STRUCTURAL AND MORPHOMETRIC CHANGES IN RUMINAL TISSUES AND PHYSIOLOGICAL EFFECTS IN WETHERS WITH PLASTIC BAG-IMPACTED RUMEN

A thesis submitted in fulfillment of requirements for Doctor of Philosophy degree of University of Nairobi (Veterinary Anatomy)

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DECLARATION

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

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DEDICATION

To my husband Richard Mills-Thompson, son Justus John, daughter Juanita,
mom Faustina Tenkorang Anarfi, father Nana Anarfi and brother William Fortune Etsey.
In memory of my late dad Emmanuel Etsey.
To GOD be the glory!

Be not afraid, only believe!
"The prophecy you believe, is the prophecy that will happen practically in your life"
(Bishop Dag Heward-Mills)

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LIST OF ABBREVIATIONS

BW	Body weight
EIA	Enzyme immunoassays
ELISA	Enzyme linked immunosorbent assays
FCM	Faecal cortisol metabolites
VFAs	Volatile fatty acids
HPA axis	Hypothalamic-pituitary-adrenal axis
PPI and PPII	Test group I and II of wethers implanted with plastic bags in the rumen through rumenotomy
NPPI and NPPII	Positive control group I and II of wethers with no plastic bags in the rumen but had rumenotomy
CPI and CPII	Negative control group I and II of wethers with neither plastic bags in the rumen nor had rumenotomy
Vv	Volume density
Sv	Surface density
S	Surface area
AR	Atrium ruminis or cranial sac of rumen
DS	Dorsal sac of rumen
VS	Ventral sac of rumen
CDB	Caudodorsal blind sac of rumen
CVB	Caudoventral blind sac of rumen

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ABSTRACT

Freely grazing ruminants are liable to both advertent and accidental ingestion of indigestible foreign materials. Rumen impaction with such materials induces stress, damages ruminal tissues and impairs ruminal functional efficiency. Quantitative data on surface area and volume depicting structural changes in ruminal tissues consequent to impaction with plastic bags is lacking. Furthermore, the physiological effects resulting from impaction have not been documented. The objectives of this study include: 1. To investigate the quantitative structural changes in ruminal tissues of wethers following experimental impaction of the rumen with plastic bags. 2. To analyze the levels of cortisol in plasma of wethers implanted with plastic bags in their rumen as an indicator of stress. 3. To estimate faecal cortisol metabolite levels as an indicator of stress in wethers with plastic bags implanted in their rumen.

The experimental set-up was in two Phases: Phase I of the experiment had a 4-week endpoint and Phase II an 8-week endpoint after implanting the rumen with plastic bags. Each phase had 3 groups of wethers consisting of 5 animals per group. The groups in Phase I were designated as test group (PPI), positive control group (NPPI) and negative control group (CPI) all of which were sacrificed at 4 weeks. Phase II groups were designated as PPII, NPPII and CPII for test, positive control and negative control groups respectively and were sacrificed at 8 weeks. The groups PPI and PPII were implanted with 166g of plastic bags in the rumen through rumenotomy. Groups NPPI and NPPII underwent rumenotomy but no plastic bags were implanted. The groups CPI and CPII had neither rumenotomy nor plastic bags. Following euthanasia, the rumen from each animal was dissected out and divided into the various sacs for gross examination, macroscopic surface area measurements by point-associated area method and

reference volume of each sac was determined by Scherle's method. Tissue blocks for histology were obtained by systematic random sampling and processed to obtain vertical sections for surface and volume density estimations. At the microscopic level, STEPanizer® stereological software was employed to estimate volume and surface densities of each ruminal sac and subsequently absolute values were calculated. Short-term (the first 72 hour period) and long-term (8-week period) plasma cortisol levels were determined for each wether, using enzyme-linked immunosorbent assay (ELISA) to indicate presence or absence of stress. Faecal cortisol metabolite levels were analyzed for each wether as an enhanced method for estimation of stress, using an 11-oxoaetiocholanolone enzyme immuno-assay (EIA I) kit.

The test groups PPI and PPII with rumen impaction showed severe histological changes particularly in the mucosal wall of the rumen compared to wethers without impaction. The papillae observed in the test group of wethers were stunted, bent over, slender and constricted and this was more severe in the cranial and ventral sacs, with the extent of severity being correlated to the duration of impaction. Stereological analysis of the entire rumen from normal wethers in CPII indicated a total mean macroscopic surface area of $0.109m^2$. Microscopically, the absolute mean absorptive surface area of the entire rumen was $0.473 \pm 0.017m^2$ of which the ventral sac (VS) was the largest at $0.186 \pm 0.010m^{2,}$ then cranial sac AR at $0.101 \pm 0.010m^2$, the dorsal sac (DS) at $0.090 \pm 0.007m^2$, the caudoventral blind sac (CVB) at, $0.079 \pm 0.007m^2$ and the smallest was the caudodorsal blind sac (CDB) at $0.016 \pm 0.001m^2$. The total mean absolute volume of ruminal tissues was $538cm^3$ of which the mucosa, submucosa, muscularis interna, muscularis externa and serosa were $162cm^3$, $107cm^3$, $158cm^3$, $98cm^3$ and $13cm^3$ respectively. Stereological evaluations revealed that the rumen impacted with plastic bags had severe loss in

