and has not been submitted for this degree or any other at this or any other institution. The dissertation does not contain another person's writing unless specifically acknowledged and accordingly referenced.

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DEDICATION

This work is dedicated to my mother Siphugu M.M, my father Siphugu M.J and the entire Siphugu's family. I hold nothing but gratitude for your patience, understanding, constant love, encouragement, support and guidance when I was constantly away from you and is much appreciated.

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ABSTRACT

The main purpose of this study was to assess the efficiency of ultrasonography in monitoring reproductive organs, pregnancy diagnosis, and foetal gender identification and to verify its reliability by laparoscopy and laparotomy, where applicable. Reproductive organs, pregnancy diagnosis and gender of the foetus were examined by A-mode ultrasound using 3.0 - 8.0 MHz trans-rectal transducer. A Sony Olympus Model laparoscope with a camera transducer was used to monitor the reproductive organs and pregnancy diagnosis. In monitoring the follicular dynamics, daily ultrasonography (ULTS) scanning was done for 17 days in sheep and for 21 days in both goats and cattle. Follicles of diameter ≥ 3 mm were selected for analysis of growth, ovulation and regression. For determining the efficiency of the techniques, laparoscopy (LAPSC) and laparotomy (LAPT) were used on days 3 and 10 of the goats and sheep oestrous cycle. The follicles were grouped into three categories according to their diameter as 3 - 4.9 mm, 5 - 7.9 mm and ≥ 8 mm, whereas the follicles of cattle were grouped as 3 - 4.9 mm, 5 - 9.9 mm and ≥ 10 mm. Early pregnancy diagnosis examinations were carried out from day 18 post insemination until pregnancy was confirmed. Foetal gender examinations were conducted from day 40 of pregnancy until the day the gender of the foetus was confirmed. Follicular development was accompanied by the occurrence of waves of follicular growth at different period of the oestrous cycle. The first follicular wave emerged on day 1.0 ± 0.4 in goats, 1.2 ± 0.4 in sheep and 2.2 ± 0.4 in cattle. The maximum diameter of the dominant follicles of observed follicular waves in goats was 7.3 ± 0.4 mm, 6.6 ± 0.2 mm, 7.3 ± 0.2 mm; in sheep was 6.4 ± 0.4 mm, 6.6 ± 0.4 mm and 6.7 ± 0.7 mm and in cattle was 13.1 ± 0.8 mm, 14.2 ± 0.6 mm and 15.7 ± 0.6 mm in wave 1, 2 and 3, respectively. However, the maximum size of the dominant follicle of the ovulatory wave in cattle was larger than the dominant follicles of both first and second waves, but in goats and sheep the dominant follicles were of similar size throughout the waves. In cattle, the ovulatory wave was shorter ($p < 0.05$) than the duration of the first and second waves, while in sheep and goats were similar throughout the waves. In goats the total number of follicles counted in right and left ovaries under category 3 - 4.9 mm was lower with ULTS and LAPSC than with LAPT method ($p < 0.05$). In sheep the mean number of follicles between 3 - 4.9 mm category in both right and left ovaries were different ($p < 0.05$) between ULTS and LAPT. However, for categories 5 - 7.9 mm and ≥ 8 mm in both goats and sheep the mean numbers of follicles observed by all techniques were similar ($p > 0.05$). In goats, pregnancy diagnosis accuracy improved from zero percent on day 18 to 100% on day 26 - 28, in sheep pregnancy diagnosis was 40% on day 18 and improved to 100% on day 20 - 22

of gestation. In cattle accuracy of pregnancy diagnosis was not possible at day 18 and gradually increased to 100% on day 30 - 32 of gestation. Out of 5 (100%) goat's fetuses whose gender was determined, the diagnosis was correct in 100% (3/3) of the male fetuses and 100% (2/2) of the female fetuses. In sheep two fetuses were sexed as males while the other three were sexed as females and were both 100%. Out of 60% (3/5) of fetuses examined in cattle, 1 (100%) was identified as male and the remaining 2 (100%) were identified as females. The results obtained confirmed that the accuracy for foetal gender by ultrasonography was 100% in all fetuses observed. The current study demonstrated that trans-rectal ultrasonography examination is an efficient method for monitoring follicular dynamics, diagnosing pregnancy and foetal gender identification and that it is as reliable as laparoscopy and laparotomy where they were applied together.

Keywords: Follicular dynamics; Pregnancy diagnosis; Foetal gender identification; Ultrasonography; Laparoscopy; Laparotomy; Sheep; Goats; Cattle.

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LIST OF ABBREVIATIONS

AI	Artificial insemination
ANOVA	Analysis of variance
ARTs	Assisted reproduction technologies
CIDR	Controlled internal drug release
CL	Corpus luteum
CO ₂	Carbon dioxide
Co	Cooperation
Cm	Centimeter
E ₂	Estradiol/Estrogen
<i>et al</i>	and others
F10 [®] SC	Veterinary disinfectant
FSH	Follicle stimulating hormone
G	Gram
GnRH	Gonadotropin releasing hormone
GT	Genital tubercle
lb	Pounds
IU	International units
Kg	Kilogram
LAPSC	Laparoscopy
LAPT	Laparotomy
LH	Luteinizing hormone
LSD	Least significant difference
Mg	Milligram
MHz	Mega Hertz
ml	Milliliter
Mm	Millimeter
N	Number of
Oz	Ounce
P ₄	Progesterone
PBS	Phosphate buffered saline
PGF _{2α}	Prostaglandin
PSPB	Pregnancy specific protein B

P-value	Probability
SD	Standard deviation
SEM	Standard error of mean
ULTS	Ultrasonography
°C	Degree Celsius
≥	Greater/equal to
<	Less than
>	Greater than
%	Percentage
±	Plus minus
≈	Almost equal to

CHAPTER 1

INTRODUCTION

1.1. Background of the study

It is predicted that by 2030, the world human population will reach 8.5 billion (Alexandratos & Bruinsma, 2012; United Nation, 2015). As a consequence of population growth, there will be a need to increase food production especially protein to meet the nutritional needs of this growing human population. A high proportion of protein consumed by human originates from livestock. Livestock production also plays a major role in shaping living standards of many people in developing countries (Delgado *et al.*, 1999). It is estimated that about 60% of people in the Southern African Development Community depend indirectly or directly on livestock production for their livelihood (Pica-Ciamarra & Otte, 2010). Given the role of livestock (especially in poor communities) it is important that the efficiency of livestock production and reproduction is continuously improved so as to enable the finite natural resources to sustainably maintain the growing population.

Reproduction of livestock especially in rural communal systems is generally low (Ngeno & Chimonyo, 2008) hence there is a need for this to improve as the small-scale and communal areas holds about 67% of the goats, 12% of the sheep and 41% of the cattle in South African livestock (Meissner *et al.*, 2013). In recent years, Assisted Reproduction Technologies (ARTs) have been introduced in these systems (particularly in rural Limpopo) with an aim to improve reproduction. These ARTs have, however, not yielded as good a result as anticipated. Under-achieved results of ARTs in communal livestock could be attributed to poor nutrition, inadequate herd management (uncontrolled breeding), congenital factors, hormonal disturbance and environmental changes which could result in animals being infertile. These factors influence the reproductive processes during ovulation, fertilization, gestation and parturition (Osmanu, 1979; Arthur, 1982; Arthur *et al.*, 1996). The abnormal and damaged reproductive organs caused by these factors has a negative influence on the onset and successful completion of the oestrous cycle (Hafez & Hafez, 2000; Bearden *et al.*, 2004; Braun, 2007) and this could reduce the reproductive potential of individual animals and consequently of the herd.

There is a limited availability of information on reproductive status of communal livestock (Webb & Mamabolo, 2004; Ngeno & Chimonyo, 2008) and preceding the application of ART's, the main source of reproductive status examination was ultrasonography scanning. In the ARTs

project, ultrasonography was used mainly for assessing ovaries and pregnancy. Application of ultrasonography for reproductive management purposes in livestock includes monitoring of ovarian structures throughout the oestrous cycle (Orita *et al.*, 2000; Evans, 2003; Simoes *et al.*, 2005), early pregnancy diagnosis (Karen *et al.*, 2001; Whittier, 2013; Raja-Khalif *et al.*, 2014) and foetal gender (Tainturier *et al.*, 2004; Santos *et al.*, 2006; Moraes *et al.*, 2009).

Information on reproduction capacity and status can be achieved by application of ultrasonography, laparoscopy and laparotomy techniques (Beal *et al.*, 1992; Goel & Agrawal, 1992; Noel *et al.*, 1993; Abubakar *et al.*, 2014). However, these techniques have their own limitations (Beal *et al.*, 1992; Abubakar *et al.*, 2014; Papazoglou & Basdani, 2015). The less than satisfactory results in the applied ART projects raised questions as to the efficiency of the ultrasonography in correctly assessing/diagnosing the reproduction status of the involved livestock. The variation in ability among the various methods to study reproduction status and capacity of live animals suggests that either of the methods could be less efficient than others in assessing reproduction status. The currently available literature on the efficiency of ultrasonography for monitoring the ovarian structures, early pregnancy diagnosis and foetal gender identification is not verified by the other two methods (laparoscopy and laparotomy) and it does not cover a broad number of livestock species.

1.2. Problem statement

Some interventions for the improvement of reproduction of livestock using ARTs have not achieved the desired results, because of the problems associated with the reproductive status of the animals. The statuses of the reproductive organs are mainly unknown and in many instances what is observed using ultrasonography is assumed to be correct. Subsequent poor results prompt verification in order to ascertain if ultrasonography is really accurate in diagnosing the reproduction capacity of livestock. The application of ultrasonography with laparoscopy and laparotomy as verification method for monitoring the ovarian structures, early pregnancy diagnosis and foetal gender identification could be useful in bridging the information gap.

1.3 Justification

Information about ultrasonography efficiency is important in designing appropriate and sustainable intervention strategies to enhance the implementation of ARTs. Verification of efficiency of ultrasonography in monitoring the ovarian structures, early pregnancy diagnosis

and foetal gender identification will improve the chances of ARTs success and consequently acceptance by livestock farming communities.

1.4 Study objectives

1.4.1. Broad objectives of the study

The main purpose of this study was to assess the efficiency of ultrasonography in monitoring the reproductive organs, pregnancy diagnosis and foetal gender identification and to verify its reliability by laparoscopy and laparotomy where applicable.

1.4.2 Specific objectives of the study

1. To determine the efficiency of ultrasonography scanning in monitoring reproductive organs when verified by laparoscopy and laparotomy.
2. To determine the efficiency of ultrasonography scanning when verified by laparoscopy for pregnancy diagnosis and ultrasonography only when gender identification of the developing foetus was done.
3. To evaluate the use of ultrasonography as compared to laparoscopy or laparotomy in different species with the emphasis on the operational time and possible surgical complications.

1.5 Hypothesis

1. Ultrasonography scanning is as efficient in monitoring reproductive organs as laparoscopy and laparotomy methods.
2. Ultrasonography scanning is as efficient during pregnancy diagnosis as laparoscopy and in foetal gender identification.
3. Ultrasonography scanning is as efficient by operational time and has surgical complications as laparoscopy and laparotomy.

CHAPTER 2

LITERATURE REVIEW

2.1. Reproduction of female farm animals

2.1.1. Morphological structure of the ovary

The ovary as the key component of reproduction of female animals is located within the abdominal cavity and is attached to the oviduct of the uterus by the ovarian ligament (Hafez, 1993). The main role of the ovary in reproduction is to produce oocytes within follicles through an exocrine process, and the secretion of oestrogen and progesterone through the corpus luteum (Schatten & Constantinescu, 2007). However, it is important to note that the ovaries have variations in terms of the shape and size depending on the species and the stage of the oestrous cycle (Hafez, 1993). The ovaries of sheep, goats and cattle appear to be almond-shaped (Hafez, 1993). Hafez (1993) indicated that the right ovaries are more active than the left ovaries. In addition, active ovaries appear to be larger than the inactive ovaries.

Structurally, the ovary is composed of the medulla and cortex surrounded by the germinal epithelium (Hafez, 1993). A dense white capsule, the tunica albuginea, covers the ovary immediately beneath the germinal epithelium (Hafez & Hafez, 2000; Schatten & Constantinescu, 2007). The loose connective tissue in both the cortex and medulla is known as stroma (Schatten & Constantinescu, 2007). The medulla of the ovary consists of irregularly arranged fibro-elastic connective tissue and extensive nerve and blood vessels system which enter the ovary through the hilus in the center of the ovary (Hafez, 1993; Schatten & Constantinescu, 2007). On the other hand, the ovarian cortex is located in intimate contact with the tunica albuginea and it contains the ovarian follicles and corpora lutea (Hafez, 1993; Hafez & Hafez, 2000; Schatten & Constantinescu, 2007). The cortex is the main site of both oocytes formation and production of oestrogen and progesterone.

2.1.2. Physiology of female farm animal reproduction

The ability of farm animals to be successfully bred, fertilized, maintain pregnancy and give birth to a healthy offspring regularly, is of outmost importance to the economics of farm herd management (Hafez & Hafez, 2000). Female reproductive physiology is associated with

the effect of hormones on the functioning of the ovary, uterus and mammary glands in order to achieve adequate fertility after breeding for the production of viable offspring (Reece, 2004). Normal female reproduction depends on hormones (Schatten & Constantinescu, 2007; Hill *et al.*, 2012). These hormones are carried and transported by the blood to different locations within the body where they perform their different functions (Hill *et al.*, 2012).

Hormones in female animals influence behavior, oestrous cycle, ovarian follicular development, oocytes maturation, ovulation and corpus luteum development, maintenance of pregnancy, parturition and lactation (Hafez, 1993; Gordon, 1997; Reece, 2004; Schatten & Constantinescu, 2007; Hill *et al.*, 2012). The hormone that plays a major role in female reproduction is Gonadotropin Releasing Hormone (GnRH) which is secreted from the hypothalamus (Hafez, 1993; Gordon, 1997; Reece, 2004; Schatten & Constantinescu, 2007; Bartlewski *et al.*, 2011; Hill *et al.*, 2012). Once the GnRH has been delivered into the portal circulation of the anterior pituitary, it stimulates the secretion of the follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Schatten & Constantinescu, 2007; Bartlewski *et al.*, 2011).

Follicle stimulating hormone and luteinizing hormone play different roles in the reproduction of female farm animals. The target tissue of FSH in female animal is the granulosa cells of the follicle of the ovary where it stimulates production of gender steroids. Gender steroids produced in the ovary are oestradiol which is produced during the follicular phase and progesterone which is produced during luteal phase. The main function of FSH is to stimulate the maturation of germ cells and follicular and oocyte development within the ovaries (Gordon, 1997; Reece, 2004). Theca cells in the ovary respond to LH stimulation by secretion of the testosterone, which is then converted into estrogen by adjacent granulosa cells. Oestrogens are produced during the follicular phase and trigger the anterior pituitary to release more FSH and LH. The concentration of LH increases during ovulation, thereby causing mature follicles to rupture (Hafez, 1993; Gordon, 1997). Ruptured follicle develops into the corpus luteum (Hafez, 1993).

2.1.3. Reproductive events that occurs in the ovaries

Female goats, sheep and cattle have to undergo the production of oocytes in order for fertilization to take place (Hafez & Hafez, 2000; Findlay *et al.*, 2009). This production of oocytes is the primary factor that determines the fertility of a female animal (Hill *et al.*, 2012). Before birth of a female animal, the ovaries contain diploid germ cells called oogonia, which divide

repeatedly by mitosis to produce many oogonia. During production of oogonia, each of them undergoes the first stage of meiosis in which its chromosomes replicate. After the replication of oogonia chromosomes, the oocyte development is arrested at prophase I stage of the oocytes, and are called primary oocytes (Bartlewski *et al.*, 2011; Hill *et al.*, 2012). Before the animal reaches reproductive maturity at puberty, the cells remain as primary oocytes without completing the first meiotic division until they are recruited for further development and possible ovulation (Schatten & Constantinescu, 2007; Bartlewski *et al.*, 2011; Hill *et al.*, 2012).

When the female animal has reached reproductive maturity at puberty, a subset of primordial follicles each with a primary oocyte is recruited to mature further during oestrous cycle in a process referred to as folliculogenesis (Schatten & Constantinescu, 2007; Bartlewski *et al.*, 2011; Hill *et al.*, 2012). Folliculogenesis can be explained as the selection, recruitment and development of the follicles, dominance/ovulation and atresia of the rest of the antral follicles in the ovary (Driancourt, 2001; Fatet *et al.*, 2011; Hill *et al.*, 2012). Early in the follicular phase, the somatic cells enclosing each primary oocyte becomes the granulosa cells, which then allows the transformation of the follicle to a primary follicle (Findlay *et al.*, 2009; Bartlewski *et al.*, 2011; Hill *et al.*, 2012).

Primary follicles are characterized by a single layer of cuboidal granulosa cells surrounding the oocyte. These cells increase in number by mitosis. When the primary follicles become secondary follicles, they have two or three layers of cuboidal granulosa cells. Then theca cells start to differentiate into steroid secreting cells and several fluid-filled cavities form in the follicles which combine during formation of the mature antral follicle (Bartlewski *et al.*, 2011; Hill *et al.*, 2012). Although many follicles become matured during the follicular phase, only some of the follicles attain full maturity. These can be explained by the development of the subordinate and dominant follicles during the oestrous cycle (Gordon, 1997; Hafez & Hafez, 2000; Fatet *et al.*, 2011). Subordinate follicles undergo the process of atresia while the dominant follicle(s) completes its first meiotic division and thereafter the follicular phase of the cycle ends with the surge of LH. A fully developed dominant follicle is known as the pre-ovulatory follicle (Bartlewski *et al.*, 2011; Fatet *et al.*, 2011; Hill *et al.*, 2012). Ovulation marks the end of the development of the follicular phase to form the luteal phase (Hafez, 1993; Gordon, 1997).

The cells of the ruptured matured follicle within the ovary are reorganized through proliferation, vascularization and other processes to form a corpus luteum or corpora lutea (Gordon, 1997). If fertilization does not take place after breeding, prostaglandin (PGF₂α) will be

produced from the uterine walls, resulting in the degeneration of the corpora lutea and a decrease in progesterone levels while new follicles develop. However, if fertilization occurs following successful breeding, the corpus luteum (CL) continues to grow further and secretes progesterone hormone which is responsible for the maintenance of pregnancy (Whittier, 1993; Findlay *et al.*, 2009; Fatet *et al.*, 2011; Hill *et al.*, 2012).

2.1.4. The oestrus cycle

The oestrous cycle of farm animals is defined as a period from the onset of one oestrus to the next oestrus (Hafez, 1993; Gordon, 1997). It can also be defined as a series of scheduled events in which the female animal has repeated opportunities to become pregnant as she is receptive to the male. Oestrus cycle can be affected by factors such as season, nutrition, lactation and illness. With the exception of season, these factors constitute management of the animal (Schatten & Constantinescu, 2007; Fatet *et al.*, 2011). Oestrous cycles in farm animals have substantial difference in the manner in which they occur. Does and ewes in the northern hemisphere are considered to be seasonally poly-oestrous due to their oestrous cycle occurring during limited period of the year and mostly late summer and early spring (Bartlewski *et al.*, 2011; Fatet *et al.*, 2011). However, in the southern hemisphere cows (Hafez & Hafez, 2000), some does (Greyling, 2000) and ewes (Shoeman & Burger, 1992) undergo the oestrous cycle throughout the year and are considered to be non-seasonal breeders.