surface area and volume estimates of the absorptive mucosa, which was more evident in Phase II wethers (PPII). The mean surface area of AR, DS, VS and CVB in PPII wethers decreased by 50.8% ($p < 0.0001$), 29.6% ($p = 0.0227$), 41.1% ($p < 0.0001$) and 37.5% ($p = 0.0227$) respectively when compared to the corresponding ruminal sacs from NPPII. The mean absolute volume of mucosae in AR, DS, VS, CDB and CVB significantly reduced (52.4%, $p < 0.0001$; 55.1%, $p = 0.0006$; 32.3%, $p = 0.0427$; 58.8%, $p = 0.0004$ and 55.0%, $p = 0.0002$ respectively) compared to the corresponding sacs in NPPII. At 8 weeks post-impaction the total mean surface area and volume of mucosal tissue in the entire rumen from PPII reduced significantly (39.7%, $p < 0.0001$; 46.9%, $p = 0.0004$ respectively) compared to rumen from NPPII. The total mean surface area of the rumen from wethers in PPII, NPPII and CPII were $0.282 \pm 0.012 m^2$, $0.467 \pm 0.017 m^2$ and $0.473 \pm 0.017 m^2$ respectively. The mean absolute volume of mucosae in the entire rumen of PPII, NPPII and CPII were $82.7 cm^3$, $155.82 cm^3$ and $162.48 cm^3$ respectively. Thus absolute volume of mucosa in the rumen of wethers in PPII formed 17% of their entire rumen volume, while that of either NPPII or CPII formed 30% of their entire rumen volume. A lower value in body-mass-standardized total surface area of $0.0113 m^2 kg^{-1}$ was recorded in PPII compared to $0.0163 m^2 kg^{-1}$ obtained for NPPII. A lower value of $3.31 cm^3 kg^{-1}$ in body-mass-standardized total volume of rumen mucosa was obtained for rumen from wethers in PPII compared to $5.45 cm^3 kg^{-1}$ recorded in NPPII.

Plasma cortisol concentration significantly ($p < 0.05$) increased about 4-fold during the first 72 hours in wethers whose rumen were implanted with plastic bags. The effect of interaction between the experimental groups and time was also significant ($p = 0.0420$). The elevated levels in plasma cortisol persisted over the next three weeks then gradually declined over the remaining 5-week period that followed. Consequently, at the end of 8-week post-implantation, mean

plasma cortisol levels had reverted to normal range. However, there was significant ($p = 0.0070$) interaction between the different groups with time. The concentration of faecal cortisol metabolites in the test group PPI significantly increased over 5-fold ($p = 0.0023$) to 3-fold ($p = 0.0294$) from baseline values during the initial 24 hour and 72 hour period post-impaction respectively. This rise in FCM levels persisted over the next 4 weeks as well as in the 6^{th} week but levels declined to normal by the 8^{th} week of experimentation.

There were no significant differences in gross, histological and stereological parameters between the positive and negative control groups. However, the positive control group had about 1.5 times increased plasma cortisol concentration 6 hours after rumenotomy, but decreased to normal levels by 72 hours. From the current study it was concluded that prolonged impaction of the rumen of wethers with indigestible plastic bags is likely to cause the following outcomes which might subsequently diminish ruminal functional efficiency and loss of health in the animal:

a) Severe loss in body weights of wethers.
b) Severe histological changes in the ruminal mucosae of wethers.
c) Progressive loss in structural quantities in the ruminal wall the extent of which is related to the duration of impaction.
d) Severe damage of the absorptive surface area and loss in volume of tissues in the rumen particularly mucosal volume may impair rumen function consequently affecting body weight in ruminants.
e) Increased levels of plasma cortisol and faecal cortisol metabolite which is more acute than chronic is indicative of stress in rumen impaction.

Therefore ingestion of waste plastic bags by small ruminants should not be underrated, because it gradually leads to rumen impaction that induces stress and loss of body weight with subsequent

structural changes in ruminal tissues that overall leads to loss of health in the animal. Creating public awareness and educating the famer on the devastating effect of ingestion of plastic bags on ruminant's health and the proper disposal of waste plastic bags are recommended. This will improve productivity and help the farmer get better returns.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background information

Optimal digestion and absorption of food are essential processes for subsequent bioavailability of macro-and micronutrients that enable an animal to have normal physiological and health maintenance functions. In this regard, the ruminant fore-stomachs are unique in that, it enables the animal to utilize cellulose from forages and other feeds through the action of normal ruminal microbes. Ruminants, particularly sheep and goats often ingest non-biodegradable foreign materials when they scavenge on waste dumping sites or graze on pastures polluted with waste plastic bags. These indigestible materials gradually accumulate and lead to rumen impaction (Ghurashi *et al.,* 2009; Abebe and Nuru, 2011; Mersha and Desiye, 2012; Khurshaid *et al.,* 2013; Otsyina *et al.,* 2015). The indigestibility of such materials interferes with normal digestive functions by blocking the luminal space of the rumen and negatively affecting gastric enzyme secretion (Igbokwe *et al.,* 2003). This may subsequently affect certain physiological functions in the body and impact negatively on the health of the animal (Abebe and Nuru, 2011).

Studies have shown that cattle, sheep and goats reared in the urban and peri-urban environments, often suffer impaction of the rumen with ingested foreign bodies such as plastic bags which interfere with flow of ingesta (Khan *et al*., 1999). This results in rumen distension with subsequent scanty or absence of feces (Abdullahi *et al.,* 1984; Igbokwe *et al.,* 2003; Remi-Adewunmi *et al.,* 2004; Okai *et al.,* 2007). The indigestible materials become intertwined with ingesta into hard compacted masses under the strong rumen motility. The compacted masses

occupy space in the rumen hence reducing the ingesta substrate available for microbial activity (Khan *et al.,* 1999). These masses of indigestible foreign bodies also exert pressure against the ruminal walls, which may culminate into gross and histological changes of ruminal tissues (Bakhiet, 2008).

Information on quantitative structural changes in impacted rumen is previously unavailable. Stereology is a quantitative tool based on geometrical and statistical considerations that allows the attainment of unbiased estimates such as volume, surface, length and number of any structure of interest using thin histological tissue sections (Mayhew, 1979; Weibel, 1979; Baddeley *et al*., 1986; Andersen *et al*., 1992; Nyengaard, 1999; Howard and Reed, 2005; Tschanz *et al*., 2011). Stereological methods have been used extensively in biomedical research to quantitatively assess structural changes in normal and pathologic tissues and have given better understanding to structural functions of tissues, cells and organs (Makanya *et al*., 1995; Andrade *et al*., 2012; Nyengaard and Alwasel, 2014; Foldager *et al*., 2015). Hence the need to apply these methods to quantitatively assess structural changes in ruminal tissues of wethers whose rumen are impacted.

Interference with flow of ingesta as a result of ingestion and accumulation of waste plastic bags leads to anorexia (Igbokwe *et al.,* 2003). The long periods that these indigestible materials remain in the rumen result in prolonged interference with feed intake that subsequently affects the health of the animal (Igbokwe *et al.,* 2003). It may also lead to stress-related physiological effects. Stress is defined as the sum total of biological reactions from intrinsic and extrinsic stimuli that result in homeostatic disturbances (Chrousos and Gold, 1992). Any environmental or biological factor that continually stimulates the Hypothalamic-pituitary-adrenal axis (HPA) for several days will lead to a chronic increase in cortisol secretion. The chronic elevation of cortisol

levels will inhibit inflammatory processes and could subsequently make the animal more susceptible to pathogens (Spraker *et al.,* 1984; Lynch *et al.,* 1992). Therefore, impaction of the rumen with indigestible foreign materials may result in physiological processes that could lead to economic losses from reduced productivity and reduced reproductive performance. It may also result in the eventual death of the animal (Radostits *et al.,* 2000; Ramin *et al.,* 2008).

Moreover, presence of indigestible materials in the rumen and reticulum hampers the absorption of volatile fatty acids and consequently reduces the rate of animal fattening (Igbokwe *et al.,* 2003), which is a critical factor in sheep production. About 80% of the energy requirements of ruminants is met by the volatile fatty acids (Bergman, 1990), which are also probably involved in the control of appetite (Martin and Walkden-Brown, 1995), hence the health of a ruminant with rumen impaction may be affected. This study was therefore designed to determine structural changes in the ruminal tissues as well as determine physiological effects in wethers whose rumen were implanted with plastic bags.

Improper disposal of non-biodegradable materials particularly waste plastic bags continues to be an environmental concern in most urban and peri-urban areas where sheep and goats scavenge on waste dumping sites, mainly as a result of diminished grazing grounds. Disposal of waste plastic bags will continue to be a problem owing to their increased use (Ramaswamy and Sharma, 2011) as a means of carrying items purchased from shops, supermarkets and open market places in most African countries. Ruminants grazing in such environments are likely to ingest these plastic bags that will gradually accumulate to cause rumen impaction. Therefore it was pertinent to carry out a controlled experimental study to determine the effects of rumen impaction with plastic bags on rumen tissue and physiological processes in sheep. This study provides useful information

that can be extrapolated to what happens in the natural ingestion of indigestible foreign materials as well as prolonged rumen impaction with normal digestive feeds. Information from this study on the negative effect of rumen impaction on animal health would serve as a basis for educating farmers on the need for proper disposal of used plastic bags that will help the farmer get better returns. This study provides relevant information and data on structural quantities in the rumen of normal wethers which could serve as a basis for assessment of ruminal disorders. This study also provides baseline data for other aspects of future research.

1.2 Hypothesis

It was hypothesized that impaction of the rumen with indigestible plastic bags leads to structural changes in ruminal tissues and elevated cortisol levels in the animal that leads to disruption of physiological functions and the overall loss of the animal's health.

1.3 Objectives

1.3.1 General objective

The general objective of the study was to investigate the effect of rumen impaction with indigestible plastic bags on structural quantities in ruminal tissues and the levels of the stress hormone cortisol in wethers whose rumen were implanted with plastic bags.

1.3.2 Specific Objectives

a) To estimate quantitatively structural changes in ruminal tissues of wethers following experimental impaction of the rumen with plastic bags.
b) To analyze changes in plasma cortisol levels in wethers whose rumen were impacted with plastic bags as an indication of stress.

c) To determine alterations in faecal cortisol metabolite levels in wethers whose rumen were impacted with plastic bags as an estimator of stress.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Benefits and economic importance of livestock production

Livestock production is an important national resource which contributes not less than 40% of the agricultural gross domestic product (GDP) globally and in developing countries about 30% of agricultural GDP (World Bank, 2009). In Kenya, livestock production plays a key role in the country's economy by contributing about 10% of the annual gross domestic product (Nyariki *et al.,* 2009) and over 40% of agricultural GDP (Republic of Kenya, 2002). A readily accessible report of Kenya's livestock population in 2003 was estimated at 9 million zebu cattle, 3.5 million exotic and grade cattle, 9.9 million sheep, 11.9 million goats, 895,000 camels, 415,200 pigs, over 25 million chicken and 470,000 rabbits (Kiptarus, 2005).

Globally, livestock production serves as a source of employment for over 1 billion people and sustains the livelihoods of about 600 million smallholder farmers in developing countries (Perry and Sones, 2007). Livestock products contribute about one fifth of the global kilocalorie and about one third of protein consumption with variations between rich and poor countries (Rosegrant *et al.,* 2009). For example, some developing countries have their per capita meat consumption under 10 kg/year, compared with an average of 80 kg/year in developed countries, indicating an annual meat consumption ratio of about 1:8 between developing and developed countries respectively. This is mainly due to low livestock production in developing countries (Food and Agriculture Organization, 2013), thus the need for increased livestock production in these countries. Livestock production is multifunctional contributing to food and nutrition for

health (Randolph *et al.,* 2007; Food and Agriculture Organization, 2009), source of investment, security and a form of insurance in many developing countries (Pell *et al.,* 2010).

Current data by Food and Agriculture Organization (2013) continue to show a global increase in livestock production particularly in developing countries for various reasons, such as increased demand for meat, eggs and dairy products. Global trends predict that food demand for livestock products in sub-Saharan Africa and South Asia will double from about 200 kcal per person per day in the year 2000 to almost 400 kcal per person per day in the year 2050 (Thornton, 2010). Several evaluations reviewed by Thornton (2010) corroborate the fact that the increase in the demand for livestock products is heavily motivated by growth in human population, increased income and urbanization and this is expected to continue for several decades. Livestock production is therefore a major window of opportunity for smallholder farmers to improve the livelihoods of their households (Biradar *et al.,* 2013).