Oestrus cycle is commonly divided into two phases namely the follicular phase and luteal phase (Farshad *et al.*, 2008; Fatet *et al.*, 2011). The follicular phase of the cycle is characterized by the growth and development of ovarian follicles and by the decline in progesterone concentrations associated with the regression of corpus luteum (Driancourt *et al.*, 1984; Gordon, 1997; Fatet *et al.*, 2011). The follicular phase of the cycle occurs between days 15 to 21 (in animals of 21 days oestrous cycle) (Whittier, 1993; Schatten & Constantinescu, 2007; Farshad *et al.*, 2008), while it occurs between days 14 to 17 (in animals of 17 days oestrous cycle) (Driancourt *et al.*, 1984) when new follicles are recruited, developed and ovulated.

The luteal phase of the cycle is characterized by the rupturing of the dominant follicle which is then transformed into a corpus luteum (Hafez & Hafez, 2000; Fatet *et al.*, 2011). This phase occurs between days 1 to 15 (in animals of 21 days oestrous cycle, e.g. cows and does) (Whittier, 1993; Schatten & Constantinescu, 2007; Farshad *et al.*, 2008), while it occurs

between days 2 to 13 (in animals of 17 days oestrous cycle, e.g. ewes) (Driancourt *et al.*, 1984) when there is luteinisation of the remnants of the follicle and subsequent regression of the corpus luteum if the female is not pregnant.

Oestrous can be divided into four stages namely the oestrus, metoestrus, dioestrus and prooestrus (Hafez & Hafez, 2000; Schatten & Constantinescu, 2007). These four stages can be grouped in terms of their occurrence. Prooestrus and oestrus stages are considered to occur during follicular phase of the cycle while the metoestrus and dioestrus occur during the luteal phase of the cycle (Schatten & Constantinescu, 2007). The oestrus phase is the period when the female is receptive to the male and the estradiol (E_2)/ oestrogen concentration is at its peak level (Farshad *et al.*, 2008; Fatet *et al.*, 2011).

Ovulation occurs at the end of oestrus in ewes and before the end of oestrus in does and at this time, oestrogen concentrations decline. In cows the oestrus also lasts a number of hours to days depending on breed. Mare oestrus period lasts for 5 days or more compared to days of the other phases of the oestrous cycle (Schatten & Constantinescu, 2007; Fatet *et al.*, 2011). Soon after oestrus, the female enters into metoestrus when estradiol level is decreasing and progesterone (P_4) level is increasing, because the corpus luteum is forming on the ovary at the site of ovulation. This phase occurs between day 1 and 5 of the cycle (Schatten & Constantinescu, 2007).

The dioestrus phase is the period that occurs after metoestrus, when progesterone level rises to its peak and estradiol is at its lowest level (Schatten & Constantinescu, 2007). In addition, maternal recognition of pregnancy occurs at this time as the embryo begins signaling the mother to maintain pregnancy and not to begin cycling again. The dioestrus phase occurs between day 6 and 15 of the cycle. (Hafez & Hafez, 2000; Schatten & Constantinescu, 2007). However, with no maternal recognition of pregnancy, the female enters the prooestrus phase. The prooestrus phase is a period which can be defined by decrease in progesterone level and an increase of estradiol, as well as the formation of the next cohort of follicles of which one or two will ovulate during the oestrus. This phase occurs between days 15 to 21 of the oestrous cycle in cows (Schatten & Constantinescu, 2007; Fatet *et al.*, 2011).

2.1.5. Follicular wave development

The process of follicular development occurs continuously in a wave-like pattern throughout the oestrous cycle as reported in goats by Medan *et al.* (2003), sheep (Seekallu *et*

al., 2010) and cattle (Muraya *et al.*, 2015). A cohort of follicles begins to grow and a follicular wave emerges (Bodensteiner *et al.*, 1996; Wiltbank *et al.*, 2002). A follicular wave can be explained as the organized development of a cohort of gonadotrophin dependent follicle cell of which initially increases in size, but most of which (subordinate follicle) subsequently regress and die by atresia (Bodensteiner *et al.*, 1996; Wiltbank *et al.*, 2002; Evans, 2003).

The development of non-invasive trans-rectal ultrasonography as a tool to monitor the growth and regression of individual ovarian follicles, repeatedly in the same animal, firmly established that antral follicles grow in cohorts in a wave-like a pattern during the oestrous cycle (Driancourt *et al.*, 1984). There are a number of terms that are particularly relevant when describing follicular waves based on trans-rectal ultrasonography observations. The day of follicle wave emergence is the first day of a follicular wave when a growing cohort of follicles is first detectable using trans-rectal ultrasonography (Wiltbank *et al.*, 2002; Evans, 2003).

During this process of follicular wave development, there are two types of follicular waves namely the anovulatory and ovulatory waves (Simoes *et al.*, 2006; Farshad *et al.*, 2008; Bartlewski *et al.*, 2011). The only difference between these two waves is that there are one to two anovulatory waves and one ovulatory wave which can be properly identified with a good timing of the wave during the oestrous cycle (Schatten & Constantinescu, 2007; Farshad *et al.*, 2008; Fatet *et al.*, 2011). During the ovulatory wave, the LH levels continue to increase which results in an LH surge causing the oocyte(s) to ovulate. However, the number of follicles that develop and ovulate depends on the species (Fatet *et al.*, 2011; Hill *et al.*, 2012).

Due to the process of follicle selection, there is a decrease in the number of growing follicles in a wave to the number of follicles that ovulate (Driancourt, 2001). This ill-defined process probably occurs over a period of time and is thought to end when the dominant follicle has been selected from subordinate follicles as observed by difference in follicle size (Bodensteiner *et al.*, 1996; Evans, 2003). A dominant follicle is one that continues to develop when the growth and development of other follicles are inhibited. Subordinate follicles are the remaining follicles that regress in the presence of a dominant follicle (Driancourt *et al.*, 1984; Driancourt, 2001; Evans, 2003)

In cows, a wave-like pattern of follicle development is very well characterized with most oestrus cycles comprising of two or three follicular waves (Sirois & Fortune, 1988; Figueiredo *et al.*, 1997; Gaur & Purohit, 2007; Muraya *et al.*, 2015). Each wave consist of one and occasionally, two dominant follicles that reaches a diameter of approximately 10 - 17 mm and

several subordinate follicles of 7 - 9 mm in diameter (Ginther *et al.*, 1996; Evans, 2003). The maximum diameter of the dominant follicle of the first wave after ovulation averages 15 - 16 mm and the dominant follicles of the subsequent waves averages 12 - 14 mm (Ginther *et al.*, 1996). There is an indication that cattle that have two follicular waves tend to have shorter oestrous cycles, to ovulate larger and older follicles and to be less fertile than cattle with three follicular waves per oestrous cycle (Gordon, 1997; Evans, 2003).

In sheep at any given time, there are about 50 antral follicles in the ovaries of an adult ewe (Driancourt *et al.*, 1984; Evans, 2003). During the oestrous cycle of the ewe, there are three follicular waves that are predominant (Noel *et al.*, 1993). Moreover, there are two waves of follicular activity found during the luteal phase and follicular phase of the oestrous cycle detectable by using ultrasonography (Noel *et al.*, 1993). However, Camp *et al.* (1983) indicated that it is not always the case that preovulatory follicles detected during mid or late luteal phase are identified as the ovulated follicles at the subsequent estrous. In studies of sheep (Evans *et al.*, 2000) there were instances whereby cycles with two to six follicular waves were achieved while Schrick *et al.* (1993) mentioned that follicular waves were not observed in sheep.

During the oestrous cycle, the anovulatory follicular wave could be defined by one or more follicles growing to at least 3 - 7 mm in diameter and not ovulating whereas the dominant follicle of the ovulatory follicles reaches a maximum diameter of at least 10 mm and ovulate in ewes (Bartlewski *et al.*, 1999a, b, c; Gibbons *et al.*, 1999; Evans, 2003). The emergence of the follicular waves 1, 2 and 3 (ovulatory wave) occurs on days 1 - 2, 7 - 8, and 14, post ovulation. The recruitment of the follicle(s) destined to ovulate takes place on day 14 of the cycle and it coincides with luteolysis. The largest follicles of follicular waves 1 and 2 have a smaller maximum diameter than the largest follicle of wave 3 (Noel *et al.*, 1993).

In goats, there are about two to six follicular waves during the oestrous cycle with three to four waves being the most prevalent (de Castro *et al.*, 1999; Medan *et al.*, 2003; Menchaca & Rubianes, 2002). However, the dynamics of follicular wave development in goats is limited as compared to cattle and sheep (Evans, 2003; Simoes *et al.*, 2006). About three to six follicles of approximately 3 to 7 mm in diameter have been recorded in the ovaries when using trans-rectal ultrasonography (Orita *et al.*, 2000; Medan *et al.*, 2003). A cohort of gonadotropin dependent antral follicles of 2 to 3 mm in diameter are recruited under the influence of LH and almost all of them atrophy before being ovulated (Fatet *et al.*, 2011). All follicles that grow in the early to

middle oestrous cycle became non-ovulatory follicles and degenerate (Orita *et al.*, 2000; Fatet *et al.*, 2011).

All the follicles that appeared in the latter half of the oestrus cycle in does will grow to approximately 6 - 9 mm in diameter and become ovulatory follicles (Orita *et al.*, 2000; Fatet *et al.*, 2011). Almost all the ovulatory follicles were detected from 5 days before ovulation, coinciding with the decline in progesterone concentrations. The plasma concentration of estradiol-17 β increased with the follicular growth until ovulation (Orita *et al.*, 2000). The emergence of the follicular waves 1, 2, 3 and 4 (ovulatory wave) occurs on days 0, 5 - 6, 10 - 11 and around day 15 post ovulation, respectively in does (Menchaca & Rubianes, 2002). Similar to sheep (Noel *et al.*, 1993), the largest follicles of the follicular waves 2 and 3 have a smaller maximum diameter than the largest follicle of wave 1 and 4 (Ginther & Kot, 1994).

2.1.6. Ovulation

Ovulation can be described as the process that involves the release of an oocyte for fertilization from a mature follicle(s) during the oestrous cycle. The ovulation rate mostly varies largely between farm animals (Hafez & Hafez, 2000; Hill *et al.*, 2012). However, the ovulation within the farm animal is influenced by many factors such as nutrition, age, genetic make-up and environment. Follicular development and ovulation are dependent on a functional microcirculatory network. A vascular wreath is a characteristic feature of the theca interna of mature ovarian follicles. The theca layer of the pre-ovulatory follicles develops a fine network of capillaries reaching the proximity of the granulosa layer (Acosta & Miyamoto, 2004; Reece, 2004; Bartlewski *et al.*, 2011). The theca interna layer and the granulosa cell layer are separated by the basal lamina. Nutrients and hormones are thereby supplied through diffusion to the granulosa cells from the capillary network through this basal lamina layer. The increase in the blood flow to the theca layer of the dominant follicle results in an increased supply of gonadotrophins and other systemic biochemical and hormonal factors necessary for follicular development (Acosta & Miyamoto, 2004; Bartlewski *et al.*, 2011).

Ovulation occurs as a result of a dynamic interaction between LH surge and intra-follicular factors including steroids, prostaglandins and peptides derived from endothelial cells. However, knowledge of the exact mechanism involved is still lagging behind (Kanitz, 2003; Acosta & Miyamoto, 2004; Reece, 2004; Hill *et al.*, 2012). The LH surge triggers structural and biochemical changes that lead to changes within the follicular wall which causes it to weaken so

that the Graafian follicle ruptures, resulting in the expulsion of the oocyte and subsequent development of the CL (Kanitz, 2003; Acosta & Miyamoto, 2004).

The collagen fibers contained in the tunica albuginea and the theca layer as well as the basal membranes contributes to the tensile strength of the follicular wall. The degradation of these collagen layers is accompanied by increase vascular dilatation and permeability, which are necessary for follicle rupture (Acosta & Miyamoto, 2004). The blood flow in the apex of ovulatory follicle decreases while increases at the base of the follicle, facilitating follicle rupture (DrianCourt *et al.*, 1984; Acosta & Miyamoto, 2004). Prostaglandins which play an important role in ovulation are produced through an interaction between the ovarian, uterine, placental and pituitary functions to regulate reproduction.

2.1.7. Corpus luteum development

The corpus luteum is a temporary endocrine organ, and its duration of occurrence occupies the metoestrus and dioestrus phases of the oestrous cycle (Gordon, 1997; Reece, 2004). Soon after the process of the ovulation has been concluded, the follicular cavity is filled with blood and lymph due to its rupture to form a corpus luteum (Reece, 2004; Hill *et al.*, 2012). The mature corpus luteum is primarily composed of the lutein cells which are arranged in columns separated by the blood vessels and connective tissue. The color of the corpus luteum differs within the species and the period of the oestrous cycle. In cattle the lutein cells contain a yellow lipochrome pigment, while in sheep and goats it is flesh colored because the granulosa cells are devoid of the lipochrome pigment (Schatten & Constantinescu, 2007; Hill *et al.*, 2012). On the other hand, the shape of the corpus luteum varies with the species. In cattle and sheep, it may primarily be either above the ovarian surface or buried in the ovarian stroma, while in goats the corpus luteum is located in the ovarian stroma (Hafez, 1993).

In goats and sheep the functional CL could be detected from day 1 to 6 days post ovulation and mostly they start degenerating on day 6 to 8 (Bartlewski *et al.*, 1999a, b, c; de Castro *et al.*, 1999; Orita *et al.*, 2000). The mature CL could be observed as a grey echogenic structures with marked boundaries (de Castro *et al.*, 1999). Before ovulation, the luteinized follicles could be detected at 3 mm in diameter on day 1. On day 2 and day 3 prior to luteinization, these follicles reach 7 and 9 mm in diameter in goats and sheep, respectively. Between days 3 and 4 post ovulation, the CL grows to about 6 - 8 mm in diameter and it reaches maximum of approximately 11 - 14 mm in diameter 6 days later (Bartlewski *et al.*,

1999a, b, c; 2001). However, ovulation during the estrous cycle could be confirmed by counting of the CL present between days 6 and 8 post ovulation as mentioned by Simoes *et al.* (2006).

2.1.8. Importance of monitoring reproductive organs

The goal of any livestock farming enterprise should be to increase efficiency and productivity of the herd as an economic trait (Martin *et al.*, 2004; Whittier *et al.*, 2009). A successful livestock production operation depends mainly on quantity being taken from the herd for sale and this means that reproduction rate is pivotal. A low reproduction rate as a result of poor fertility indicates that a problem exists in a herd, but does not indicate the cause of the problem or where it occurs (Mokantla *et al.*, 2004). Low fertility can be attributed to abnormal ovaries, which in most cases are caused by the ovarian follicular cysts (Silva *et al.*, 2002; Youngquist & Threlfall, 2007). Culling of infertile and sub-fertile females is done only after considerable efforts of diagnosing the problem has failed or when the animal has proven to be infertile (Muller *et al.*, 2014) and this is mostly after the animal was bred and time has been wasted. There are various methods for monitoring the reproductive organs of the livestock but all have an aim to observe the structural soundness, abnormalities and functionality of the reproduction organs. Monitoring of reproductive organs for abnormalities and functionality can be useful in ensuring that animals suspected of infertility or subfertility can be detected early and are not selected for breeding.

2.2. Pregnancy diagnosis in farm animals

2.2.1. Importance of early pregnancy diagnosis

Early pregnancy diagnosis is of considerable value to livestock industry in a sense that it improves reproductive management of the herd. Early pregnancy diagnoses play an important role in shortening the inter-calving/lambing/kidding period and also assist farmers in identification of animals that require infertility treatment and repeat inseminations. When early pregnancy is conducted, non-pregnant animals could be sold to reduce feed expenses, labour, medication and result in a well-managed, profitable operation. However, non-pregnant animals can also be carried to the next breeding season depending on the type of operational management (Ashok *et al.*, 2013; Whittier, 2013; Raja-Khalif *et al.*, 2014; Karadaev, 2015).

Animals that are pregnant require to be separated from non-pregnant animals so as to reduce the probabilities of reproduction and production losses in the form of abortions and

production of weak off-spring (Karen *et al.*, 2001; Ferrari *et al.*, 2009; Karadaev, 2015). Furthermore, accurate information about the gestational stage would be important when lactating females are being dried off at an appropriate period of time, and to be able to monitor females that are near giving birth (Doize *et al.*, 1997). Karen *et al.* (2001) and Tanturier *et al.* (2004) reported that diagnosing pregnancy earlier allows the appropriate nutritional management in the late gestation period, which could assist in preventing pregnancy toxemia, optimizing birth weight, weaning weight and survivability of the foetuses and could also reduce the incidences of dystocia.

2.2.2. Embryonic development stages

To accurately diagnose and monitor pregnancy, the knowledge of stages of embryonic development is required. Table 1 summarizes early embryonic development of cattle, goats and sheep. Early embryonic development period post insemination/breeding in livestock is accompanied by series of events which begins with fertilization and is completed by implantation stage (Ashock *et al.*, 2013). However, it is important to note that the embryonic development period would differ from species to species and breed within species.

Table 1: Important series of events during the early embryonic period of the goats, sheep and cattle

Days of pregnancy	Series of events
Day 0-1	Fertilization, single cell embryo (zygote) in oviduct.
Day 2	Early cleavages in the oviduct (up to 8 cell stages), activation of embryonic genome.
Days 3-4	Embryo enters the uterus.
Days 5-6	16-32 cell zona-enclosed embryo progressing into compact morula stage.
Days 7-8	Formation of blastocoel with differentiation of embryonic cells.
Days 9-10	Blastocyst expansion and hatching from the zona pellucida
Days 11-15	Blastocyst elongation from a tubular to a filamentous structure.
Days 14-19	Maternal recognition of pregnancy.
Days 19-20	Implantation begins.
Day 21	Caruncle-cotyledons appear.
Days 22-41	Implantation progresses.
Day 42	Implantation completed.

Ashock *et al.*, 2013

2.2.3. Period of pregnancy diagnosis in farm animals

A positive diagnosis could be explained as a detection of the presence of the embryonic vesicle as a non-echogenic fluid and then embryo proper in the uterine lumen then later the foetus heartbeat (Padilla-Rivas *et al.*, 2005; Amer, 2010; Romano *et al.*, 2006; Tavana *et al.*, 2014). The embryo proper can be observed as an echogenic structure within the embryonic vesicle separated from the uterine cavity by the embryonic membrane which appears as a very narrow, hypo-echoic line. The embryonic vesicle in this regard could be observed as a non-echoic accumulation of the fluid in the lumen of the uterine horn (Goel & Agrawal, 1990; Changsheng Dong *et al.*, 2013; Tavana *et al.*, 2014). However, at that early stage, it is important to be able to differentiate pregnancy from the urine bladder (Karen *et al.*, 2001; Medan *et al.*, 2004; Suguna *et al.*, 2008; Ferrari *et al.*, 2009).

When using trans-rectal ultrasonography for pregnancy diagnosis, positive signs of pregnancy in sheep could be diagnosed as early as day 12 (Karen *et al.*, 2001; Ferrari *et al.*, 2009), however, Garcia *et al.* (1993) and Doize *et al.* (1997) indicated that positive signs could be observed on day 18 post mating. Embryo proper and foetal heartbeat could be detected from day 19 to day 24 of gestation (Garcia *et al.*, 1993; Karen *et al.*, 2001; Karen *et al.*, 2009), however, Ferrari *et al.* (2009) indicated that the embryo proper and foetal heartbeat could be detected as early as day 15.