2.2. Importance of small ruminants in Africa

Small ruminants (sheep and goats) are of considerable importance in the developing countries for food (meat, milk), income and employment (Hailat *et al.,* 1993; Hailat *et al.,* 1996). Small ruminants in Africa represent about 21% of the world's small ruminant population. The sheep population in Africa represents 17% of their total world population while the goats represent 30% of their total world population (Ibrahim, 1998). More than 95% of the goat population is found in developing countries (Food and Agriculture Organization, 2006), where they provide subsistence for low income households from the sale of meat, skins, live animals (Morand-Fehr *et al.,* 2004) and wool products (Devendra and Mcleroy, 1987). Milk and meat products as a

source of animal proteins, supplement the nutritional needs of the rural population, particularly regions with rapid human population growth (Boyazoglu *et al.,* 2005; Devendra, 2007). A study by Ibrahim (1998) indicated the importance of animal proteins in the diets of pastoral people, which aids in correcting amino acid deficiencies particularly in small children within sub-Saharan Africa.

In Ghana, small ruminants are a major source of livelihood and food security for the smallholder farmers especially in the northern part of the country. These farmers depend on their herds for multiple reasons including; using them as a form of insurance, meat to celebrate religious festivals and primarily for sustenance during 'critical times' of food shortage (Amankwah *et al.,* 2012). It is similar in Kenya where apart from small ruminants being kept for tangible benefits such as money from their direct sale or sale of their products, they are also kept as assets in cases of emergencies, cultural and ceremonial purposes (Kosgey *et al.,* 2008).

2.3 Health risks to small ruminant production in developing communities

Urban and peri-urban dwellers as well as nomads raising small ruminants usually have a semi-residential system where animals are kept in the house (El-Amin, 1975). In the wet season, small ruminants utilize natural pastures and during the dry season they are moved from place to place in search of pastures and water (Mohammed, 1989; Verbeek *et al*., 2007). The long distance these animals travel in search of water and pasture in addition to stress from starvation affects their health (Nyariki *et al.,* 2009). In the process of searching for food and water, they are exposed to polluted environments where they may ingest waste plastic bags as well as other indigestible foreign bodies scattered particularly within the urban or peri-urban areas (Otsyina *et*

al., 2015). These may have negative effects on digestion, subsequently on the health of the animals and consequently reducing their productivity and impacting negatively on the livelihoods of the dependent households.

2.4 Ruminant digestive anatomy and physiology

2.4.1 Structure and function of ruminant stomach

The stomach of the ruminant is subdivided into four compartments namely, rumen, reticulum, omasum and abomasum. The rumen, reticulum and omasum form the fore-stomach, of which the tunica mucosa is covered with keratinized stratified squamous epithelium. In contrast, the tunica mucosa of abomasum is lined by a simple glandular epithelium similar to the stomach of monogastric species (Scala *et al.,* 2011) with the ability to secrete enzymes for digestion of ingesta that has already been broken down in the fore-stomach. Various mucosal projections are present in each fore-stomach compartment, which include ruminal papillae, reticular folds and omasal laminae respectively. The reticulum and omasum also have papillae in their mucosal lining (Yamamoto *et al.,* 1998). These morphological features of the mucosae play an essential role in rumination. The ruminal papillae help increase the surface area for biochemical activities such as absorption of volatile fatty acids, water and electrolytes (Dobson *et al.,* 1956; Henrikson and Habel, 1961; Cerny, 1977; Singh *et al.,* 1983). Presence of nitrous oxide (NO) in the fore-stomach mucosa prolongs the life of mucosal cells by delaying the onset of cellular apoptosis, hence playing a role in production of cell energy (Scala *et al.,* 2011).

The physical and biochemical processes related to rumination, including fermentation by symbiotic bacterial that digest cellulose takes place in the compartments of the fore-stomach.

The rumen which is the largest of the compartments is primarily for microbial fermentation. The ruminal microbes ferment and break down plant material into their carbohydrate fractions and produce volatile fatty acids (VFAs) such as acetate, priopionate and butyrate absorbed in the rumeno-reticular wall which provide as much as 70% of the ruminant's total energy requirements (Bergman, 1990). The reticulum is important for mechanical breakdown of ingesta while the omasum is essential for absorption of water and electrolytes in sheep and goats (Engelhardt and Hauffe, 1975).

2.4.2 Morphology of the rumen

The entire rumen is sac-like in shape with internal ruminal pillars dividing the rumen into 5 distinct compartments or ruminal sacs namely; atrium ruminis (cranial sac), dorsal sac, ventral sac, caudodorsal blind sac and caudoventral blind sac. Right and left longitudinal pillars border the ventral and dorsal sacs whereas well-developed cranial and caudal pillars border the atrium ruminis and the two blind sacs respectively (Yamamoto *et al.*, 1998). The internal or mucosal surface of the rumen has projections known as ruminal papillae which increase the absorptive surface area for biochemical activities such as absorption of volatile fatty acids, water and electrolytes (Steven and Marshall, 1970; Singh *et al*., 1983). In sheep the ruminal papillae are generally conical or tongue shaped (Poonia *et al*., 2011). The ruminal papillae however, show variations in size, shape and density between the different ruminal sacs (Dobson *et al*., 1956; Yamamoto *et al*., 1998; Liebich, 1999; Poonia *et al*., 2011). The ruminal papillae in the atrium ruminis are well developed (approximately 4.8 mm in height, 1.1 mm in width) and densely distributed (Berg and Edvi, 1976; Yamamoto *et al.*, 1998) followed by the ventral and caudal

blind sacs with a reduced papillae density in the dorsal sac (Dellmann, 1971; Tamate *et al*., 1971).

The morphology of ruminal mucosa is affected by both the dietary components as well as the internal environment of the rumen (Flatt *et al.,* 1958; Harrison *et al.,* 1960; Tamate *et al.,* 1962 and1963; Nockels *et al.,* 1966; McGavin and Morrill, 1976), which could affect other compartments of the fore-stomach (Harrison *et al.,* 1960; Lauwers, 1973). Study by Abdelaal and El-Maghawry (2014) indicated that a large size indigestible foreign body in the rumen leads to stunted and sloughed ruminal papillae. This may imply that the type of diet and its duration in the rumen has effects on function (Steele *et al.,* 2011; Liu *et al.,* 2013). Hence rumen impaction with indigestible materials such as plastic bags is likely to have effects on ruminal tissue and is a pertinent point of study.