In goats, Martinez *et al.* (1998) detected positive signs of pregnancy as early as day 15 which differs from the day 19 as reported (Padilla-Rivas *et al.*, 2005; Amer, 2010; Suguna *et al.*, 2008; Raja-Khalif *et al.*, 2014). The embryo proper and foetal heartbeat could be detected between days 20 and 24 of gestation as suggested by Martinez *et al.* (1998); Padilla-Rivas *et al.* (2005); Amer, (2010); Raja-Khalif *et al.* (2014). However, Suguna *et al.* (2008) differed with the latter mentioned reports by indicating that the embryo proper and heartbeat could only be detected on day 28 of gestation. Restall *et al.* (1990) diagnosed pregnancy later in gestation between days 51 and 60.

In sheep Roman & Christian (2008) detected pregnancy between days 16 and 20 post insemination using a 7.5 MHz transducer probe on Suffolk ewes. In Awassi and Merino ewes study (Karen *et al.*, 2004), accurately diagnosed pregnancy between day 18 and day 24, respectively, using 5 MHz probe. Roman & Christian (2008) found the use of trans-rectal probe (7.5 MHz) to be more accurate and precise than 5 MHz probe as also reported by Karen *et al.*

(2004). Karen *et al.*, (2004) mentioned that the effect of age and breed played dominant role in detecting pregnancy between days 18 and 24. Using transabdominal ultrasonography probe Ganaie *et al.* (2009) detected pregnancy between 15 and 30 days post insemination. However, Padilla-Rivas *et al.*, (2005) explained that although transabdominal probe may be more convenient, trans-rectal probe remains preferred. The latter further indicated that trans-rectal probe is preferred for pregnancy diagnosis because transabdominal probe detect pregnancy 4 - 5 days later.

In cattle, some pregnancy associated structures had been observed as early as between day 9 and day 21 post insemination (Curran *et al.*, 1986; Boyd *et al.*, 1988; Whittier, 2013) and the embryo proper and heartbeat had been observed between days 19 and 24 (Fricke, 2002; Broaddus & de Vries, 2005) and between days 26 and 30 (Pieterse *et al.*, 1990; Nation *et al.*, 2003; Naikoo *et al.*, 2013). Although the embryo proper can be detected earlier during gestation, it is mostly practical to scan females which are expected to have embryos that are more than 26 days of age in order to reduce embryonic losses (Curran *et al.*, 1986). Pregnancy diagnosis by method of palpation per rectum can be done as early as between day 30 and day 35 post insemination, although much practice is necessary in order to determine pregnancy at that stage (Ashok *et al.*, 2013).

By using transabdominal ultrasonography in small ruminants, the embryo proper and foetal heartbeat could be detected between days 33 and 75 of gestation as reported by (Suguna *et al.*, 2008; Raja-Khalif *et al.*, 2014; Karadaev, 2015). However, as much as the presence of the embryonic vesicle can be observed as a positive sign of pregnancy diagnosis, foetal heartbeat is preferred as an indicator of pregnancy, since it provides conclusive proof of the presence of a live foetus (Padilla-Rivas *et al.*, 2005). Pregnancy diagnosis could be done as early as between day 17 and day 28 post mating, as it is difficult to visualize genital organs in heavy small ruminants with much omental fat (Goel & Agrawal, 1992).

2.3. Early foetal gender identification

2.3.1. Importance of early foetal gender identification

Early foetal gender identification is important for breeding as it enables for identification of the gender of the foetus while still *in-utero* for use in beef and dairy herds (Tainturier *et al.*, 2004; Santos *et al.*, 2006; Santos *et al.*, 2007a, b; Moraes *et al.*, 2009). This enables the livestock producers to plan their livestock management in advance in terms of nutrition,

diseases, breeding and marketing of their animals at early age, and as well have replacement herd (Santos *et al.*, 2007a, b). This primary application would allow separation of females in dairy herd and males in meat herds of goats, sheep and cattle (Santos *et al.*, 2007a, b). Santos *et al.* (2006) and Ali & Fahmy (2008) noted that in livestock production systems of goats, sheep and cattle, early gender identification would permit the identification of male and female co-twins offspring's when they are still *in utero* which could lead to a female freemartin.

2.3.2. Foetal features important for gender determination

During examination for foetal gender identification by trans-rectal ultrasonography scanning, there are various important anatomic foetal features that are critical in achieving proper orientation of the foetus (Tainturier *et al.*, 2004; Stroud, 2005). The heartbeat and the body structures such as the umbilical cord, spinal cord, both front and hind legs and the head are the landmark features that should be visible before the gender of the foetus can be determined. However, when the body is still developing, it is sometimes difficult to differentiate the hind legs from the front legs if the anatomic features are not well identified (Stroud, 2005).

After observing the anatomical features, the genital tubercle (GT) development and migration of the primordial germ cells can be seen (Dyce *et al.*, 2002). The migration indicates whether the foetus is male or female. The genital tubercle is the embryonic structure that is formed from the cloaca during embryonic development and it differentiates into the penis and prepuce in the male foetus and to the vulva and clitoris in the female foetus. During the differentiation, GT moves from the initial position between the hind legs towards the umbilical cord in males and towards the tail in females. (Coubrough & Castell, 1998; Tainturier *et al.*, 2004; Moraes *et al.*, 2009; Yotov *et al.*, 2011). Beal *et al.* (1992) and Dyce *et al.* (2002) indicated that the development of genital tubercles in livestock animals begins during the embryonic development with the migration of the primordial germ cells in the caudal area and the formation of the gonadal ridge. Furthermore, Dyce *et al.* (2002) reported that the development and migration of the genital tubercles are the basis of the processes of identifying the foetal gender during the embryonic development. However, Santos *et al.* (2006) argues that identification of the appropriate day for genital tubercle migration is still one of the main challenges of foetal gendering in ruminants animals.

Foetal structures such as the penis, and prepuce, scrotum and gonads should be clearly visible when identifying male foetus (Tainturier *et al.*, 2004; Stroud, 2005). The penis is

visualized in the ventral-caudal abdomen, just caudal to the root of the umbilical cord. The penis is partially or completely enclosed within the prepuce. However, it can often appear fully extended. The foetal scrotum displays a composite echo density between the hind legs as the scrotal lodges appear as two symmetrical, oval and less echo dense areas. The gonads of the male foetus appears uniformly echo dense (Bucca, 2005).

In contrast to the male foetus, the female foetus features includes the mammary glands, nipples/teats, vulva/clitoris and gonads. The mammary glands of the foetus can be visualized in the pubic area and appear triangular or trapezoidal in shape and uniformly echo dense. Nipples/teats emerge from the mammary gland ventral border as two large hyper-echo dense dots. There are no relevant structures that can be visualized on the ventral perineum as opposed to the male foetus where the urethra inside the penis runs the entire length (Bucca, 2005). The foetal clitoris is an echogenic structure that emerges out of the buttocks. It is positioned high up in the perineum and should not be confused with the anus which is located adjacent to the root of the tail. According to Santos *et al.* (2006) anatomical features are vital parameters and are prone to change as the foetus grows, and could as well lead to the wrong identification of the male foetus as the female foetus. Buerstel *et al.* (2002) accepted that a foetus is the male when the presence of a penis and scrotum is established, and the female, when there are rudimentary teats with clear visibility.

2.3.3. Stages of foetal gender identification

The examination of foetal gender is grouped in three different stages, which are early foetal gender identification, intermediate foetal gender identification and late foetal gender identification in cattle, goats and sheep. Figure 1 as adopted from Tainturier *et al.* (2004) shows the main stages at which diagnosis can be done. Early foetal gender identification is carried out from day 55 to day 65 whereas intermediate foetal gender identification is carried between day 65 to day 80, and late foetal gender identification is carried out from day 80 to day 100 of pregnancy by using the trans-rectal ultrasonography scanner to determine the location of the genital tubercle (GT) (Curran, 1992; Tainturier *et al.*, 2004). It is also important to note that foetal gender identification using ultrasonography scanning is impossible after day 120 of pregnancy, since the foetus would have moved deep down out of reach and the size has significantly increased which makes it difficult to obtain the required anatomic features (Tainturier *et al.*, 2004).

Tainturier *et al.* (2004) and Oliveira *et al.* (2005) mentioned that foetal gender identification cannot be easily performed before day 50 of the pregnancy, since the genital tubercles cannot be seen by using ultrasonography before day 45. This indicates that between day 45 and day 50, the genital tubercles are very difficult to visualize between the hind legs and the umbilical cord. Tainturier *et al.* (2004) further indicated that the genital tubercles are initially located between the hind legs in both genders, but after day 50 of pregnancy they begin to migrate towards the umbilicus in the male foetus and towards the tail root in the female foetus. However, after day 50 the genital tubercle effectively begins to move halfway towards their initial position (hind legs) and their final position (umbilical cord or tail) and reaches their final position on day 60. During this period the difference between a male foetus and a female foetus becomes clearly visible (Santos *et al.*, 2006).

It is indicated by Curran (1992) and Azevedo *et al.* (2009) that it is easier to visualize the genital tubercles between day 60 and day 65 when they look like a bilobular, ovoid structure, which are a few millimeters in size and highly echogenic. Additionally, it is best to visualize the genital tubercle in either the frontal or transverse view. In the frontal view, the possible locations of genital tubercle are visualized at once, while in the transverse view the possible locations of the genital tubercle is visualized separately and the probe has to be moved from the head to the tail for clear visibility (Tainturier *et al.*, 2004 & Oliveira *et al.*, 2005). Curran (1992) and Azevedo *et al.* (2009) stated that between day 70 and day 75 of pregnancy the genital tubercle in the male foetus becomes hypo-echogenic as it forms the penis, therefore it becomes particularly difficult to identify because genital tubercle losses its bilobate structure. Mari *et al.* (2002) reported that after day 75 of pregnancy the penis is fully developed while Curran (1992) emphasized that during this stage the male scrotum is fully visible between the hind legs.

Contrary to the male foetus, mammary teats become visible in the inguinal region of the female foetus from day 80, where they appear as four highly echogenic dots arranged in a square as described by Tainturier *et al.* (2004) and Yotov *et al.* (2011). After day 100 of pregnancy, the genital tubercles develop into the clitoris which looks like globules and echogenic structures in the triangle shape located under the tail as described by Ali & Fahmy (2008), which can as well be visible in a frontal section (Oliveira *et al.*, 2005). However, Tainturier *et al.* (2004) argued that it is very rare to clearly visualize the typical triangle of the clitoris because the foetus is too deep into the abdominal cavity, which makes it difficult to see on the ultrasound.

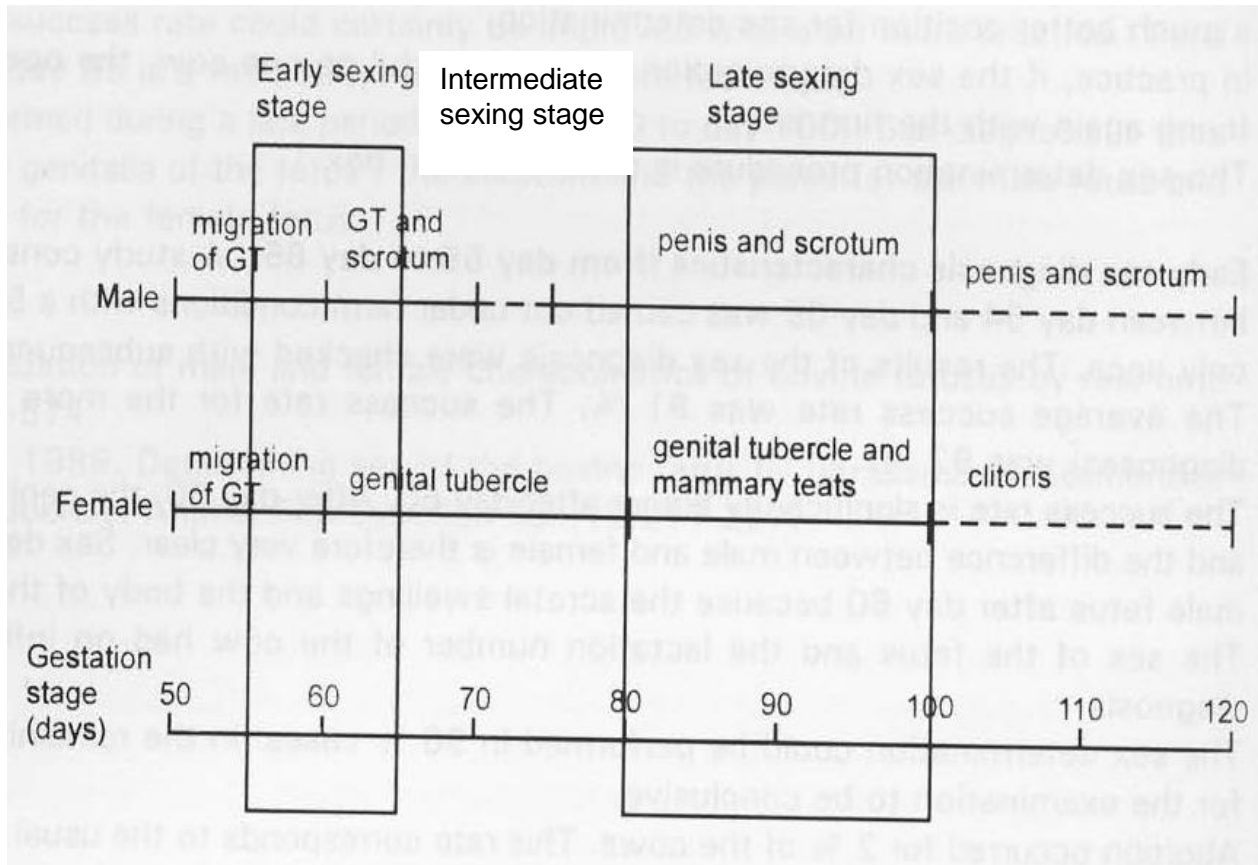


Figure 1: Indicates the three main different stages of foetus gender identification goats, sheep and cattle as adopted from Tainturier *et al.*, (2004)

2.4. Methods of monitoring reproductive organs, pregnancy diagnosis and early foetal gender identification

Utilization of an accurate and easily applicable method for reproductive organs monitoring, pregnancy diagnosis and early foetal gender identification might improve reproduction management of the herd. However, methods used in livestock must be applicable, feasible and practical under field conditions; otherwise they are not well received as suitable by farmers and practitioners (Fricke, 2002; Ferrari *et al.*, 2009; Karadaev, 2015).

Method of reproductive organs monitoring that is applicable, feasible and practical under farm conditions is ultrasonography. There is however methods like laparoscopy and laparotomy

which can be used for reproductive organs monitoring in small ruminants although not practical under farm conditions. There are methods of pregnancy diagnosis that are applicable, feasible and practical under farm conditions such as trans-rectal ultrasonography scanning, palpation per rectum and return to oestrus. However there are methods such as laparoscopy, milk progesterone tests and pregnancy specific protein B (PSPB) tests are reliable although not practically applicable to small farms. In early foetal gender identification the Method widely used is ultrasonography.

2.4.1. Ultrasonography scanning

The application of real-time ultrasonography for studying reproductive functions, pregnancy diagnosis and gendering of developing foetus in animal sciences is no longer a technique for few breeders, but a widely used tool in reproductive management and research (Terzano, 2012). Ultrasonography is a simple, reliable and non-invasive imaging technique without secondary effects (Ribadu & Nakao, 1999; Medan & Abd El-Aty, 2010; Terzano, 2012; Grizelj *et al.*, 2013). In the 1980s, trans-rectal ultrasonography scanner was developed for use in domestic animals (Ginther, 1995).

Real-time, B-mode ultrasound is a display mode in which the signal echoed from a tissue is displayed as a white dot on a black background. The ultrasound probe acts to send and receive high frequency ultrasonic waves. When an electric field is applied to the crystals in the probe, they change shape and vibrate while creating sound waves (Ginther, 1995; Fricke, 2002). Fluid such as blood or follicular fluids does not reflect sound waves and no image appears on the screen. Trans-rectal B-mode ultrasonography scanning using a linear array 5.0 to 7.5 MHz transducer is the most common technology used for trans-rectal pregnancy diagnosis and monitoring of reproductive viability on farm animals (Beal *et al.*, 1992).

The application of trans-rectal ultrasonography in veterinary practice has been developed to become the most efficient diagnostic tool for managing livestock reproduction (Medan & Abd El-Aty, 2010; Terzano, 2012). Thus, ultrasonography application has developed from a limited use for pregnancy diagnosis (Medan & Abd El-Aty, 2010) and detection of some pathological conditions in female and male animals (Terzano, 2012) to foetal gender determination (Medan & Abd El-Aty, 2010). Trans-rectal ultrasonography enables the determination of the ovarian activities in terms of follicular development, ovulation rate and time and corpus luteum development (Orita *et al.*, 2000; Wiltbank *et al.*, 2002; Medan & Abd El-Aty,

2010; Terzano, 2012; Grizelj *et al.*, 2013). However, a routine identification of ovaries and their structures could be difficult or not possible in some stages of sheep and goats' (Simoes *et al.*, 2005) oestrous cycle. Nevertheless, trans-rectal ultrasonography method for monitoring of the ovarian structures, early pregnancy diagnosis and gender determination should be simple, reliable, not time consuming and applicable under normal farm conditions. In order to be accepted by breeders and used in large-scale herd trans-rectal ultrasonography method should not bring difficulties to daily herd management (Ferrari *et al.*, 2009).

During trans-rectal ultrasonography application in monitoring reproductive organs pregnancy diagnosis and foetal gender identification female animals are examined in their standing position and a probe is inserted into the rectum of the female animal and positioned above the uterus (Ginther & Kot, 1994; Simoes *et al.*, 2005; Karen *et al.*, 2014). Before the probe is inserted through the rectum, faecal material in the rectum is removed to allow clear visibility of the organs. The probe is lubricated with water mixed with cooking oil and inserted through the rectum. To find the reproductive organs, the probe is inserted pointing downwards so that the bladder and uterine horns can be observed in a longitudinal direction. After the probe has been inserted into the rectum, it is then rotated from the 3 o'clock position past the 6 o'clock position to the 9 o'clock position to scan the entire area below the probe (Ginther & Kot, 1994; Simoes *et al.*, 2005; Karen *et al.*, 2014). This procedure allows the structures under observation to be fully visible on the scanner screen. In addition, this technique enables a quicker ultrasonographic examination in small ruminants because, usually, it is not necessary to remove the faecal material (Simoes *et al.*, 2005) as compared to cattle. Thus, a larger number of examinations per hour can be performed.

Real-time ultrasonography scanning enables the study of the ovarian structures in small ruminants such as sheep and goats (Bartlewski *et al.*, 1999a, b, c; Orita *et al.*, 2000; Simoes *et al.*, 2005) and is routinely done in cattle (Ginther *et al.*, 1996; Wiltbank *et al.*, 2002; Evans, 2003). It also permits the monitoring of their growth and development through successive observations (Simoes *et al.*, 2005). As a non-invasive method, it offers great advantages when compared to the invasive methods such as laparoscopy and laparotomy in small ruminants because it is less stressful and it does not require administration of anaesthetics (Simoes *et al.*, 2005; Grizelj *et al.*, 2013).

In cattle, ultrasonography has been used to monitor ovarian activities (Carroll *et al.*, 1990), morphological uterine involution throughout the early post-partum period and mechanisms responsible for ovarian disorders such as ovarian cyst (Youngquist & Threfall, 2007). In goats (de Castro *et al.*, 1999; Menchaca & Rubianes, 2002; Simoes *et al.*, 2005) and sheep (Bartlewski *et al.*, 1999a, b, c; Gibbons *et al.*, 1999; Bartlewski *et al.*, 2001; Cox *et al.*, 2015) ultrasonography was used to demonstrate change in ovarian follicles, ovulation rate, antral follicular dynamics, ovulatory follicles, CL development and measuring of follicular diameter. As a result, it proved that ultrasonography is more reliable for evaluating ovarian activities in all ruminants than rectal palpation in cattle (Beal *et al.*, 1992; Hanzen *et al.*, 2000).

The widespread application of ultrasonography for effective reproductive management includes detection of early pregnancy, embryo proper, foetal heartbeat, foetal development, single and multiple pregnancies, embryonic death and gendering of the foetus (Ginther & Kot, 1994; Garcia *et al.*, 1993; Padilla-Rivas *et al.*, 2005; Karen *et al.*, 2014). Pregnancy is detected by observing enlarged uterine lumen, with a dark spherical amniotic cavity just beyond the urinary bladder and is later confirmed by the visibility of foetal heart beats. During the scanning procedure the visibility of urinary bladder serves as a landmark. However, it is possible to confuse amniotic vesicle with the urinary bladder at that early stage thus repeated examinations are required (Padilla-Rivas *et al.*, 2005). On the other hand, Moura (1993) stated that determination of the foetal gender is based on the identification of the GT relative to the location of umbilical cord attachment or tail or by identification of other external genitalia from different scanning positions. The latter also indicate that the foetus is diagnosed as a male when the GT is located immediately caudal to umbilical cord or as female when the rudimentary teats are positioned directly below the tail. For ultrasound transducer to yield desired results it should be manipulated to produce a frontal, cross-section and sagittal images of the ventral body surface of the foetus. However, the biggest challenge of foetal gender using trans-rectal ultrasound in small ruminants compared to horse and cattle is the reduced ability to manipulate the uterus during the examination and this affects the accuracy of the diagnosis (Buerstel *et al.*, 2002; Reichenbach *et al.*, 2004; Oliveira *et al.*, 2005). In addition, Buerstel *et al.* (2002); Reichenbach *et al.* (2004) and Santos *et al.* (2007a, b) suggested that for the foetal gender to be identified during the examination, repeated examinations of the same animal are required to accurately identify the foetal gender.

The main advantage of ultrasonography in veterinary practice is that repeated examinations of animal's reproductive tract can be performed without impairing its breeding potential or having adverse effect on the conceptus (Ribadu & Nakao, 1999; Simoes *et al.*, 2005; Grizelj *et al.*, 2013). The main disadvantages of using ultrasonography scanning are associated with the cost and time involved with the use of the technique. Ultrasonography machines are relatively expensive. Generally, the factors that affect the speed of ultrasonographic examinations are the proficiency of the operator, availability and restraint of the animals (Fricke, 2002; Terzano, 2012).

2.4.2. Laparoscopy

Laparoscopy is a minimally invasive surgical technique for viewing the internal structures of the abdominal cavity by means of a telescope through a small incision made in the abdominal wall (Kumar *et al.*, 2011; Patel *et al.*, 2014). Laparoscopy is a method originally used in human gynecology to diagnose various abnormalities of the genital tract and to study physiological conditions of genitalia for female sterilization. Its use in veterinary science started around 1968 to examine the genitalia of ewes (Goel and Agrawal, 1992; Patel *et al.*, 2014).

Water and feed have to be restricted from goats and sheep for at least 12 to 24 hours prior to the procedure (Kumar *et al.*, 2011; Dovenski *et al.*, 2012; Abubakar *et al.*, 2014; Patel *et al.*, 2014). Goats and sheep should be injected with about 1 ml of Xylavet 2% 3 minutes prior to the procedure as an intravenous sedative to calm them during the procedure. For examination to take place without difficulties, animals should be placed on the cradle, in dorsal recumbency (on its back) with the head tilted down (Trendelenburg position) and all four legs tied (Barry *et al.*, 1990; Kumar *et al.*, 2011; Dovenski *et al.*, 2012; Abubakar *et al.*, 2014). In addition, this position allows the gravity to pull the abdominal viscera towards the diaphragm (Patel *et al.*, 2014).

Once the animal is in the Trendelenburg position, about four fingers (7 - 10 cm) are placed anterior to the udder and 5 - 10 cm on each side of the midline (*linea alba*) (Dovenski *et al.*, 2012). This allows the removal of the hair by carbon steel surgical blade and a pair of scissors on the abdomen. Water and soap are used to clean the proposed area prior to shaving. A concentration of both F10[®]SC and alcohol is used to clean and disinfect the shaved area. After the completion of shaving, about 5 ml of Lignocaine 2% is injected on the outside of the shaven area as local anaesthetic to block the nerves along the proposed incision on both sides

to infiltrate subcutaneous and muscular tissues (Abubakar *et al.*, 2014; Patel *et al.*, 2014; Papazoglou & Basdani, 2015).

Then a number 15 scalpel blade is used to make a small incision of 5 mm on the abdominal cavity of the animal that allows the trocar and cannula to be inserted through and be pushed inside the abdominal cavity. The depth of the trocar and cannula penetration is controlled by the hand with the middle finger pointing towards the sharp end to prevent organs from being injured (Dutta *et al.*, 2010; Kumar, 2014). Limited abdominal exploration for laparoscope guided organ examination permits precise and accurate site localization of the various internal organs (Kumar *et al.*, 2011; Patel *et al.*, 2014).

Once the cannula and trocar are inside the abdominal cavity, the trocar is removed while air is blown into the abdominal cavity using a plastic tube with a valve attached to it to control air flow (Barry *et al.*, 1990; Dovenski *et al.*, 2012; Kumar *et al.*, 2011; Kumar, 2014). After the abdominal cavity has been inflated, the laparoscope with light source connected to the computer screen is inserted through the cannula. The laparoscope is then moved around to visualize the reproductive organs for counting and measuring of the ovarian structures (follicles and corpora lutea) and for pregnancy diagnosis. (Barry *et al.*, 1990; Dutta *et al.*, 2010; Dovenski *et al.*, 2012; Kumar, 2014).

When follicles and corpus luteum are not clearly visible, forceps is used to lift or move the uterus around until when the ovary is clearly visible. This also helps to keep the uterus in a stable position, since it is continuously in motion (Kumar *et al.*, 2011; Patel *et al.*, 2014). The pregnant uterus of small ruminants could be visualized by laparoscopy as having black spot that indicates the developing embryo. However, Kumar *et al.* (2011) stated that a light reddish colored gravid uterus could be clearly observed. The difference in uterine size can also be visualized during the examinations. After the removal of the laparoscope, air is deflated and the cannula is removed. The small skin wound is sutured using a non-absorbable suture material. Wound spray (antibiotic aerosol) is used to prevent infections (Kumar, 2014; Patel *et al.*, 2014).

In modern days, this technique is often performed on small ruminants such as goats and sheep for insemination, pregnancy diagnosis and embryo transfer (Goel and Agrawal, 1992; Kumar *et al.*, 2011; Nuha *et al.*, 2012; Kumar, 2014). However, in sheep and goats this technique has generated much useful information on the development and regression of the follicles, but do not allow the dynamics of the process to be evaluated fully during a complete

oestrous cycle. In addition, there is no agreement on when, during the oestrous cycle, the pattern of the follicular wave growth occurs (Noel *et al.*, 1993). Furthermore, the accuracy of laparoscopy in pregnancy diagnosis post breeding increases with gestational period. Although an experienced technician requires less time to conduct the examination, it is often difficult to observe the reproductive organs in heavy females with much omental fat (Goel and Agrawal, 1992).

Unlike ultrasonography examination, laparoscopy is not applicable in cattle because of their larger body size, handling difficulties and that the reproductive organs are situated far from reach. Thus, laparoscopic examination is only used in small ruminants such as sheep and goats. The advantages of laparoscopic procedure when compared to the exploratory method such as laparotomy, is that it allows direct visualization of genitalia of non-pregnant and pregnant females with improved animal recovery, reduced infection rate, less pain, shortened anesthetic time and reduced blood loss during operational procedures (Nuha *et al.*, 2012; Kumar, 2014; Patel *et al.*, 2014). The disadvantages of laparoscopic procedure under field conditions is that it requires time for training, specialized equipment and skilled personnel (Karadaev, 2015).

2.4.3. Laparotomy

Laparotomy is an invasive technique in which an incision is made into the abdominal wall of an animal (Abubakar *et al.*, 2014; Papazoglou & Basdani, 2015). In females, this technique is mostly used to visualize and examine the reproductive organs such as ovaries, oviduct, cervix and uterine horns in small ruminants (Abubakar *et al.*, 2014). Surgical exploration provides information through direct inspection and palpation of reproductive organs and also hollow organ luminal mucosa observations (Goel and Agrawal, 1992; Papazoglou & Basdani, 2015).

Prior to examination, water and feed have to be restricted from goats and sheep for at least 12 to 24 hours prior to the procedure (Dovenski *et al.*, 2012; Abubakar *et al.*, 2014; Patel *et al.*, 2014). Goats and sheep should be injected with about 1 ml of Xylavet 2% 3 minutes prior to the procedure as intravenous sedative to calm them during the procedure. For examination to take place without difficulties, the animals are subjected to positioning and procedures as described for laparoscopy. However, after the completion of shaving, about 10 ml of Lignocaine 2% is injected as local anaesthetic to block the nerves along the proposed incision on both

sides to infiltrate subcutaneous and muscular tissues (Abubakar *et al.*, 2014; Patel *et al.*, 2014; Papazoglou & Basdani, 2015).

In laparotomy, an incision of about 5 to 10 cm which is long enough to allow four fingers to enter the abdominal cavity is made (Gonzalez de Bulnes *et al.*, 1999; Papazoglou & Basdani, 2015). This incision has a potential to result in post-surgical secondary effects. Using fingers, the reproductive organs are lifted above the cavity for direct observation unlike ultrasonography and laparoscopic examinations. During the observations, the reproductive organs are continually lubricated with phosphate buffered saline (PBS) for them not to become dry (Abubakar *et al.*, 2014). In this method, measurements of the reproductive organs and counting of ovarian structures such as follicles and corpora lutea is much easier. On completion of the examination of the reproductive organs, catgut is used to suture the muscles while a non-absorbable suture material is used to suture the skin.

The use of laparotomy as an invasive surgical procedure for observing reproductive organs, have considerable welfare implications if the animals are subjected to general anaesthesia or deep sedation (Simoes *et al.*, 2005; Grizelj *et al.*, 2013; Gurjar *et al.*, 2014). Moreover, this technique cannot be used for repeated examination of reproductive organs during oestrous cycle as it could lead to development of infection if animals are not closely monitored post examination (Gonzalez de Bulnes *et al.*, 1999; Gurjar *et al.*, 2014). Laparotomy cannot be conducted in monitoring the reproductive organs of cows due to their larger body size, difficulties in handling and that their reproductive organs are far from reach. However, laparotomy could be used for pregnancy diagnosis in small ruminants and its accuracy increases with gestational period. Although this method is quite accurate in diagnosing pregnancy, it could also result in animals being subjected to stress which might lead to abortion and possibly reduction of the fertility of the operated animals (Goel and Agrawal, 1992). Under field conditions Laparotomy procedure requires time for training, specialized equipment and skilled personnel (Karadaev, 2015).

2.4.4. Rectal palpation

Palpation of the uterine content through the rectum is probably the most commonly used traditional method for diagnosing pregnancy in cows (Kastellic *et al.*, 1988; Romano *et al.*, 2006; Whittier, 2013; Ashock *et al.*, 2013) than in small ruminants. This is because small ruminants have a smaller rectum that cannot allow the hands to go through. In addition, trans-rectal

palpation for monitoring the ovarian structures may result in incorrect diagnosis of the CL and follicles when compared to trans-rectal ultrasonography (Beal *et al.*, 1992; Hanzen *et al.*, 2000).

During palpation, several structures that are palpable within the uterus are indications of pregnancy. The structures within the reproductive tract of cows allow the trained operator to detect the characteristics of the tract that assist to determine either pregnancy or non-pregnancy. The signs such as the displacement of the chorio-allantoic membrane, different sizes of the uterine horns, palpation of the amniotic vesicle, palpation of the foetus and palpation of the placentomes (cotyledon and caruncle) in the wall of the pregnant uterus confirm pregnancy (Kastellic *et al.*, 1988; Romano *et al.*, 2006; Whittier, 2013; Ashock *et al.*, 2013).

Rectal palpation has the advantages of being accurate, fast and a relatively cheap traditional method that is less labour intensive and that has ability to allow an estimation of the pregnancy stage (Whittier, 2013; Ashock *et al.*, 2013). Nonetheless, training is necessary and examination should be conducted by a qualified veterinarian or by an experienced herdsman or technician. The main disadvantage of conducting rectal palpation is that it is performed later in the gestational period as compared to trans-rectal ultrasonography scanning and other methods of pregnancy diagnosis. Although trans-rectal palpation is the cheapest method of pregnancy diagnosis, it could cause the risks of embryonic loss if cows are examined earlier in the gestation (Ashock *et al.*, 2013; Ott *et al.*, 2014).

Table 3 summarizes the foetal size and characteristics used in determining pregnancy through palpation per rectum during various stages of gestation in cows. In this test, pregnancy status of each cow was determined by per-rectal palpation 70 days after AI. Projected gestation length was determined based on days of gestation and projected calving date (Whittier, 2013). In the Table 3 below, measurements of weight were converted from ounces (oz.) and pounds (lb) to gram (g) and kilogram (kg) and inches were converted to centimeter (cm).

Table 2: Foetal size and characteristics used in determining pregnancy by rectal palpation

Foetal Size				
Stage	Days of Gestation	Weight	Length (cm)	Identifying Characteristics
I	30	0.28g	1.00	One uterine horn slightly enlarged and thin; embryonic vesicle size of small marble. Uterus in approximate position of non-pregnant uterus. Foetal membranes may be slipped between fingers from 30 to 90 days.
	45	3.54-7.09g	2.54-1.81	Pregnant uterine horn somewhat enlarged, thinner walled and prominent. Embryonic vesicle size of small egg.
	60	7.09-14.17g	7.88	Pregnant uterine horn 7.88 to 13.19 cm in diameter; fluid filled. Foetus size of mouse.
	90	85.05-170.10g	12.7-15.24	Both uterine horns are swollen (10.16 to 12.7 cm in diameter). Foetus is size of rat. Uterine artery 0.32 to 0.48 cm in diameter. Cotyledons 1.91 to 2.54 cm across, but very soft.
II	120	0.45-0.91kg	25.4-30.48	Similar to 90-day but foetus more easily palpated. Foetus is size of small cat with head the size of a lemon. Uterine artery 0.64 cm in diameter. More noticeable and 3.27 cm in length. Horns are 12.7 to 17.78 cm in diameter.
	150	1.81-2.72kg	30.48-40.64	Difficult to palpate foetus. Uterine horns are deep body cavity with foetus size of large cat – horns 167.64 cm in diameter. Uterine artery 0.64 to 0.95 cm and

III	180	4.54-7.26kg	50.80-60.96	Cotyledons 5.08 to 7.88 cm in diameter. Horns with foetus still out of reach. Foetus size of dog. Uterine artery 0.95 to 1.27 cm in diameter. Cotyledons more enlarged. From sixth month until a movement of foetus may be elicited by grasping feet, legs or nose.
	210	9.07-13.61kg	60.96-81.28	From 7 months until parturition foetus may be felt. Age is largely determined by increase in foetus size. The uterine artery continues to increase in size at 210 days, 1.27 cm in diameter; at 240 days, 1.27 to 1.59 cm diameter; at 270 days, 1.27 to 1.91 cm in diameter.
	240	18.14-27.21kg	71.12-91.44	
	270	27.21-45.36kg	71.12-96.52	

Whittier, 2013.

2.4.5. Non-return to oestrus

The oestrus period of animals is known as the period of sexual receptivity. The length of the oestrous cycle in does and cows is 21 days; while in ewes is 17 days, depending on the breed (Hafez & Hafez, 2000). Non-return to oestrus post breeding or insemination is one of the signs of pregnancy in farm animals, especially in rural farming, where there is unavailability of infrastructure. Animals will return to oestrus as a result of failure to conceive, while those that do not return to oestrus may have conceived. However, it is important to note that it is not always the case that animals that do not return to oestrus are pregnant. The non-return to oestrus could be caused by other factors that influence the fertility of farm animals such as lactation stress, environmental conditions, nutrition and diseases like metritis or endometritis (Goel and Agrawal, 1992).

Non-return to oestrus following breeding may be suggestive of pregnancy, but pathological conditions of uterus or ovaries; physiological anoestrus late in the breeding season and being out of breeding season may cause anoestrus in non-pregnant ewes and does (Ishwar, 1995). Non-return to oestrus is a reliable tool for pregnancy diagnosis in cows, but not

ewes under farm conditions, because cows are known to be non-seasonal breeders, whereas some breeds of does and ewes are seasonal breeders (Goel and Agrawal, 1992).

Ishwar (1995) argued that under commercial livestock farming, traditional methods of pregnancy diagnosis such as visual observation, abdominal palpation, service records and non-return to oestrus are not reliable means of diagnosing early pregnancy. The latter study also mentioned that non-return to oestrus is an unreliable method when ewes or does are synchronized and bred during the non-breeding season. Just like rectal palpation in cows, non-return to oestrus as a tool of pregnancy diagnosis is mostly recommended for traditional farm owners that do not have facilities for performing pregnancy diagnosis (Goel and Agrawal, 1992) with techniques such as trans-rectal ultrasonography and laparoscopy.

2.5. Efficiency of ultrasonography in monitoring reproductive organs, pregnancy diagnosis and early foetal gender identification

Because of impediments in the extensive livestock sector where the facilities are not suitable for application of other techniques (laparotomy and laparoscopy), the application of ultrasonography is more practical, applicable and feasible for the purpose of monitoring of ovarian structures, pregnancy diagnosis and foetal gender identification. The use of ultrasonography as a tool for monitoring the ovarian structures has been demonstrated in various species of domestic farm animals such as cattle (Carroll *et al.*, 1990; Ginther *et al.*, 1996; Wiltbank *et al.*, 2002), sheep (Baird *et al.*, 1991; Bartlewski *et al.*, 1999a, b, c; Gibbons *et al.*, 1999) and goats (Ginther & Kot, 1994; Evans, 2003; Medan *et al.*, 2003). Among the examined aspects were detection of ovulation rate (Gibbons *et al.*, 1999; Simoes *et al.*, 2006; Cox *et al.*, 2015), presence of non-ovulatory and ovulatory follicles (Ginther *et al.*, 1996; Orita *et al.*, 2000), pre-ovulatory follicles (Camp *et al.*, 1983; DrianCourt, 2001; Fatet *et al.*, 2011), presence of larger follicles (de Castro *et al.*, 1999; Wiltbank *et al.*, 2002; Medan *et al.*, 2003) and the CL development (de Castro *et al.*, 1999; Orita *et al.*, 2000; Cox *et al.*, 2015) during the oestrous cycle of ruminants.

Simoes *et al.* (2005) evaluated the accuracy of ultrasonography by comparing with only a referral method such as laparoscopy, laparotomy or slicing in small ruminants. Simoes *et al.* (2005) stated that in all the ovaries observed the total number of follicles counted per ovary was lower with ultrasonography (7.7 ± 2.5) than with slicing (12.7 ± 4.7) methods. However, when the latter classified follicle size by category, only a significant difference in < 2 mm category

persisted between those methods and no significant differences were found between all the other size categories. The correlation coefficient between ultrasonography and slicing method of the ovary for the number of follicles greater or equal to 3 mm was higher (Simoes *et al.*, 2005). However, Camp *et al.* (1983) and Noel *et al.* (1993) used laparoscopic examination to evaluate the ovarian activities on their studies. Noel *et al.* (1993) found that the mean number of small, medium-sized and large follicles did not vary significantly between the waves. Moreover, Grizelj *et al.* (2013) indicated that the relationship between the number of CL and follicles obtained by ultrasonography was higher than those counted by laparotomy.