2.4.3 Histological organization of the ruminal wall

The ruminal wall from external to internal is made up of tunica serosa, tunica muscularis, tunica submucosa and mucosa. Tunica serosa is rich in adipose tissue and covers the rumen except a portion of the dorsal sac which attaches to the abdominal roof from the oesophageal hiatus of the diaphragm where tunica adventitia is present (Mosimann and Kohler, 1990; Liebich, 1999; Dyce *et al.,* 2002). Tunica muscularis consists of a thicker inner circular and thinner outer longitudinal smooth muscle layer important for mechanical mixing of ingesta, removal of gas through eructation, contractions and also regurgitation (Liebich, 1999; Poonia *et al.,* 2011). Tunica submucosa consists of loose connective tissue. Tunica mucosa of the rumen is lined by keratinized stratified squamous epithelium (Ramkrishna and Tiwari, 1979; Scala *et al.,* 2011; Poonia *et al.,* 2011) responsible for important physiological functions such as animals' total

energy balance through the transport and metabolism of rumen-derived volatile fatty acids (VFAs) (Baldwin, 1998). Additionally, the rumen epithelium acts as a protective barrier between the rumen environment and portal circulation (Graham and Simmons, 2005).

2.4.4 Functional organization of ruminal mucosa

Morphologically, four distinct cell strata can be distinguished in the epithelium of the rumen mucosa. From the lumen surface to the basolateral membrane they include: stratum corneum, stratum granulosum, stratum spinosum, and stratum basale with each stratum performing a unique role to promote the overall structural function (Steven and Marshall, 1970; Graham and Simmons, 2005; Poonia *et al.,* 2011). The stratum corneum which is the most external cell layer in direct contact with the lumen comprises of flat, cornified keratinocytes that frequently undergo sloughing and replacement (Lavker and Matoltsy, 1970; Graham and Simmons, 2005). Its primary role is physical protection of underlying strata from the physical internal environment of the rumen (Graham and Simmons, 2005). Stratum granulosum, located beneath the stratum corneum, is comprised of granular cells and plays an important role in regulation of volatile fatty acid absorption. The stratum spinosum which is located next to the stratum granulosum consists of metabolically active cells. The primary role of stratum spinosum is metabolism of VFAs and subsequent transport of the end products to adjacent cells for cellular energy or to the adjacent stratum basale for extrusion of metabolites into arterial circulation (Graham and Simmons, 2005). The stratum basale is the layer of cells immediately adjacent the basal lamina and is in close contact with arterioles. It is comprised of columnar cells that rest on a basement membrane and are linked to stratum spinosum cells via desmosomes (Graham and Simmons, 2005). Similar to spinosum cells, stratum basale cells have larger nuclei and abundant mitochondria that

indicate their high capacity for metabolic activity and also replace the cells of other layers (Graham and Simmons, 2005; Steele *et al.,* 2011).

2.5 Effects of environmental pollution with plastics on ruminant health

2.5.1 Plastic waste generation

The use of plastic products including plastic bags has steadily increased globally, resulting in massive generation of plastic material waste, which eventually constitutes a great proportion of the solid waste in large cities of Sub-Saharan Africa (World Bank, 1996; Yankson, 1998). In Ghana polyethylene bags contribute not less than 70% of the plastic material waste generated in the municipal (Fobil, 2000). These polyethylene bags are in the form of "ice water" sachets, ice-cream wrappers, black and transparent plastic bags seen virtually everywhere in choked drains, heaped refuse by the roadside and dumping sites. These accumulated waste plastic bags pose a serious public health concern (Thompson *et al.,* 2009; Rustagi *et al.,* 2011) and are unsightly, thus having negative effects on tourism (Bashir, 2013).

2.5.2 Prevalence of indigestible foreign bodies in the rumen

Ruminants particularly sheep and goats reared in the limited land resource of the urban and peri-urban areas end up ingesting waste plastic bags scattered in the environment as they browse for feeds on the roadsides and scavenge on waste dumping sites. Numerous studies have established the prevalence of rumen impaction with indigestible foreign materials in ruminants, which also affects their health and production (Ghurashi *et al.,* 2009; Abebe and Nuru, 2011; Mersha and Desiye, 2012; Khurshaid *et al.,* 2013; Otsyina *et al.,* 2015).

A recent study in Kenya reported about 11% prevalence of indigestible foreign bodies in the rumen of sheep and goats of which plastic bags constituted 72% as the highest recovered foreign body (Otsyina *et al*., 2015). In Pakistan, about 60% occurrences of indigestible foreign bodies in the rumen and reticulum of cattle has been reported (Khurshaid *et al*., 2013). Prevalence of indigestible foreign bodies in the rumen has been reported at 20% in sheep in Nigeria (Igbokwe *et al*., 2003) and about 80% in goats in Sudan (Ghurashi *et al*., 2009). Impaction of the rumen with indigestible foreign bodies in ruminants have also been reported in Ghana (Okai *et al.*, 2007), Egypt (Abdelaal and El-Maghawry, 2014) and Ethiopia (Abebe and Nuru, 2011).

The pollution of the environment with plastic bags due to careless, inappropriate disposal or inadequate waste management systems has been incriminated as one of the main causes of rumen impaction (Mohammed, 2012; Abdelaal and El-Maghawry, 2014; Otsyina *et al.*, 2015). The plastics accumulate in the rumen due to their indigestible nature and may affect the health of the animal, subsequently decreasing productivity and reproductive performance (Igbokwe *et al.*, 2003; Abebe and Nuru, 2011; Khurshaid *et al.*, 2013). Occasionally, death may occur due to rumen impaction (Singh 2005; Kumar and Dhar, 2013).