The use of ultrasonography as a tool for pregnancy diagnosis has been demonstrated in various species of domestic farm animals such as cattle (Kastelic *et al.*, 1989; Fricke, 2002; Broaddus & de Vries, 2005; Romano *et al.*, 2006;), sheep (Garcia *et al.*, 1993; Karen *et al.*, 2001; Karen *et al.*, 2004; Romano & Christians, 2008; Ferrari *et al.*, 2009) and goats (Martinez *et al.*, 1998; Doize *et al.*, 1997; Amer, 2010; Karen *et al.*, 2014; Raja-Khalif *et al.*, 2014). Ultrasonography examination has been reported to detect pregnancy as early as day 9 in cattle (Whittier, 2013), day 12 in sheep (Karen *et al.*, 2001; Ferrari *et al.*, 2009) and day 19 in goats (Padilla-Rivas *et al.*, 2005; Amer, 2010; Suguna *et al.*, 2008) into gestation. Other reports have, however disputed those claims and emphasized that the accuracy of ultrasonography examination in pregnancy diagnosis between day 9 and 16 is not significantly better than a random guess (<50%) (Kastelic *et al.*, 1989; Garcia *et al.*, 1993; Romano & Christians, 2008). However, the accuracy of pregnancy diagnosis increased significantly from day 18 of gestation onwards and could be explained by the rapid increase in the volume of the embryonic vesicle, and caruncles development and heartbeat at a later stage (Romano *et al.*, 2006; Romano & Christians, 2008; Ferrari *et al.*, 2009; Karen *et al.*, 2014).

Foetal gender identification is one of the modern trends in reproduction science (Yotov *et al.*, 2011). Early foetus gender diagnosis by ultrasonography method is both accurate and safe (Ali, 2004; Tainturier *et al.*, 2004; Quintela *et al.*, 2011). However, the factors with the highest impact over the method's accuracy are the gestational age, position of the foetus, foetus gender, type and breed of the animal under investigation (Ali, 2004; Yotov *et al.*, 2011). In small ruminants, Santos *et al.* (2007a, b) attributed the impact over the ultrasonography accuracy to the decreased ability to manipulate the uterus during examination. It is also important to note the difference in total number of animals used during the examination and a period of foetal gender identification by ultrasonography.

During ultrasonography examination for foetal gender identification, the success rate (accuracy) is significantly higher (> 60 - 100%) after day 60 of gestation since the GT has reached its final position and the differences between male and female foetus are clear (Ali, 2004; Tainturier *et al.*, 2004, Santos *et al.*, 2007a; Dias *et al.*, 2009; Moraes *et al.*, 2009; Yotov *et al.*, 2011). Yotov *et al.* (2011) indicated that the highest number of unidentified gender could be detected between days 28 and 63 of gestation. In small ruminants, the accuracy of ultrasonography in determining single foetus, twin foetuses and triplet foetuses between days 40 and 60 is significantly higher (100%) as explained by Santos *et al.* (2007a, b). Accuracy of determining the female foetus and male foetuses varies from study to study (Beal *et al.*, 1992; Coubrough & Castell 1998; Dias *et al.*, 2009; Quintela *et al.*, 2011) as compared after birth. Many studies have been conducted to determine the specific period for foetal gender diagnosis, however, little has been done to determine the accuracy of ultrasonography method for gender diagnosis.

CHAPTER 3

METHODOLOGY

3.1. Study site

The study was conducted at Mara Research Station and University of Venda in the Limpopo Province of South Africa. Mara Research Station is located \approx 54 km west of Makhado town in the arid sweet bush-veld area. The annual rainfall is 452 mm with summer temperatures of 25.7 °C – 30.5 °C and winter temperatures of 10 °C – 22.6 °C (Acocks, 1998). University of Venda is located at Thohoyandou which is \approx 70 km east of Makhado within a subtropical climate region with the summer temperatures of 22.7 °C – 35.5 °C and winter temperatures of 10 °C – 24 °C. The annual rainfall is 650 mm (Niederwieser, 2004).

3.2. Animals used and their management

Animals that were used in this study were five Boer goats (does) (2 to 4 years old), five White Persian sheep (ewes) (2 to 4 years old) and five Nguni cattle (cows) (2 to 4 years old). Does and ewes were kept at the University of Venda experimental farm and cows were kept at Mara Research Station. Animals at the University of Venda experimental farm were provided with a balanced diet daily while animals at Mara Research Station were kept extensively with supplementary feeding provided. Water was provided *ad libitum* for animals. The health of the animals was monitored by conducting physical examination observation on a daily basis. All animals were not starved prior to ultrasonography (ULTS) scanning examinations, however, on the days that laparoscopy (LAPSC) and laparotomy (LAPT) were conducted, does and ewes were starved for 16 to 24 hours prior to the examination.

3.3. Synchronization and breeding of does, ewes and cows

Prior to pregnancy diagnosis the estrous of all study animals were synchronized in preparation for artificial insemination (AI). The process of synchronisation included the following activities; restricting of animals to a standing position. The area around the vulva was cleaned thoroughly with water and paper towel. Does, ewes and cows were synchronized using CIDR (Eazi-Breed[®] CIDR, Pfizer Animal Health, Auckland, New Zealand). A CIDR is a T-shaped nylon insert molded with a silicon rubber skin containing 0.3 g progesterone. The wings of the CIDR were folded together towards the front for intra vaginal insertion using the applicator. After

insertion of the CIDR, the wings of the CIDR returned to their original T-shape and applied pressure to the vaginal walls and therefore, holding the CIDR in place. The blue nylon string (tail) of CIDR was cut to prevent does, ewes and cows from pulling their own or another's CIDR from the vagina with their mouths.

The CIDRs were gently removed after 7 days. At removal of the CIDR, does, ewes and cows were injected 10 mg of PGF_{2α} (Lutalyse[®] dinoprost tromethamine) Sterile Solution. At 24 hours after removal of the CIDR, does, ewes and cows were injected in the muscles with 300 IU of Equine chorionic gonadotrophin (Folligon[®], 1000IU) [MSD Animal Health. INTERVET.SA. (Pty) Ltd] to synchronize the follicular development and ovulation. Then they were observed for signs of standing heat after 10 hours and they were inseminated 12 hours after being confirmed to be in standing heat.

3.4. Techniques used for examinations

3.4.1. Ultrasonography method

The reproductive organs of the animal, pregnancy diagnosis and gender of the foetus were examined using an A5 Sonoscope A-mode ultrasound (Sonoscope Co Ltd, Shenzhen P.R China, 50 - 60 Hz) connected to a L561V linear array 3.0 - 8.0 MHz sonoscope trans-rectal transducer. During trans-rectal ultrasonography application, animals were restrained in their standing position (Ginther & Kot, 1994; Simoes *et al.*, 2005; Karen *et al.*, 2014). Before the probe was inserted through the rectum, the faeces in the rectum were removed to allow clear visibility of the organs. The probe was lubricated with water mixed with cooking oil and was inserted into the rectum positioned above the uterus. In order to find the reproductive organs, the probe was inserted with the scanning section pointing downwards to observe the bladder and uterine horns in a longitudinal direction. After the probe was inserted into the rectum, it was rotated from the 3 o'clock position through the 6 o'clock position to the 9 o'clock position to scan the entire area below the probe (Ginther & Kot, 1994; Simoes *et al.*, 2005; Karen *et al.*, 2014). This procedure allowed the structures under observation to be fully visible on the scanner screen. In addition, this technique enabled a quicker ultrasonographic examination in small ruminants, because, usually it is not necessary to remove the faecal material (Simoes *et al.*, 2005) as compared to cattle.

3.4.2. Laparoscopic method

Reproductive organs of the animal and pregnancy diagnosis were examined using a Sony Olympus Model OTV-S5C and CLH-250 (Olympus Optical Co., Ltd. Made in Japan) connected to a camera transducer Olympus A50374A Germany. Water and feed were restricted from does and ewes for at least 16 to 24 hours prior to the procedure (Dovenski *et al.*, 2012; Abubakar *et al.*, 2014). Animals were injected with about 20 mg of xylazine (Xylavet 2%), 3 minutes prior to the procedure as sedative in the jugular vein to sedate them during the procedure. Each doe and ewe was placed on the cradle, in dorsal recumbency (on its back) with the head tilted down (Trendelenburg position) and all four legs tied (Barry *et al.*, 1990; Abubakar *et al.*, 2014; Patel *et al.*, 2014). In addition, this position allowed the gravity to pull the abdominal viscera towards the diaphragm (Patel *et al.*, 2014).

Once the animal was in the Trendelenburg position, about four fingers of the examiner (\approx 7 cm) were placed in front of the udder and about 5 cm on each side of the midline (*linea alba*) (Dovenski *et al.*, 2012). Upon the completion of the measurement of the possible area to be shaved, the examiner's fingers were removed. Water and soap were used to clean the proposed area prior to shaving. This allowed the removal of the hair by carbon steel surgical blade. Concentrations of both F10[®]SC and alcohol were used to clean and disinfect the shaved area. After the completion of shaving, about 5 ml of Lignocaine 2% injection was injected as local anaesthetic on both sides about 5 cm from the midline to block the nerves on the lateral side of the proposed incision to infiltrate subcutaneous and muscular tissues (Abubakar *et al.*, 2014; Papazoglou & Basdani, 2015).

A number 15 scalpel blade was used to make a small incision of 0.5 cm through the skin on the abdominal cavity of the animal that allowed the trocar and cannula to be inserted through and be pushed into the abdominal cavity. The depth of the trocar and cannula penetration was controlled by the middle finger of the examiner's hand holding the cannula pointing towards the sharp end to prevent organs from being injured (Dutta *et al.*, 2010). Limited abdominal exploration for laparoscope guided organ examination permitted precise and accurate site localization of the various internal organs (Kumar *et al.*, 2011; Patel *et al.*, 2014).

Once the trocar and cannula were inside the abdominal cavity, the trocar was removed. Air was blown into the abdominal cavity using a plastic tube with a valve attached to it to control air flow. After the abdominal cavity was inflated, the laparoscope with light source connected to the computer screen was inserted through the cannula. The laparoscope was then moved

around to visualize the reproductive organs for counting and measurement of the ovarian structures and for pregnancy diagnosis.

When the follicles and corpus luteum were not clearly visible, the procedure that was conducted in the first incision as mentioned above was followed during second incision. The second incision was used to insert a forceps through a cannula that was used to lift or move the uterus around until when the ovaries were clearly visible. This also helped to keep the uterus in a stable position, since it was continuously in motion. The ovary size, number of follicles, size of follicles and pregnancy (visualized as a black spot in the uterine horn) were observed. When completed the laparoscope was removed, air was deflated and the cannulas were removed. The small skin wound was sutured using A non-absorbable suture material. Wound spray (antibiotic aerosol) was sprayed over the wound to prevent infections.

3.4.3. Laparotomy method

The preparatory steps of laparotomy are similar to those of laparoscopy except for the amount of Lignocaine injected and also the size and position of incision. During laparotomy examinations about 10 ml of Lignocaine 2% injection was injected as local anaesthetic on both sides of the midline to block the nerves along the proposed incision to infiltrate subcutaneous and muscular tissues (Abubakar *et al.*, 2014; Papazoglou & Basdani, 2015).

During the examination the incision was done for about 7 cm from the udder by a number 15 scalpel blade on the ventral midline of the abdominal cavity which was long enough to allow four fingers of the examiner to enter the abdominal cavity (Gonzalez de Bulnes *et al.*, 1999; Papazoglou & Basdani, 2015). Once the fingers were inside, reproductive organs were lifted above the cavity for clear direct observation unlike ultrasonography and laparoscopic examinations. During observations, reproductive organs were continuously kept wet with phosphate buffered saline (PBS) to prevent drying (Abubakar *et al.*, 2014). Measurements of reproductive organs and counting of ovarian structures were performed. In completion of the examination catgut was used to suture the muscles while a non-absorbable suture material was used to close the skin. Animal was injected with 10 ml of Duplocillin, a long acting penicillin antibiotic. Wound spray (antibiotic aerosol) was used on the incision to prevent infections. The animal was then lifted down from the cradle. Animals were placed in clean and calm environment and were observed until they stand on all four legs. Duplocilin was administered in three days interval after examination until full recovery of animals.

3.5. Data collection

3.5.1. Follicular dynamics

Ultrasonography technique was used to monitor the ovarian structures development for 17 days in sheep and for 21 days in goats and cattle. Follicles of diameter ≥ 3 mm were selected for analysis of growth, ovulation and regression. Follicular monitoring was achieved by counting of all the follicles visible during examination on each day. For follicular wave development patterns the average of the largest and second largest follicle observed were recorded daily. Throughout the cycle of goats and sheep the number and size of the follicles were considered to be small between 3 - 4.9 mm, medium 5 - 7.9 mm and large at ≥ 8 mm. In cattle, the number and size of the follicles were considered to be small between 3 - 4.9 mm, medium 5 - 9.9 mm and large at ≥ 10 mm in diameter. The dominant follicles of the waves were outlined as the follicles that exceeded ≥ 5 mm in goats and sheep, ≥ 10 mm in cattle and were the largest compared to other follicles of each wave when measured. The day of ovulation was marked as day 0. Regression of a dominant follicle of the last wave was the beginning of new oestrous cycle. For counting number and measurement of the follicles with ease, the ultrasonography scanner screen was frozen then follicular diameters were measured using the millimeter scale.

3.5.2. Ovarian observations by different methods

Does, ewes and cows were used for monitoring of ovarian structures (follicles and corpus luteum) and for ovarian shape observation using ultrasonography (ULTS), laparoscopy (LAPSC) and laparotomy (LAPT) techniques, where applicable, respectively. ULTS method was used in all three species, whereas LAPSC and LAPT techniques were used in does and ewes. Data on follicular diameters, ovarian size and shape were recorded for each observed ovary (right and left). LAPSC and LAPT examinations were conducted on day 3 and day 10 in does and ewes. Due to poor recovery from previous examinations, LAPSC and LAPT were not continued for third and fourth examinations.

3.5.3. Pregnancy diagnosis

Early pregnancy diagnosis by ultrasonography was initiated from day 18 post artificial insemination. The examinations were repeated after every 2 days until the day pregnancy was confirmed. Foetal heartbeat was used as a conclusive confirmation of pregnancy and this was

when a positive diagnosis was recorded. LAPSC examinations were conducted on the day that the foetal heartbeat was visualized in each doe and ewe. In addition, a single LAPSC examination was used to reduce chances of animals being stressed and aborting.

3.5.4. Foetal gender identification

Foetal sexing was conducted from day 40 of pregnancy by ultrasonography technique until day the gender of the foetus was confirmed and recorded. The examinations were repeated every 5 days. Foetal gender was determined by observing the genital tubercle (GT) (scrotal bag and rudimentary teats). The final position of GT in both male and female foetuses was used as a conclusive confirmation of foetal gender. The foetus was reported as a male when the scrotum was clearly visible between the hind legs and as a female when the rudimentary teats were clearly observed. The correctness of ULTS for gender identification of each foetus was confirmed after birth.

3.6. Statistical analysis

3.6.1. Follicular dynamics

Data on mean follicle size on specific days of the oestrus cycle were plotted using Microsoft excel (Microsoft, 2010). The analysis of variance was applied to test for difference in follicular wave parameters (emergence, first day of dominant follicle emergence, and maximum size of dominant follicles and duration of follicular waves) among the three waves in goats, sheep and cattle. Resulting means (Means \pm SEM) were separated using LSD where significant difference was found. Days in oestrus and average diameter of two largest follicles were plotted to depict the wave pattern.

The model for analysis of variance for comparison of three waves was as follows:

$$Y_i = \mu + M_j + e_{ij}$$

Where:

Y = Observation of i^{th} trait (emergence, first day of dominant follicle emergence, maximum size of dominant follicles and duration of follicular waves)

μ = Mean of observations

M = Fixed effect of the j^{th} wave (waves first, second & third)

e = Residual variance

3.6.2. Efficiency of ultrasound

To compare ultrasound and the other two techniques (laparoscopy and laparotomy) data on mean number of follicles (in their respective categories) were subjected to analysis of variance (ANOVA) using SAS 2004 software package (SAS software, version 9.1.3. 2004. SAS Institute Inc., Cary, NC, USA). The means of ultrasonography measured parameters were compared to those of laparotomy and laparoscopy for verification of their accuracy, where applicable. The means comparison was of the following categories by size: 3 - 4.9 mm, 5 - 7.9 mm and ≥ 8 mm in goats and sheep. The mean of cattle measurement was not compared, but were arranged in categories by size: 3 - 4.9 mm, 5 - 9.9 mm and ≥ 10 mm. Least significant difference (LSD) was used to separate means at $P < 0.05$ probability level. Where no significant difference of mean number of follicles per category was found, it would mean that the three methods were measuring the same thing hence equally efficient.

Pregnancy diagnosis was analyzed as percentages for identification of the day in which pregnancy can be detected using ultrasonography verified by laparoscopy. Foetal gender identification was analyzed for percentages for identification of differences between observed proportions (genital tubercle, scrotum and rudimentary teats) using ultrasonography.

The model for analysis of variance for comparison of ULTS and LAPSC and LAPT was follows:

$$Y_i = \mu + M_j + e_{ij}$$

Where:

Y = Observation of i^{th} trait (mean follicle number)

μ = Mean of observations

M = Fixed effect of the j^{th} method of monitoring/ measurement

e = Residual variance

CHAPTER 4

RESULTS

4.1. Follicular dynamics

Follicular development was accompanied by the occurrence of waves of follicular growth starting at different periods of the oestrous cycle with first and second waves occurring during the luteal phase and third wave occurring during the follicular phase. All follicles that developed during the luteal phase atrophied, while during the follicular phase one or two follicles ovulated. The third wave of the oestrous cycle was named as the ovulatory wave of the cycle. The patterns of follicular waves were marked by the development of follicles with a diameter of ≥ 3 mm to 5 mm then followed by selection, development and atresia of the subordinate and dominant follicles. The number of follicles with a diameter of ≥ 3 mm in three follicular waves of the cycle that appeared daily differed per day ($p < 0.05$). During the examination by ultrasonography method about 3 to 10 follicles could be observed at any given day of the cycle in goats, sheep and cattle. The follicular patterns of three follicular waves in goats, sheep and cattle throughout the cycle are shown in Figure 2, 3 and 4, respectively.