2.6 Symptoms of rumen impaction with indigestible foreign bodies

Plastic bags are made mainly of polyethylene and polyvinyl chloride (Thompson *et al.*, 2009). The ingested polythene may hinder the process of fermentation and mixing of ingesta contents, which could lead to indigestion (Singh, 2005). If not removed through surgery, the effects may lead to deteriorating health which could result in death of the animal (Ghurashi *et al.*, 2009).

Clinically, impaction of indigestible foreign bodies in the rumen may be characterized by the animal developing inappetance, pale mucous membranes, reduced milk yield, cessation of rumination, reduced ruminal motility or atony and scanty feces (Vanitha *et al.,* 2010). Abnormal bulging of the left paralumbar fossa may be seen, which could signify rumen tympany or impaction (Ramaswamy and Sharma, 2011). Other additional symptoms include rough hair coat, intermittent rumen tympany and distended abdomen (Sileshi *et al.,* 2013). Abdelaal and El-Maghawry (2014) observed that foreign body impaction in ruminants led to depression, weakness, inappetence, ruminal atony, hard pelleted mucus coated feces and sunken eyes.

2.7 Stress in farm animals

Stress in farm animals is very common and this may be manifested in farming practices (Hough *et al.*, 2013). Selye (1946) defined "stress" as the disease of adaptation, where the mechanisms to cope with stressors become overextended and eventually break down. Since then, many definitions of stress have evolved and one of the authors defined stress at three levels with respect to how the animal responds to a stressor. These levels are stress, overstress and distress (Ewbank, 1985). In this regard, stress is when an animal copes with a stressor within its capacity at an adaptive and harmless level. Overstress is when the coping mechanism is extended, but still remains sufficient to counteract the stressor. Distress is when a stressor stretches the coping mechanism beyond its limits to the point where the response is non-adaptive and has effect on health of the animal (Ewbank, 1985; Moberg, 2000). Potent stressors such as disease, pain, aggression, restraint and chronic stressors such as isolation and social instability could affect normal physiological functions (Lay, 2000). However, the effect of stress on an animal depends on the type, duration, and intensity of the stressor along with susceptibility of the animal

(Ferguson *et al*., 2008). When duration of exposure to stressor is short, the animal copes better (Lay, 2000), possibly due to the stressor eliciting stress response by generating adaptive mechanisms. The source of stress could be deviations in physiological homeostatic, environmental or emotional conditions. The indigestibility of plastic bags causes them to remain in the rumen for long periods with eventual accumulation, could disturb homeostasis and induce stress in the animals. Sheep have been reported to be one of the species most sensitive to emotional factors (Stephens, 1980), hence the necessity to evaluate how impaction of the rumen with indigestible plastic bags influences stress.

2.7.1 Physiology of stress

The Hypothalamic-pituitary-adrenal (HPA) axis together with autonomic nervous system and behavioral adaptation, mediates stress responses (Manteuffel, 2002). The main active stress hormone in the HPA axis response for most mammals is cortisol (Mormede *et al.,* 2007), which is secreted by the adrenal cortex. Under the influence of a stressor the hypothalamus releases corticotropin-releasing hormone (CRH), which triggers the anterior lobe of the pituitary gland (pas distalis) to release adrenocorticotropic hormone (ACTH). The ACTH stimulates the adrenal cortex to synthesize and secrete cortisol. Increased concentration of plasma cortisol then inhibits further release of ACTH through negative feedback mechanisms (Norman and Litwack, 1987; Nussey and Whitehead, 2001; Smith and Dobson, 2002).

However, the continuous activation of this stress response becomes detrimental to the health of the animal (Wingfield, 2001). The prolonged increase in cortisol levels due to chronic stress (prolonged stressor), is detrimental to animal health because of suppressing inflammatory

processes and immune responses, which greatly increase animal susceptibility to pathogens (Spraker *et al.,* 1984; Lynch *et al.,* 1992). Cortisol has multiple effects particularly on metabolism, immune function, inflammatory processes, and brain function (Manteuffel, 2002). Therefore, uncontrolled prolonged elevation of cortisol levels may increase susceptibility to disease, decrease reproductive capacity and impair growth in mammals (von der Ohe and Servhee, 2002). It has been shown that starvation and cold exposure are some of the factors that stimulate HPA axis and have been associated with difficult parturition, relatively low birth weight as well as lamb deaths (Alexander, 1984). This implies that the concentration of glucocorticoids in blood is useful for stress assessment and has been used as an index of stress in animals in various studies (Boonstra *et al*., 1998; Hopster *et al.,* 2002; Hackländer *et al.,* 2003; Romero and Reed, 2005; Kitaysky *et al.,* 2007; Sheriff *et al.,* 2009).

2.7.2 Measurement of stress using plasma cortisol and faecal cortisol metabolites levels

Measurement of circulating cortisol in blood is universally accepted as the gold standard of evaluating stress and welfare in animals, since it reflects the responsiveness of the HPA axis to a stressor (Mormede *et al.,* 2011). Other biological samples such as saliva, urine, feces and milk have also been used successfully to measure HPA axis function as an estimate of stress (Palme, 2005; Cook, 2011).

Many studies have successfully used plasma or serum levels of cortisol or corticosterone to quantify the stress response in ruminants (Kannan *et al.,* 2000; Moolchandani *et al.,* 2008; Van de Walt *et al.,* 2009) (Table 2.1). Cortisol in circulation exists either bound to proteins or as unbound (free) form. At basal conditions about 90-95% of the total blood cortisol circulates in bound form, being tightly bound to proteins such as corticosteroid-binding globulin (CBG) and

albumins. The CBG binds more than 80% of blood cortisol with high affinity and low capacity, and albumin binds 10-15% of cortisol with low affinity and high capacity (Lewis *et al.*, 2005). The remaining 5% of the total blood cortisol is the only free form which is biologically active to permeate into cells (Rosner, 1990; Perogamvros *et al.*, 2012). The half-life of circulating cortisol is known to be about 100 minutes (Kerrigan *et al.*, 1993). The measurement of total plasma cortisol concentration or the active free (unbound) plasma cortisol has been shown to have excellent correlation (Greenwood and Shutt, 1992), thus any of the two forms could be assayed using enzyme-linked immunosorbent assays (ELISA) (Hay and Mormede, 1997). In general domestic animals have little corticosteroid-binding activity compared to humans (Gayrard *et al*., 1996; Breuner and Orchinik, 2002).