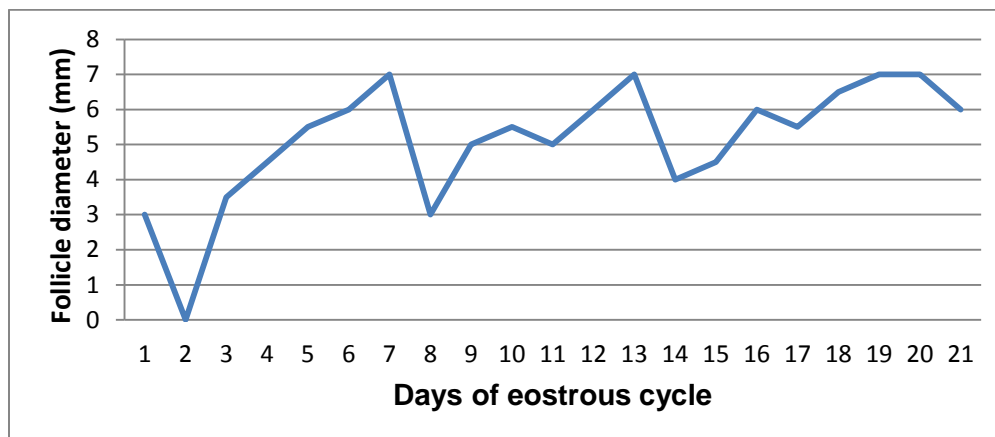


Figure 2: The pattern of three follicular waves observed in Boer goats throughout the oestrous cycle

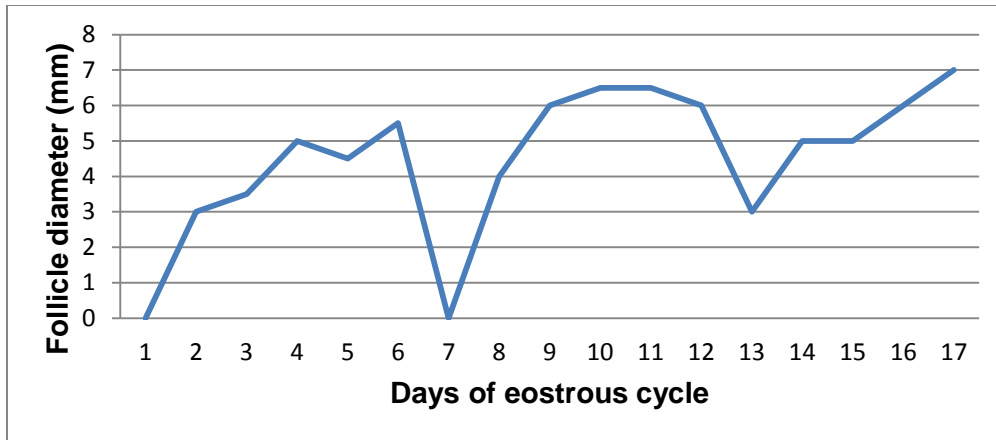


Figure 3: The pattern of three follicular waves observed in White Persian sheep throughout the oestrous cycle

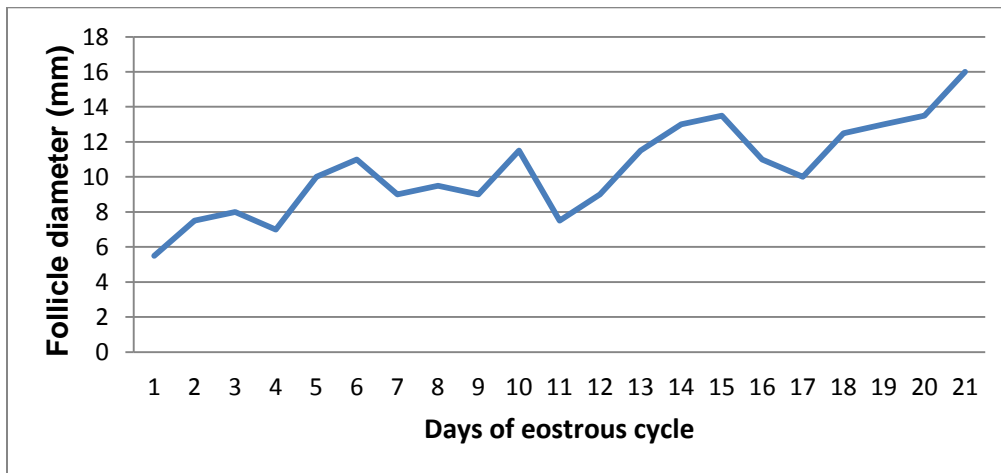


Figure 4: The pattern of three follicular waves observed in Nguni cattle throughout the oestrous cycle

In Boer goats, the mean (Mean \pm SEM) days of the follicular wave emergence after the oestrus cycle were 1.0 ± 0.4 , 8.0 ± 0.8 and 14.8 ± 0.8 for wave one, two and three, respectively. The dominant follicles of the three follicular waves observed attained similar maximum size throughout the cycle, but the dominant follicle of wave one and wave two regressed. The duration of the waves were not significantly different ($p > 0.05$) throughout the cycle, but the third wave was quantitatively longer than both first and second waves. The differences in follicular wave parameters in Boer goats with three follicular waves are shown in Table 3.

Table 3: Day of follicular wave emergence, day of dominant follicles emergence and duration of follicular waves in Boer goats

Number of waves per cycle	Wave	Follicular wave emergence (day/s) after oestrus	Dominant follicles emergence (day/s) after oestrus	Maximum dominant follicle size (mm)	Duration of the follicular waves (day/s)
Three	First	1.0 ± 0.4	2.6 ± 0.4	7.3 ± 0.4	6.2 ± 0.6
	Second	8.0 ± 0.8	9.2 ± 0.6	6.6 ± 0.2	5.0 ± 0.4
	Third	14.8 ± 0.8	17.2 ± 0.4	7.3 ± 0.8	6.4 ± 0.8

In White Persian sheep, the mean days of the follicular wave occurrence after the oestrus cycle were 1.2 ± 0.4 , 6.4 ± 0.2 and 12.6 ± 0.4 in the first, second and third waves, respectively. The onset of the dominant follicle of the first, second and third waves were 2.0 ± 0.8 , 7.8 ± 0.6 and 13.8 ± 0.8 , respectively. There was no significant difference ($p > 0.05$) between the maximum dominant follicles of the three waves observed. The length of the waves was not significantly different ($p > 0.05$) throughout the cycle, but first wave was shorter than the second and third waves of the cycle. The differences in follicular wave parameters in White Persian sheep with three follicular waves are shown in Table 4.

Table 4: Day of follicular wave emergence, day of dominant follicles emergence and duration of follicular waves in White Persian sheep

Number of waves per cycle	Wave	Follicular wave emergence (day/s) after oestrus cycle	Dominant follicles emergence (day/s) after oestrus	Maximum dominant follicle size (mm)	Duration of the follicular waves (day/s)
Three	First	1.2 ± 0.4	2.0 ± 0.8	6.4 ± 0.4	4.6 ± 0.2
	Second	6.4 ± 0.2	7.8 ± 0.6	6.6 ± 0.4	5.2 ± 0.2
	Third	12.6 ± 0.4	13.8 ± 0.8	6.7 ± 0.7	5.2 ± 0.4

In Nguni cattle, the first follicular wave and selection of the dominant follicle occurred on day 2.2 ± 0.4 and day 2.8 ± 0.8 . The dominant follicle of wave one attained the maximum diameter of 13.1 ± 0.8 mm. The second wave emerged on day 10.6 ± 0.8 and reached maximum diameter of 14.2 ± 0.6 mm, whereas the third wave appeared on day 16.2 ± 0.8 with the selection of dominant follicle occurring on day 16.6 ± 0.4 . There was no significant difference ($p > 0.05$) between the dominant follicles of the three waves observed. However, the size of the dominant follicle of the ovulatory wave was larger than the dominant follicles of both first and second waves and this did not change throughout the cycle. Third (ovulatory) wave was shorter ($p < 0.05$) than the duration of the first and second waves, whereas first wave was

the longest wave of the cycle. The differences in follicular wave parameters in Nguni cattle with three follicular waves are shown in Table 5.

Table 5: Day of follicular wave emergence, day of dominant follicles emergence and duration of follicular waves in Nguni cattle

Number of waves per cycle	Wave	Follicular wave emergence (day/s) after oestrus cycle	Dominant follicles emergence (day/s) after oestrus	Maximum dominant follicle size (mm)	Duration of the follicular waves (day/s)
Three	First	2.2 ± 0.4	2.8 ± 0.8	13.1 ± 0.8	6.4 ± 0.4
	Second	10.6 ± 0.8	12.2 ± 0.8	14.2 ± 0.6	6.2 ± 0.2
	Third	16.2 ± 0.8	16.6 ± 0.4	15.7 ± 0.6	4.8 ± 0.2

4.2. Follicles observations by different methods

To measure the accuracy of trans-rectal ultrasonography (ULTS) technique in the observation of the follicular development, ULTS observation data was compared to that of laparoscopy (LAPSC) and laparotomy (LAPT) which were considered as the reference methods. The comparison was done for Boer goats and White Persian sheep. In all animals under study, the shape of each ovary and follicles on right and left ovaries were clearly and repeatedly identifiable when using ULTS, LAPSC and LAPT, where applicable. The ovaries of goats, sheep and cattle appeared to be almond-shaped. Typical images of the ovary shape and follicles observed by different methods in goats, sheep and cattle are presented in Figure 5, 6 and 7. In both goats and sheep, there was no statistical difference ($p > 0.05$) when observing the ovarian size (length vs. breath) by ULTS, LAPSC and LAPT (Table 6 and 7). The mean size observed by LAPT also reflects the accuracy of the actual size of the ovaries which may be affected by the resolutions of ULTS scanner and LAPS camera during examinations. In this regard, ULTS was shown to operate just as good as the reference methods in observing the size of the ovaries. The results of ULTS during the observation of the ovarian size in cattle are presented in Table 8.

Table 6: Mean ovary size of Boer goats studied using different methods on day 3 and day 10 of observations

Methods	Right ovary size (mm)		Left ovary size (mm)	
	Length	Breath	Length	Breath
ULTS	15.60	11.10	14.00	10.50
LAPSC	15.50	11.50	14.90	11.70
LAPT	14.70	12.20	15.50	11.80

Table 7: Mean ovary size of White Persian sheep studied using different methods on day 3 and day 10 of observations

Methods	Right ovary size (mm)		Left ovary size (mm)	
	Length	Breath	Length	Breath
ULTS	17.90	13.80	18.40	15.20
LAPSC	17.40	14.90	18.50	14.80
LAPT	17.50	13.30	16.80	12.50

Table 8: Mean ovary size of Nguni cattle studied using ULTS method on day 3 and day 10 of observations

Methods	Right ovary size (mm)		Left ovary size (mm)	
	Length	Breath	Length	Breath
ULTS	16.90	14.80	17.40	15.10



Figure 5: Ultrasonography images of the ovaries with follicles observed on day 10 of the oestrous cycle in (a) Boer goats, (b) White Persian sheep and (c) Nguni cattle

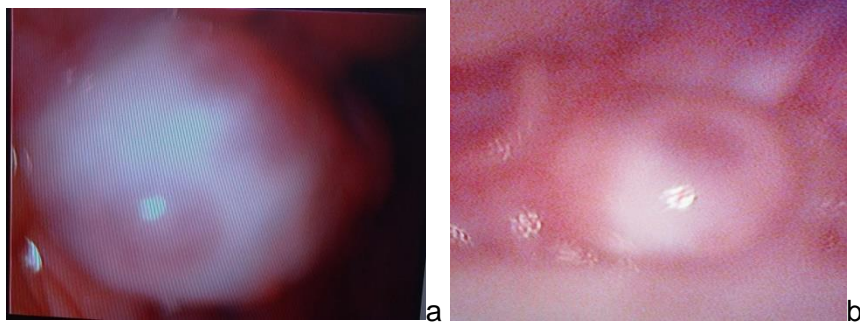


Figure 6: Laparoscopic images of the ovaries with follicles observed on day 10 of the oestrous cycle in (a) Boer goats and (b) White Persian sheep

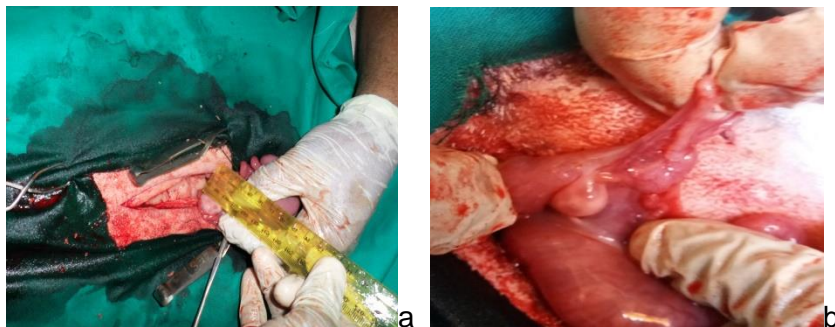


Figure 7: Laparotomy images of the ovaries with follicles observed on day 10 of the oestrous cycle in (a) Boer goats and (b) White Persian sheep

In goats, the mean numbers of follicles in the observed ovaries in all the categories on both day 3 and day 10 combined are presented in Table 9. In the right ovaries, the observed total number of follicles counted under category 3 - 4.9 mm was lower with ULTS and LAPSC than with LAPT method ($p < 0.05$). However, there was no significant difference ($p > 0.05$) when observing follicles with a diameter of ≥ 8 mm by all three methods. The mean numbers of different follicles categories observed in the left ovaries were similar ($p > 0.05$) to follicles observed in the right ovaries.

Table 9: Mean number of follicles by categories of size for the right and left ovaries of the studied Boer goats ovaries using different methods on day 3 and day 10 of the observations

Methods	Categories of size (mm)					
	Right ovaries			Left ovaries		
	3 - 4.9 mm	5 - 7.9 mm	≥ 8 mm	3 - 4.9 mm	5 - 7.9 mm	≥ 8 mm
ULTS	0.50 ^b	0.70 ^b	0.70 ^a	0.40 ^b	0.50 ^b	0.70 ^a
LAPSC	0.90 ^b	1.30 ^b	0.70 ^a	1.00 ^{ab}	1.40 ^a	0.70 ^a
LAPT	1.70 ^a	2.00 ^a	1.30 ^a	1.70 ^a	1.90 ^a	1.00 ^a

^{a, b} Means with the different superscripts are different (P < 0.05)

In sheep, the mean number of follicles in the right and left ovaries of different categories are given in Table 10. Mean numbers of follicles between 3 - 4.9 mm category in both right and left ovaries were significantly different ($p < 0.05$) between ULTS and LAPT. However, for categories 5 - 7.9 mm and ≥ 8 mm in both ovaries the mean numbers of follicles observed were similar ($p > 0.05$).

Table 10: Mean number of follicles by categories of size for the right and left ovaries of the studied White Persian sheep ovaries using different methods on day 3 and day 10 of the observations

Methods	Categories of size (mm)					
	Right ovaries			Left ovaries		
	3 - 4.9 mm	5 - 7.9 mm	≥ 8 mm	3 - 4.9 mm	5 - 7.9 mm	≥ 8 mm
ULTS	0.80 ^b	0.90 ^b	0.60 ^a	0.60 ^b	1.10 ^a	0.80 ^a
LAPSC	0.90 ^b	1.40 ^a	0.40 ^a	1.10 ^a	1.00 ^a	0.60 ^a
LAPT	1.70 ^a	1.20 ^{ab}	0.60 ^a	1.50 ^a	1.40 ^a	0.60 ^a

^{a, b} Means with the different superscripts are different (P < 0.05).

In cattle, mean numbers of follicles of both the ovaries are presented in Table 11. There was no difference in ULTS identifying the follicles between all the categories on right and left ovaries. ULTS was applied in Nguni cattle during the experiment, however, its accuracy was not verified by any other method as they could not be applied. The inability to apply laparoscopy (LAPSC) and laparotomy (LAPT) techniques in cattle was due to size constraint.

Table 11: Mean number of follicles by categories of size for the right and left ovaries of the studied Nguni cattle ovaries using ULTS method on day 3 and day 10 of the observations

Methods	Categories of size (mm)					
	Right ovaries			Left ovaries		
	3 - 4.9 mm	5 - 9.9 mm	≥ 10 mm	3 - 4.9 mm	5 - 9.9 mm	≥ 10 mm
ULTS	0.80	1.60	1.70	0.30	1.60	1.50

The operational time and surgical complications were recorded between ULTS, LAPSC and LAPT. In goats, the operational time of ULTS was \approx 8 minutes/animal, LAPSC \approx 15 minutes/animal and LAPT \approx 42 minutes/animal. In sheep the operational time was shorter than in goats. Time consumed by ULTS was \approx 5 minutes/animal, LAPSC \approx 10 minutes/animal and LAPT \approx 33 minutes/animal, respectively. In cattle, ULTS operational time was \approx 7 minutes per animal. The ovaries were generally not difficult to locate by all three methods during the examinations. In addition, visualization and manipulation of the genital tract were considerably easier to perform during the examinations.

Surgical complications were observed with LAPT as it is an invasive method. Both goats and sheep were taking longer to recover especially after the second examinations. Animals experienced discomfort and required treatment of surgical wounds and regular monitoring post examinations. The surgical wounds limit the use of LAPSC for subsequent examinations as the animals have a layer of omental fat that attaches to the abdominal wound to protect their abdominal cavity from infections. After the surgical operations were stopped, all animals improved slowly until they fully recovered.

4.3. Pregnancy diagnosis

The accuracy of pregnancy diagnosis by trans-rectal probe in Boer goats does was not possible on day 18, but improved significantly to 100% on day 26 - 28 of gestation (Table 12). On the other hand, in White Persian ewes, trans-rectal probe accuracy for pregnancy diagnosis improved significantly from 40% on day 18 to reach maximum of 100% on day 20 - 22 of the examinations (Table 13). In Nguni cows, accuracy of pregnancy diagnosis was very low (zero) at day 18, but gradually improved to 100% on day 30 - 32 of gestation (Table 14). In this study no multiple pregnancies were detected on any animals used for examinations. This was confirmed at birth of kids, lambs and calves.

Table 12: Days to accurately diagnosing pregnancy in Boer goats by ULTS and percentage correct diagnosis

Interval (Days)	Pregnancy diagnosis (n) (%)	% diagnosed correctly
18	-	-
20	-	-
22	-	-
24	-	-
26	(5) (100)	100
28	(5) (100)	100

Table 13: Days to accurately diagnosing pregnancy in White Persian sheep by ULTS and percentage correct diagnosis

Interval (Days)	Pregnancy diagnosis (n) (%)	% diagnosed correctly
18	(2) (40)	100
20	(5) (100)	100
22	(5) (100)	100

Table 14: Days to accurately diagnosing pregnancy in Nguni cattle by ULTS and percentage correct diagnosis

Interval (Days)	Pregnancy diagnosis (n) (%)	% diagnosed correctly
18	-	-
20	-	-
22	-	-
24	-	-
26	-	-
28	-	-
30	(5) (100)	100
32	(5) (100)	100

During examinations an animal was considered to be pregnant when a fluid-filled gestational sac lining the uterus lumen and foetus heartbeat was observed. In addition, with the trans-rectal probe, the first thing that could be visualized during examination was a small non-echogenic amniotic vesicle in the uterine lumen just beyond the urinary bladder (Figure 8). However, it was important not to declare animal to be pregnant on the basis of the amniotic vesicle since at that early stage it could be confused with the urinary bladder. In this regard, positive pregnancy was confidently confirmed on the presence of the foetal heartbeat (Figure 8) on day 26 - 28 in does, 20 - 22 in ewes and 30 - 32 in cows, Table 15, 16 and 17, respectively.

Laparoscopy technique was used to verify the pregnancy of both does and ewes on day 28 and day 22, respectively. Laparoscopic technique was not conducted in cows due to size constraint. During laparoscopic examinations, non-echogenic structure like a black spot along the uterine lumen symbolizing the developing embryo was observed (Figure. 9) and was used as the confirmation of pregnancy. Increased uterine size and change of shape from flat to round were observed during the examinations.