Although, plasma glucocorticoids are frequently measured as parameters of stress (Möstl and Palme, 2002; Mormède *et al.*, 2007), collection of blood samples in itself is a source of stress that may interfere with the results (Hopster *et al.*, 1999). Cortisol in blood should therefore be used for stress with caution because it fluctuates as time progresses (Möstl and Palme, 2002). Taking blood samples within 2 to 3 minutes of catching the animal then allowing a period of acclimatization to sampling procedures is recommended before actual experimental cortisol measurements. Advances in methodology have been made for these reasons and some authors have used other specimens such as saliva, milk, urine, hair and faeces (Berman *et al.*, 1980; Möstl and Palme, 2002; Palme, 2005; Mormède *et al.*, 2007; Cook, 2011; Peric *et al.*, 2013). Among these, faecal sample is preferred because of its additional merits, which include the fact that it can be collected easily and over long period of time, individuals can serve as their own controls using baseline values and its collection is nearly physiological feedback-free or stress-free and non-invasive (Möstl and Palme 2002; Palme, 2005).

Faecal cortisol metabolites (FCM) as parameters of stress have already been utilized in domestic livestock including ruminants such as cattle and sheep (Palme *et al.,* 1999 and 2000; Merl *et al.,* 2000; Möstl *et al.,* 2002; Touma and Palme, 2005). Two group specific 11-oxoaetiocholanolone enzyme immunoassays (EIA) developed by Palme and Möstl (1997) and Möstl *et al.* (2002) have been used successfully to quantify cortisol metabolites in the faeces of ruminants (Palme and Möstl, 1997; Palme *et al.*, 1999; Dehnhard *et al.,* 2001; Huber *et al.,* 2003; Touma and Palme, 2005; Pesenhofer *et al,* 2006; Kleinsasser *et al.,* 2010), cats and dogs (Schatz and Palme, 2001), ponies and pigs (Möstl *et al.*, 1999), horses (Merl *et al.*, 2000) and snowshoe hares (Sheriff *et al.*, 2010).

Cortisol is metabolized by the liver and excreted as conjugates through the kidney into urine or through the bile into the gut (Fig 2.1) (Möstl and Palme, 2002; Palme *et al.*, 1996). In the gut some of the conjugated metabolites are further metabolized, deconjugated by bacterial enzymes (Taylor, 1971; Palme *et al.*, 1996) and partially reabsorbed into enterohepatic circulation (Lindner, 1972; Palme *et al.*, 2005) and eventually cortisol metabolite is excreted in the faeces. Thus the metabolism of cortisol results in metabolites of cortisol in faeces and absence or very minimal of the actual cortisol molecule. A study by Palme and Möstl (1997) where 1g of ^{14}C-cortisol was intravenously administered to sheep, demonstrated that no actual cortisol molecules were excreted in the faeces. It was concluded from that study that it is metabolites of cortisol that are predominantly present in faeces, although significant differences exist in the particular metabolite formed depending on the species (Möstl and Palme, 2002). In ruminants, at least 21 cortisol metabolites have been detected in faecal samples using high performance liquid chromatography (HPLC) / mass spectroscopy (Möstl *et al.*, 2002; Palme *et al.*, 2005).

It is important to note that faecal cortisol metabolites which are actually a reflection of the status of blood cortisol level only become detectable in the faeces with some delay or time lag. This time lag depends mainly on the transient time of passage of cortisol metabolite from the duodenum to the rectum and this is species-specific (Palme *et al.*, 1996 and 2005). The time lag may range from less than 30 minutes to more than 24 hours depending on the species (Palme, 2005; Touma and Palme, 2005). In sheep, the time lag for the excretion of cortisol metabolites into faeces is about 12 hours (Palme *et al.*, 1999; Möstl and Palme, 2002; Touma and Palme, 2005).

Table 2.1: Results of experiments by various researchers over time showing plasma cortisol levels as a measure of stress in sheep under different stressors

Type of Experimental Test	**Blood Cortisol concentration (ng/ml)**	**Authors**
Artificial Insemination	50-350	Khalid *et al*., 1998
Basal	10-15	Parker *et al.,* 2003
Handling	22-78	Pearson *et al.*, 1977
Isolation	20-100	Apple *et al.,* 1993
Loading	2-13	Parrott *et al.*, 1998
Shearing	58-79	Hargreaves and Hutson, 1990
Shearing	20-80	Mears *et al.*, 1999
Transport	15-54	Parrott *et al.*, 1998
Transport	21-27	Hall *et al.*, 1998
Various	10-100	Mears and Brown, 1997
Weaning	9-15	Orgeur *et al.*, 1998
Weaning	7-29	Sowinska *et al.*, 2001

Adapted from Archer (2005)