Table 15: Accuracy of pregnancy diagnosis in Boer goats

Methods						
Structure observed	ULTS			LAPSC		
	Observed (n)	Kidded (n)	Accuracy (%)	Observed (n)	Kidded (n)	Accuracy (%)
Amniotic vesicle	5	5	100	-	-	-
Foetal heartbeat	5	5	100	-	-	-
Embryo	-	-	-	5	5	100

Table 16: Accuracy of pregnancy diagnosis in White Persian sheep

Methods						
Structure observed	ULTS			LAPSC		
	Observed (n)	Lambded (n)	Accuracy (%)	Observed (n)	Lambded (n)	Accuracy (%)
Amniotic vesicle	5	5	100	-	-	-
Foetal heartbeat	5	5	100	-	-	-
Embryo	-	-	-	5	5	100

Table 17: Accuracy of pregnancy diagnosis in Nguni cattle

Method			
Structure observed	ULTS		
	Observed (n)	Calved (n)	Accuracy (%)
Amniotic vesicle	5	5	100
Foetal heartbeat	5	5	100

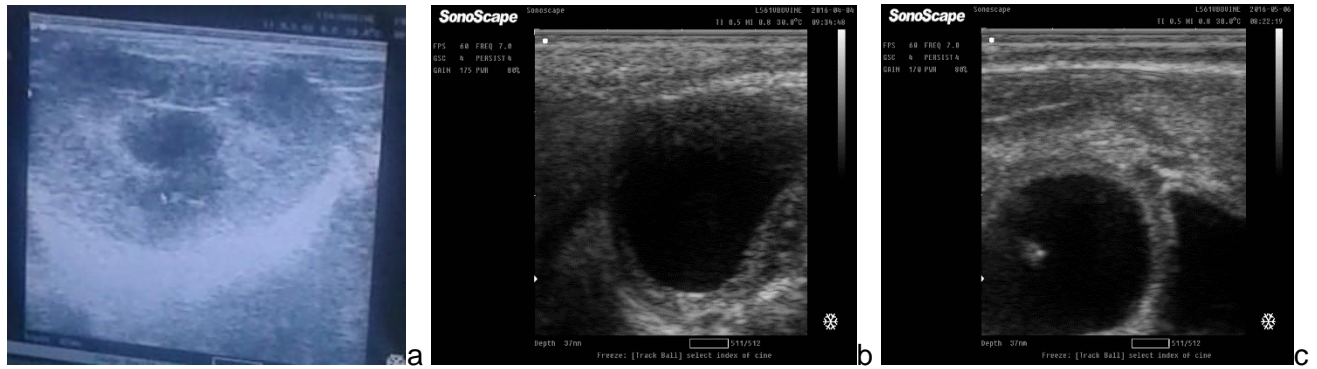


Figure 8: Ultrasonography images representing (a) non pregnant uterus of a goat, (b) cattle urinary bladder and (c) sheep pregnant uterus with the foetal heartbeat surrounded by amniotic vesicle

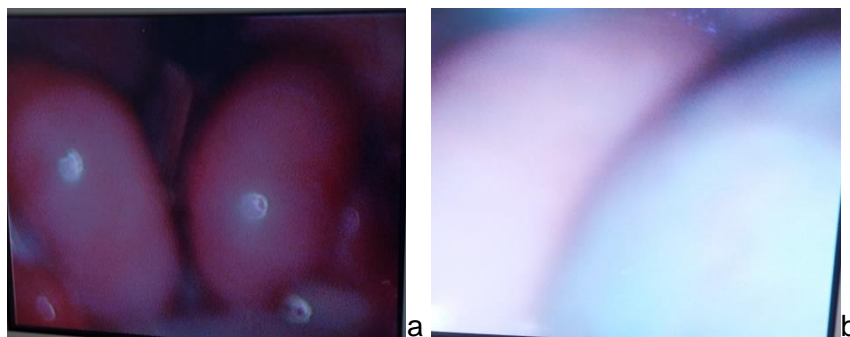


Figure 9: Laparoscopic images of a sheep (a) non pregnant uterus and (b) goat pregnant uterus visualized with a black spot

The time for diagnosis of pregnancy in does and cows by trans-rectal ultrasonography was different for both amniotic vesicle and foetal heartbeat. On the other hand, in ewes the diagnosis of pregnancy by the scanner was not different for both amniotic vesicle and foetal heartbeat as they were both observed on the same and subsequent days of examinations. The mean time required for the trans-rectal ultrasonography for pregnancy diagnosis examination in does, ewes and cows was ≈ 4.2 , ≈ 3 and ≈ 5.6 minutes per animal, respectively. Whereas with laparoscopic examination, the mean time required for pregnancy diagnosis examination in does and ewes was ≈ 12.7 and ≈ 8.8 minutes per animal, respectively. During pregnancy diagnosis, no surgical complications were recorded and all animals recovered quickly from laparoscopic examinations. Despite the fact that the introduction of a rectal probe may cause slight discomfort, no injury or damage was inflicted on the animals.

4.4. Foetal gender identification

Trans-rectal ultrasonography examination for foetal gender identification was initiated from day 40 of gestation after the does, ewes and cows were earlier detected to be pregnant. The examinations were repeated every 5 days until the confirmation (day 85) of foetal gender through conclusive evidence of visibility of scrotum and rudimentary teats as shown in Figure 10. The genital tubercle (GT) structures consisting of the scrotum and rudimental teats were successfully observed between the hind legs and tail as their final position. The results obtained during the examinations, indicated that from day 40 until day 70 of pregnancy in all animals under observations, there was no clear indication of the signs that could allow accurate gender diagnosis.



Figure 10: Ultrasonography images showing the foetal gender of the female cattle foetus by appearance of rudimentary teats between the hind legs (a) and scrotum of male foetus between the hind legs (b) all observed between day 75 and day 85 of gestation

During examination for foetal gender identification by trans-rectal ultrasonography scanning, there were various important anatomic foetal features that were critical in achieving proper orientation of the foetuses. The heartbeat and the body structures such as the umbilical cord, spinal cord, both front and hind legs and the head were the landmark features observed before the gender of the foetus could be determined. However, when the body was still developing, it was sometimes difficult to differentiate the hind legs from the front legs if the anatomic features are not well identified. The accuracy for ultrasonic foetal gender identification was achieved between day 75 and day 85 of gestation in goats, sheep and cattle when the scrotum and rudimentary teats were clearly visible as presented in Table 18, 19 and 20.

Table 18. Days to accurately identify foetal gender in Boer goats by ULTS and percentage correct diagnosis

Interval (Days)	Gender Identified (n) (%)	% gender identified
40	-	-
45	-	-
50	-	-
55	-	-
60	-	-
65	-	-
70	-	-
75	(5) (100)	100
80	(5) (100)	100
85	(5) (100)	100

Table 19. Days to accurately identify foetal gender in White Persian sheep by ULTS and percentage correct diagnosis

Interval (Days)	Gender Identified (n) (%)	% gender identified
40	-	-
45	-	-
50	-	-
55	-	-
60	-	-
65	-	-
70	-	-
75	(5) (100)	100
80	(5) (100)	100
85	(5) (100)	100

Table 20. Days to accurately identify foetal gender in Nguni cattle by ULTS and percentage correct diagnosis

Interval (Days)	Gender Identified (n) (%)	% gender identified
40	-	-
45	-	-
50	-	-
55	-	-
60	-	-
65	-	-
70	-	-
75	(3) (60)	60
80	(3) (60)	60
85	(3) (60)	60

In does, pregnancies were diagnosed to be carrying single foetuses in all animals observed. Out of 5 (100%) doe's foetuses whose genders were determined, the diagnosis was correct in 100% (3/3) of the male foetuses and 100% (2/2) of the female foetuses. At birth, the gender of the foetuses was recorded and compared to the ULTS results. The results after birth were related to those reported by ULTS in Table 21 and 22.

Table 21: Foetal gender of Boer goat foetuses by ULTS examination between days 40 and 85 of pregnancy

Gestational type	Sexed foetus (n)	Unsexed foetuses (n)	Gender at birth (n)	Accuracy of diagnosis (n) (%)
Single	5	-	5	(5) (100)
Total	5	-	5	(5) (100)

Table 22: Gender of Boer goats foetuses by ULTS examination vs actual birth

Gestational type	Gender scanned		Gender at birth		Accuracy (%)	
	Male (n)	Female (n)	Male (n)	Female (n)	Male (n)	Female (n)
Single	3	2	3	2	(3) (100)	(2) (100)
Total	3	2	3	2	(3) (100)	(2) (100)

Similar to does, the examination was conducted on 5 (100%) ewes and they were also carrying single foetus pregnancies. The gender diagnosis was correct in 2/2 (100%) of the male fetuses and was correct in 3/3 (100%) of the female fetuses observed. The information obtained from ewe's birth confirmed the results observed during ultrasonographic scanning. From the results obtained during scanning examination and observations at birth, it can be confirmed that the accuracy for foetal gender identification in ewes by ultrasonography was 100% in all foetuses observed as shown in Table 23 and 24, respectively.

Table 23: Foetal gender of White Persian sheep foetuses by ULTS examination between days 40 and 85 of pregnancy

Gestational type	Sexed foetus (n)	Unsexed foetuses (n)	Gender at birth (n)	Accuracy of diagnosis (n) (%)
Single	5	-	5	(5) (100)
Total	5	-	5	(5) (100)

Table 24: Gender of White Persian sheep fetuses by ULTS examination vs actual birth

Gestational type	Gender scanned		Gender at birth		Accuracy of diagnosis (%)	
	Male (n)	Female (n)	Male (n)	Female (n)	Male (n)	Female (n)
Single	2	3	2	3	2 (100)	(3) (100)
Total	2	3	2	3	2 (100)	(3) (100)

In cows, examined fetuses were three out of five (3 of 5) animals examined with single pregnancies. In this regard, out of 60% (3/5) of fetuses examined, 1 (100%) foetus was identified as male and the remaining 2 (100%) fetuses were identified as females. Two cows aborted during the study. Abortion of one of the fetuses occurred between the period after pregnancy diagnosis and the beginning of the foetal gender identification. The foetal gender of the foetus could not be differentiated between male and female at that early period because the genital structures were not at their final position. Another foetus was aborted during the period of foetal gender identification. However, the foetus was not found so it could be identified as a male or female. It was concluded that the foetus may have been eaten by predators/scavengers since the cows were kept at the camps. Abortion rate was recorded at 40% (2/5) at the end of the experimental examination. The observation at birth reports suggest that ULTS was accurate in identifying the genders of the different fetuses as indicated in Table 25 and 26, respectively.

Table 25: Foetal gender of Nguni cattle fetuses by ULTS examination between days 40 and 85 of pregnancy

Gestational type	Sexed foetus (n)	Unsexed fetuses (n)	Abortion (n)	Gender at birth (n)	Accuracy of diagnosis (n) (%)
Single	3	-	2	3	(3) (60)
Total	3	-	2	3	(3) (60)

Table 26: Gender of Nguni cattle fetuses by ULTS examination vs actual birth

Gestational type	Gender scanned		Abortion (n)	Gender at birth		Accuracy (%)	
	Male (n)	Female (n)		Male (n)	Female (n)	Male (n)	Female (n)
Single	1	2	2	1	2	(1)(100)	(2) (100)
Total	1	2	2	1	2	(1)(100)	(2) (100)

The mean time required for the trans-rectal ultrasonography for foetal gender identification examination in does, ewes and cows was \approx 9 minutes/animal, \approx 7 minutes/animal and \approx 13 minutes/animal, respectively.

CHAPTER 5

DISCUSSION

5.1. Follicular dynamics

Results of the current study explain follicular dynamics of Boer goats, White Persian sheep and Nguni cattle as monitored daily by ultrasonography technique. According to the current study the mean length of the interovulatory interval in sheep (17.01 ± 0.7 days), goats (21.2 ± 0.6 days) and cattle (21.01 ± 0.8 days) is similar to the results of other investigators such as Seekallu *et al.* (2010), Medan *et al.* (2003) and Adams *et al.* (2008). Results demonstrate that the interovulatory interval is characterized by a wave-like pattern of follicular development as reported in goats by Medan *et al.* (2003), sheep (Seekallu *et al.*, 2010) and cattle (Muraya *et al.*, 2015). A follicular wave can be explained as the organized development of a cohort of dependent follicles which initially increases in size, but most of which subsequently regress and die by atresia (subordinate follicle) (Bodensteiner *et al.*, 1996; Wiltbank *et al.*, 2002; Evans, 2003).

In the current results, all the goats, sheep and cattle observed had three follicular waves throughout the cycle similar to studies of (Evans *et al.*, 2000; Menchaca & Rubianes, 2002; Muraya *et al.*, 2015). However, there are other studies in goats (Orita *et al.*, 2000; Simoes *et al.*, 2006) and sheep (Seekallu *et al.*, 2010) that achieved more than three follicular waves per cycle. In the studies of goats (Orita *et al.*, 2000), sheep (Evans *et al.*, 2000) and cattle (Muraya *et al.*, 2015) there were instances in which cycles with two follicular waves were observed. Different follicular wave patterns within species indicate that breeds have great influence on how the cycle is arranged. In the current study the number of follicles with a diameter of ≥ 3 mm that appeared daily and subsequently grew to ≥ 5 mm in diameter differed daily in all the three waves. Medan *et al.* (2003) and Evans *et al.* (2000) alluded the appearance of follicles with diameters of ≥ 3 mm as a demonstration of progressive growth of follicles in a wave-like pattern.

In goats, the current results on the days of the follicular wave emergence are similar to those reported by Simoes *et al.* (2006) in Serrana goats and de Castro *et al.* (1999) in Saanen goats. In oestrous cycle of dairy goats as reported by Menchaca & Rubianes (2002) the first (0.0 ± 0.4) and third (13 ± 0.3) follicular waves emerged earlier than observed in the current study, but second (7.3 ± 0.3) follicular wave emerged on the similar days. All the follicular waves

observed by Medan *et al.* (2003) emerged much earlier than those in the current study. The latter reported that the mean days of follicular wave emergence of first, second and third waves were 0.3 ± 0.5 , 6.5 ± 0.2 and 12.1 ± 0.4 , respectively. Ovulatory (3rd) wave that appeared around 14.8 ± 0.8 days of the cycle as found in the current study is in agreement with the reports of de Castro *et al.* (1999) and Simoes *et al.* (2006).

In the current study, the days of the dominant follicles emergence were 2.6 ± 0.4 , 9.2 ± 0.6 and 17.2 ± 0.4 for the first, second and third waves, respectively. However, Simoes *et al.* (2006) observed the emergence of maximum dominant follicle of the first (4.4 ± 2.1) and third (19.8 ± 1.1) waves much later than the current study, but in the second wave, the maximum dominant follicle emerged within the range observed in the current study. In Saanen goats de Castro *et al.* (1999) also observed the maximum dominant follicle of the first (day 5) and third (day 19) later than in the current study while the latter observed similar day of the second wave dominant follicle emergence. The size of the maximum dominant follicles of the first, second and third waves of the cycle observed in the current study were also larger than those observed by Medan *et al.* (2003). However, the latter observed the maximum size of the dominant follicle of wave one (6.6 ± 0.1) and wave two (6.2 ± 0.1) to be smaller than in the current study, but achieved the larger size in wave three (8.0 ± 0.1). These differences could be attributed to the breeds and possibly ages of the goats used for the observation in the above mentioned studies.

In sheep, Noel *et al.* (1993) reported the occurrence of three follicular waves similar to the current study. However, the current study disagrees with the study of Schrick *et al.* (1993) who mentions that follicular waves were not observed in sheep. Toosi *et al.* (2009) observed days of follicular waves emergence similar to the current study in Western White face ewes. However, in an earlier study of Noel *et al.* (1993), follicular waves appeared much later in wave two (7.6 ± 0.8 days) and wave three (14.0 ± 0.6), but wave one was similar to the current study. In Suffolk-Cross ewes (Evans *et al.*, 2000), the dominant follicle of wave one appeared on similar days, but in wave two (6.9 ± 0.3) and wave three (11.7 ± 0.3) the dominant follicle appeared much earlier than in the current study. Contrary to the current results, Noel *et al.* (1993) observed the emergence of the dominant follicle much later in all three waves (5.9 ± 1.0 , 12.0 ± 1.1 and 16.5 ± 0.6) in Suffolk ewes. According to the current study the maximum dominant follicles observed in the first and second follicular waves were greater when compared to those observed in wave one (5.4 ± 0.1 mm) and wave two (5.1 ± 0.2 mm), but similar maximum size was attained in follicular wave three as reported by Evans *et al.* (2000).

Seekallu *et al.* (2010) obtained dominant follicle size in first wave and third wave similar to this study, however, the latter obtained different size diameter in second wave (5.4 ± 0.2 mm) in Western White face ewes. In the current study all the dominant follicles of the luteal phase (wave one and two) atrophied, but the dominant follicle of the follicular phase (third wave) ovulated. These observations are consistent with the results of Noel *et al.* (1993) in Suffolk ewes. Noel *et al.* (1993) and Seekallu *et al.* (2010) stated that the length of the follicular waves was approximately 6 days. However, the current results indicate that the length of the follicular waves was shorter than the results obtained in the studies above. Similar to goats, reports vary widely because of different conditions in which the ultrasonography examinations were performed, experience of the operator, the breeds and possibly ages of the sheep used for the observations.

In cattle, the current study affirms that there are three follicular waves in the cycle as previously reported by Sirois & Fortune (1988), Gaur & Purohit (2007) and Muraya *et al.* (2015). In the current results, the days of the follicular wave occurrence are similar to those reported by Sirois & Fortune (1988) in Holstein heifers and Adams *et al.* (2008). However, Adams *et al.* (2008) observed the first follicular wave emergence earlier (day 0) than the current study. In the oestrous cycle of Kenyan Boran cows (Muraya *et al.*, 2015) and Rathi cows (Gaur & Purohit, 2007), all the follicular waves occurred much earlier than the current study. Gaur & Purohit (2007) reported that days of wave emergence were 0.78 ± 0.44 , 7.11 ± 1.05 and 13.22 ± 2.44 , while Muraya *et al.* (2015) mentioned that follicular waves emerged on days 0.42 ± 0.16 , 8.15 ± 0.56 and 15.00 ± 0.98 for wave 1, 2 and 3, respectively.

The current study indicates that the ovulatory wave appeared around 16.6 ± 0.4 days of the cycle supporting the results reported by Sirois & Fortune (1988) and Adams *et al.* (2008). In the current study, the days of the dominant follicles emergence were 2.8 ± 0.8 , 12.2 ± 0.8 and 16.6 ± 0.4 for the first, second and third waves, respectively. However, Muraya *et al.* (2015) observed the emergence of maximum dominant follicle of the first wave much later (4.13 ± 0.99) than the current study, but in the second and third waves, the maximum dominant follicle emerged within the range observed in the current study. In addition, Figueiredo *et al.* (1997) observed the maximum dominant follicle of first (1.57 ± 0.20), second (9.14 ± 0.47) and third (15.14 ± 0.48) much earlier in Nelore beef cattle breed than in the current study. The size of the maximum dominant follicles of the first, second and third waves of the cycle observed in the current study were larger than those observed in the studies of Holstein heifers (Sirois & Fortune, 1988) (12.3 ± 0.2 , 10.2 ± 0.5 and 12.8 ± 0.3), Rathi cattle (Gaur & Purohit, 2007)

(11.78 ± 1.20 , 10.44 ± 2.13 and 12.44 ± 1.59) and Kenyan Boran cows (Muraya *et al.*, 2015) (10.95 ± 0.34 , 10.63 ± 0.58 and 13.52 ± 0.50) for waves 1, 2 and 3, respectively.

Adams *et al.* (2008) further mentioned that the dominant follicles of the successive waves undergo atresia under the influence of progesterone while the dominant follicle of the ovulatory wave undergoes ovulation. In the current study the length of the ovulatory wave was shorter than the length of follicular waves one and two. Contrary to current study, Gaur & Purohit (2007) found the length of the first (13.00 ± 1.58), second (11.44 ± 2.19) and third (7.67 ± 1.80) follicular waves to be longer than the current results. In addition, in Nelore beef cattle breed Figueiredo *et al.* (1997) reported the length of the first (13.00 ± 1.58), second (11.50 ± 0.42) and third (6.86 ± 0.21) follicular wave to be longer than in the current investigation. The current study agrees with the findings of Gaur & Purohit (2007) and Figueiredo *et al.* (1997) that the ovulatory wave was shorter than the first and second waves. However, Muraya *et al.* (2015) found the ovulatory wave to be longer than follicular wave one and two in Kenyan Boran cows. The latter also reported the longer length of the first (7.08 ± 0.40), second (7.77 ± 0.77) and third (8.62 ± 0.72) follicular wave than in the current study. Similar to goats and sheep these differences could be attributed to the breeds and possibly ages of the cattle used for the observation in the above mentioned studies.

5.2. Follicles observations by different methods

The current study reveals that ultrasonography technique provides for efficient and accurate observation of ovarian structures as concluded in studies by Driancourt (1984) and Simoes *et al.* (2005). To our knowledge, this is the first study to be conducted in goats, sheep and cattle to compare the efficiency and accuracy of ultrasonography to laparoscopy and laparotomy in observing ovarian structures.

The results of the current study are in agreement with Hafez, (1993) that the ovaries of the goats, sheep and cattle appeared to be almond-shaped. However, the shape of the ovaries could be altered by the growing follicles and corpora lutea. To our knowledge, there is also no study that reported about the measurements of the ovarian size when using ultrasonography, laparoscopy and laparotomy. However, there is a study conducted by Mohammadpour (2007) measuring the ovarian dimension using caliper device after the slaughter of Iranian Lori-Bakhtiari sheep and Native goats. In the current study, the actual sizes of the ovaries were clearly observed by laparotomy technique than with ultrasonography. On the other hand,

modification of the laparoscopic camera transducer and ultrasonography scanner limited the ability and ease of measurement of ovarian size.

In the current study, follicles in does and ewes were categorized in size as 3 - 4.9 mm; 5 - 7.9 mm and ≥ 8 mm similar to the study of Simoes *et al.* (2005). On the other hand, Wiltbank *et al.* (2002) grouped the follicles according to their emergence (3 - 4.9 mm), deviation (5 - 9.9 mm) and ovulation (≥ 10 mm) in cows similar to the current study. Analysis of the mean numbers of follicles categorized in different size classes indicates that changes in antral follicles population in goats, sheep and cattle were caused mainly by numbers of small antral follicles (Bartlewski *et al.*, 2006). Noel *et al.* (1993) indicated that in Suffolk ewes the growth of antral ovarian follicles ≥ 2 mm occurs in three waves. During each wave, follicles with diameters of ≥ 2 mm developed from a pool and a few of them continued maturations and growth. These follicles either ovulated or became atrophied (Noel *et al.*, 1993). Driancourt *et al.* (1984) reported that follicles reaching ovulation were recruited from those of any size greater than 2 mm at the time of natural luteolysis. In the current study, for greater consistency in identifying follicles, only follicles with a diameter of ≥ 3 mm were selected, measured and classified as applied by Bartlewski *et al.* (2000), Medan *et al.* (2003), Simoes *et al.* (2005) and Simoes *et al.* (2006). The selection of follicles with diameters of ≥ 3 mm may be attributed to the under-estimation by ultrasonography to detect follicles < 3 mm, possibly because the resolutions of the 7.5 MHz probe limits our ability to discover these small follicles as indicated by Simoes *et al.* (2005).

In goats and sheep, differences in the mean number of follicles observed in the category of 3 – 4.9 mm were significantly different between ultrasonography and laparotomy. However, in the same category laparoscopic and laparotomy techniques were consistent in observing the follicles. The means of category 5 – 7.9 mm between the goats and sheep as observed by laparoscopy and laparotomy were not different, but in goats laparotomy recorded high mean number of follicles than any other method. In the current study, the mean count of the follicles in category ≥ 8 mm was observed to be similar by all three methods, but laparotomy counted numerically more follicles in goats. Similarity of the mean follicular count could be attributed to the occurrence of follicles with diameters of ≥ 8 mm per ovary. These could also be attributed to juxtaposition of some of those follicles in the category 5 - 7.9 mm as expressed by Simoes *et al.* (2005).

In cattle the observations were made by ultrasonography only. The follicle counts were not verified but seemed to be within range. It is important to note that with the use of

ultrasonography there is a possibility of under counting of certain categories of follicles. McDougall *et al.* (1995) reported an under evaluation of the follicles between diameters of 3 mm and 5 mm.

Simoes *et al.* (2005) were able to observe the corpora lutea during the examination using ultrasonography, laparoscopy and laparotomy. However, in this study no corpus luteum was observed by any of the methods used during the examination. Simoes *et al.* (2005) alluded the absence or inability to observe corpus luteum to their more internal location in the ovaries, hindering visualization on the ovary surface. In addition, in agreement with Simoes *et al.* (2005) further research is needed to monitor the development of corpus luteum by ultrasonography, laparoscopy and laparotomy to allow accurate observation or identification of corpus luteum.

During examinations it was not difficult to observe the genital tract of the goats and sheep by any of the three methods used. However, Teixeira *et al.* (2011) reported that it was particularly difficult to visualize and manipulate the genital tract in Santa Ines sheep by laparotomy method than with laparoscopy. Furthermore, the latter reported that the difficulties in observing the genital tract prompted intense traction to withdraw the ovaries from the abdominal cavity. To our knowledge, the operational time required to observe the ovarian structures in goats, sheep and cattle has not been reported in the previous studies. In the current results laparoscopic technique required ≈ 15 and ≈ 10 minutes per animal in goats and sheep, respectively, while laparotomy required ≈ 42 and ≈ 33 minutes per animal in goats and sheep, respectively. The results obtained in this study indicate that laparoscopy and laparotomy examinations were considerably much shorter than overall time taken by Teixeira *et al.* (2011) to complete examination per animal when using laparoscopy (27.5 ± 2.9 minutes) and laparotomy (75.8 ± 29.5 minutes), respectively.

5.3. Pregnancy diagnosis

Reliable techniques for early pregnancy diagnosis are important tools in separating pregnant from non-pregnant animals. The methods of pregnancy diagnosis in goats, sheep and cattle will depend upon the availability of equipment, trained personnel and known number of days post insemination. Of the 5 does, 5 ewes and 5 cows used in the current study all were detected to be pregnant. A 100% overall accuracy of ultrasonography was achieved for diagnosing pregnancy in goats, sheep and cattle. The overall accuracy results in the current study are in agreement with the results obtained in the goats (Medan *et al.*, 2004), sheep

(Ferrari *et al.*, 2009) and cattle (Romano *et al.*, 2006). Overall accuracy for diagnosing single pregnancies obtained in the current study was confirmed during kidding, lambing and calving period. The amniotic vesicle could be detected much earlier before the foetal heartbeat similar to other studies in goats (Padilla-Rivas *et al.*, 2005), sheep (Gonzalez de Bulnes *et al.*, 1998) and cattle (Romano *et al.*, 2006).

In goats, results demonstrate that 100% accuracy for pregnancy diagnosis could be achieved between days 26 and 28 of gestation by observing a heartbeat when using a trans-rectal probe. The current results are consistent with the results reported by Karadaev (2015) between days 25 and 30 post insemination. Amer (2010) achieved 100% overall accuracy in dairy goats between days 19 and 22 while in Egyptian native goats (Karen *et al.*, 2009) 100% overall accuracy was obtained between days 16 and 22 much earlier than in the current investigation. Slightly different from the results of the current study, an earlier study in Thai native goats (Restall *et al.*, 1990) reported 100% overall accuracy, while Goel & Agrawal (1990) obtained 90% accuracy between days 51 and 60, respectively. Whether the breeds and animal ages contribute towards the differences, is open for conjecture as alluded to by Padilla-Rivas *et al.* (2005).

In sheep, results show that pregnancy diagnosis could be detected starting from day 18 of gestation based on the presence of positive signs of pregnancy by trans-rectal ultrasonography probe. In the current study, on day 18 accuracy of pregnancy detection was recorded to be 40% while the accuracy gradually improved to 100% on day 24 post insemination. Contrary to the current study, Roman & Christian (2008) detected pregnancy earlier (day 16) in Suffolk ewes with the accuracy of 26.7%, whereas on day 18 the accuracy was reported to be 90% far higher than the current study on the same day. The latter achieved 100% accuracy on day 20 similar to the current study using similar probes (7.5 MHz). In Awassi and Merino ewes study (Karen *et al.*, 2004), accuracy of diagnosing pregnancy between day 18 and day 24 was lower (21.8% and 46%) in both group 1 and 2, respectively, using 5 MHz probe. Roman & Christian, (2008) found the use of trans-rectal probe (7.5 MHz) to be more accurate and precise than 5 MHz probe as reported by Karen *et al.*, (2004). Karen *et al.*, (2004), mentioned that the effect of age and breed played an important role in detecting pregnancy between days 18 and 24. Using transabdominal ultrasonography probe Ganaie *et al.* (2009) reported accuracy of 68% between 15 and 30 days post insemination. However, Padilla-Rivas *et al.* (2005) explained that although transabdominal probe may be more convenient, trans-rectal probe remains preferred. The latter further indicated that for trans-rectal probe to be

preferred for pregnancy diagnosis is because transabdominal probe detect pregnancy 4 - 5 days later.

In cattle, in the current study the accuracy of ultrasonography scanning was very low (zero) on day 18, but improved gradually on day 30 and day 32 obtaining maximum accuracy of 100%. Boyd *et al.* (1988) and Curran *et al.* (1986) were able to successfully diagnose pregnancy between days 9 and 21 of gestation, but the accuracy of pregnancy detection was not reported at that early stage. In addition, Kastelic *et al.* (1989) indicated that accuracy of pregnancy diagnosis before day 18 is nothing more than just a guess. In dairy cattle, Kastelic *et al.* (1989), Pieterse *et al.* (1990) and Romano *et al.* (2006) were able to accurately diagnose pregnancy much earlier than the current study. Kastelic *et al.* (1989) reported the accuracy of 85% on day 18, whereas Pieterse *et al.* (1990) were able to obtain 44.8% between days 21 and 26 while Romano *et al.* (2006) achieved the accuracy of 74.5% on day 24 in cows and 50% in heifers on day 21. Moreover, in the study of Naikoo *et al.* (2013) in Mehsana Buffaloes, pregnancy was accurately (100%) diagnosed on day 26 of the gestation. In the studies mentioned above, the accuracy of diagnosing pregnancy gradually improved as the gestation period progressed. This was probably due to an increase in the visibility of the foetuses heartbeat making it easier for pregnancy to be confirmed.

The accuracy (100%) obtained in the current study is in agreement with the results reported by Romano *et al.* (2006) in dairy cows on day 30 of pregnancy. Although Pieterse *et al.* (1990) and Nation *et al.* (2003) obtained the accuracy of 97.7% (between days 26 and 33) and 96% (between days 28 and 35) in diagnosing pregnancy, the latter were not specific on the day of pregnancy detection, however, their accuracy is within the range of the current study. Romano *et al.* (2006) mentioned that the difference among the reports vary widely due to different conditions in which ultrasonography was performed, breed and experience of the operator working with the equipment. The current investigation supports the assumption made by the latter. To our knowledge, the accuracy of diagnosing pregnancy by ultrasonography in beef cattle has not been reported previously, however, the accuracy has been determined in many research conducted in dairy cows. This warrants the establishment of further research on indigenous beef cattle of South Africa to determine the accuracy of ultrasonography in detecting early pregnancy.

In the current examination, in both goats and sheep laparoscopy achieved 100% of the overall accuracy confirming the results of trans-rectal ultrasonography scanning by visualizing a

black spot lining the uterus lumen. Similar to the study of Kumar *et al.* (2011) increased uterine horn size and change of shape from flat to round were observed during the examinations. There was no difference between the current study and the study of Goel & Agrawal (1992) in terms of accuracy in detecting pregnancy by laparoscopic technique. Laparoscopy technique could therefore be used for early pregnancy diagnosis in both goats and sheep similar to ultrasonography. In the current study, there were no difficulties in observing the genital organs in both goats and sheep by ultrasonography and laparoscopy. In addition, no major surgical complications were observed during the investigation. The mean time required to diagnose pregnancy during this study in does, ewes and cows was ≈ 4.2 , ≈ 3 and ≈ 5.6 minutes per animal, respectively. The reported time consumed during pregnancy diagnosis examination in the current study was different from the study of Padilla-Rivas *et al.* (2005) in goats (≈ 2.5 minutes) using trans-rectal probe. However, to our knowledge, the time required to conclude pregnancy diagnosis examination in sheep and cattle has not been reported in previous studies. The mean time required for laparoscopic method to carry out the examination per animal was ≈ 12.7 (does) and ≈ 8.8 (ewes) minutes which is in agreement with the review study by Goel & Agrawal (1992).

5.4. Foetal gender identification

The period of foetal gender identification was outside the range of day 40 and day 70 of gestation for goats (Santos *et al.*, 2006; 2007a, b; Amer, 2010) and sheep (Coubrough & Castell, 1998). In goats and sheep, during the examination of animals, there was no clear indication of the signs that could allow accurate gender diagnosis as a result of the GT structures migration period not being known. Amer (2010) mentioned that the accuracy of foetal gender identification in small ruminants depends highly on the period of GT structures migration, particularly in goats. Santos *et al.* (2007b) reported that the GTs migration occurs between days 45 and 53 of pregnancy. Although they occur from day 40 onwards, it is suggested that the examinations should be performed between days 55 and 70 of the pregnancy (Amer, 2010).

In goats, the accuracy of foetal gender identification between day 40 and day 70 of gestation was considerably very low (zero) in single pregnancies as compared to accuracy achieved by Santos *et al.* (2006) in Boer goats, Santos *et al.* (2007b) in unknown goats breed and Amer (2010) in dairy goats. In the above mentioned studies, the accuracy of the foetal genders was achieved much earlier than in the current study. In the study of Santos *et al.* (2006), the overall accuracy of foetal gender identification between days 40 and 60 of gestation

was reported to be 100% (8/8) in the first experiment done by daily ultrasonography examination. In addition, the latter obtained 94.4% overall accuracy (17/18) between days 45 and 60 of gestation through single examination. Santos *et al.* (2007b) obtained overall accuracy of 100% (15/15) through daily examination between days 45 and 60 while with single examination the latter achieved 81% (13/16) between days 55 and 70 of gestation. Amer (2010) reported overall accuracy of 100% (16/16) and 83.3% (15/18) of all animals examined between days 40 and 60 and between days 61 and 70 of gestation in dairy goats.

In the current investigation, the overall accuracy gradually increased to 100 percent between days 75 and 85 of gestation in all animals examined. Out of 5 doe's, foetuses whose genders were determined, the diagnosis was correct in 100% (3/3) of the male foetuses and 100% (2/2) of the female foetuses. The current results differ from those reported by Santos *et al.* (2007b) who achieved 37% correct diagnosis of the female foetuses observed and 48% correct diagnosis of the male foetuses in experiment 1 (between days 45 and 60). The latter achieved 32% and 50% correct diagnosis in male and female foetuses, respectively in experiment 2 (between days 55 and 70). The difference between the current investigation and that of Santos *et al.* (2007b) could be because in the latter study, does that were examined had single and multiple pregnancies. To the best of our knowledge, the accuracy of determining the foetal gender in goats between days 75 and 85 (late foetal gender developmental stage) has not been reported before, however, the accuracy of determining the foetal gender (male vs female) has been reported during early foetal gender developmental stage by Santos *et al.* (2007b). Santos *et al.* (2006) alluded the achievement (to allow a quick and accurate) of early foetal gender identification to the availability of a skilled technician and use of adequate ultrasonography equipment. The current investigation supports the sentiments mentioned by the latter authors. However, there is a need for the establishment of further research to determine the accuracy of ultrasonography in diagnosing foetal gender between days 75 and 85 of gestation in goats.

In sheep, the overall accuracy (100%) of diagnosing the gender of the foetuses was obtained in the current study between days 75 and 85 of gestation. The overall accuracy is similar to the study of Dias *et al.* (2009) in Santa Ines ewes (98.1%), but Coubrough & Castell (1998) achieved the overall accuracy lower (89%) than in the current study. During the foetal gender examination of the current investigation, the gender diagnoses was correct in 2/2 (100%) of the male fetuses and was correct in 3/3 (100%) of the female fetuses observed. The results of the current study are similar to the results of Coubrough & Castell (1998) who

achieved 100% correct diagnosis in 14 of 14 male foetuses observed, but the latter achieved 77% correct diagnosis in 10 of 13 of the female foetuses observed between days 60 and 69 of gestation. The accuracy of the female foetuses achieved by the latter was lower compared to the percentage achieved in the current study. Dias *et al.* (2009) obtained the accuracy of 82.1% (23/28) and 75.6% (31/41) in correctly diagnosing the male and female foetal gender. The accuracy achieved by Dias *et al.* (2009) for diagnosing both the male and female foetuses is considerably lower than in this study, although the examinations were conducted between days 49 and 59 of gestation.

In cattle, the overall accuracy obtained was 60 percent between days 75 and 85 of gestation. The overall accuracy achieved in this study is considerably much lower compared to overall accuracy of 86.3 percent in dairy cows as reported by Quintela *et al.* (2011) in the same period of examination with the current investigation. In the current study there were three out of five (3 of 5) animals examined with single pregnancies. The lower overall percentage achieved in the current study could be alluded to abortion of the foetuses during the period of the examination. In this regard, out of 60% (3/5) of correctly diagnosed foetuses, 1 (100%) was identified as male foetus and the remaining 2 (100%) were identified as female foetuses. Ali (2004) and Quintela *et al.* (2011) achieved overall accuracy of 97.3% and 99.3% higher in dairy cows than the current study. However, the latter achieved similar results (100%) in determining the correct diagnosis of the male and female foetuses between day 76 and day 85 of the gestation. The correct diagnosis of the male and female foetuses at this stage could be attributed to clear visibility of scrotum and rudimentary teats on their final positions between the hind legs during the examinations.

In sheep, (Coubrough & Castell, 1998) and cattle (Tainturier *et al.*, 2004) reported the mean time required for the trans-rectal ultrasonography examinations to identify foetal gender to be ≈ 9 and was $\approx 5 - 10$ minutes per animal, respectively. The results reported above were not similar to the results of the current investigation. To our knowledge, the time required to conclude gender identification examination in sheep has not been reported in previous studies. Prolonged time consumed during the examinations of goats and sheep could be attributed to a small rectum that limited the manipulation of the probe (Coubrough & Castell, 1998; Azevedo *et al.*, 2009), while in cows, could be due to the position of the reproductive organs that moves deep down the abdominal cavity as the pregnancy progresses.

CHAPTER 6

6.1. Conclusions

The current results show that ultrasonography method using 7.5 MHz trans-rectal probe can accurately monitor follicular development throughout the oestrous cycle of Boer goats, White Persian sheep and Nguni cattle. Although the oestrous cycles of examined animals are similar to those of their counterpart breeds, predominantly three follicular waves occur. The current study demonstrated that ultrasonography and laparoscopic techniques are equally accurate in diagnosing early pregnancy. Ultrasonography proved to be efficient in identifying foetal gender. Ultrasonography demonstrated no post examination (surgical) complication and required less time and labour to complete the observations compared to the reference methods.

6.2. Recommendations

Although laparotomy and laparoscopy techniques are efficient and accurate, they have their limitations with regards to extensive livestock. In this sector where facilities are not suitable for application of laparotomy and laparoscopy, the application of ultrasonography is more practical, applicable and feasible for monitoring of ovarian structures, pregnancy diagnosis and foetal gender identification.

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