Note: The concentration of cortisol in plasma ranged from 2-350 ng/ml

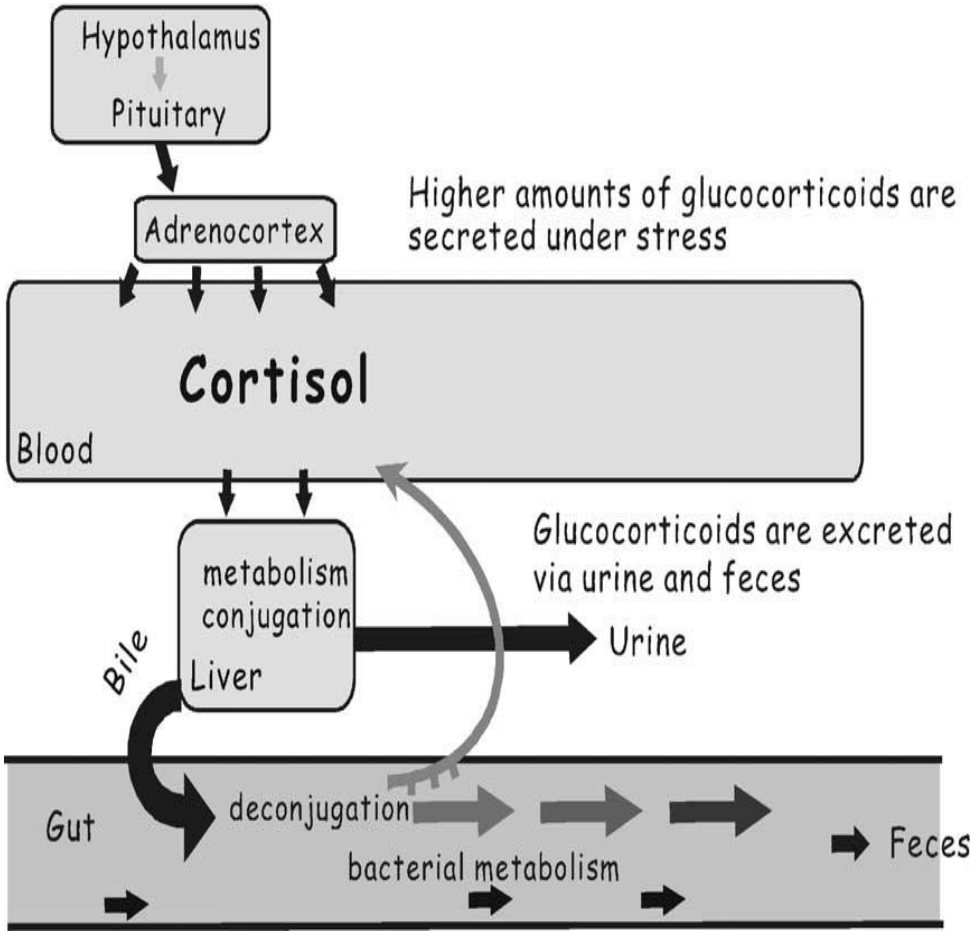


Figure 2.1: Pattern-flow for secretion, metabolism and excretion of glucocorticoids. Adapted from Möstl and Palme (2002).

2.8 Volatile fatty acids in ruminants

2.8.1 Volatile fatty acids as a primary energy source

Volatile fatty acids (VFAs) also known as short chain fatty acids (SCFAs), are low-molecular mass carboxylic acids; C_2-C_7 mono-carboxylic aliphatic acids (Siedlecka *et al.*, 2008). They serve as important intermediates or metabolites in biological processes. These include acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, 2-methylbutyric, hexanoic, and heptanoic acids. In addition, small amounts of other organic compounds, such as methane, carbon dioxide, lactate, and alcohol are produced in several parts of the gastrointestinal tract of humans and animals through the processes of microbial fermentation. Acetic, propionic, and butyric acids with carbon chain lengths of 2, 3 and 4 respectively are the predominant forms of VFAs produced mainly from the fermentation of plant materials, such as celluloses, fiber, starches, and sugars (Bergman, 1990). Ruminants (sheep, cow and deer) have the highest total VFAs concentration in the rumen relative to other mammalian species (Elsden *et al*., 1946). Furthermore VFAs are the primary energy source for ruminants (Bergman *et al.*, 1965), contributing about 70% of their daily energy requirements (Bergman, 1990).

2.8.2 Absorption of volatile fatty acids by ruminal epithelium

It is reported that approximately 88% of the total VFAs produced in the rumen are absorbed across the ruminal epithelium (Bergman, 1990; Kristensen *et al.*, 1998) via three known pathways which include, passive diffusion (Bugaut, 1987), bicarbonate-dependent active transport (Sehested *et al.*, 1999), and bicarbonate-independent active transport (Aschenbach *et al.*, 2009; Penner *et al.*, 2009). However, the type of feed plays a major role on the absorption of VFAs across the ruminal epithelium due to its influence on VFAs concentration and ruminal pH

(Allen 1997; Aschenbach *et al.,* 2011). Therefore an imbalance in the production and absorption of VFAs could lead to ruminal acidosis, which is detrimental to the health of the ruminants (Schurmann, 2013).

2.8.3 Volatile fatty acids analysis for assessment of ruminant health

Acetic acid or acetate is the main volatile fatty acid present in all mammals and it is also produced in higher concentrations than all other VFAs combined (Bergman, 1990). It is followed by propionate and then butyrate, although the type of diet can affect VFAs concentration. The molar ratios of acetate to propionate to butyrate vary from 75:15:10 to 40:40:20. Butyrate has been shown to have positive influence on rumen epithelial proliferation and rumen development (Mentschel *et al.,* 2001; Górka *et al.,* 2011) through the activation of hormones and growth promoters, including glucagon (Gálfi *et al.,* 1993), insulin (Sakata *et al.,* 1980; Gálfi *et al.,* 1993), insulin-like growth factor-1 (IGF-1) (Shen *et al.,* 2004), and epidermal growth factor (EGF) (Baldwin, 1999).

In addition, butyrate may possess anti-apoptosis factors, hence low ruminal butyrate concentration results in cell apoptosis, subsequently leading to degradation of the ruminal epithelium (Mentschel *et al*., 2001). Thus it is important to analyze VFAs in studies of health and diseases of the gastro-intestinal tract in ruminants (Tangerman and Nagengast, 1996) particularly when there is rumen impaction. Various metabolic diseases occur in ruminants as a result of types and toxic levels of feed, nutritional problems of inadvertent or unintentional feeding toxic substances. Analysis of Volatile fatty acids can be a reflection of the functional status of the rumen microbial population (Jagoš *et al.,* 1977; Jagoš and Dvorak, 1990; Dvorak *et al.,* 1997).

2.8.4 Determination of volatile fatty acids by gas chromatographic method

Chromatographic and Electromigration methods are the two most widely used methods for analyzing VFAs in veterinary and agricultural practices (Filipek and Dvorak, 2009). Among the chromatographic methods, filling and capillary gas chromatography (GC) are mostly used (Baše and Bartoš 1970; Kmošťák and Kolouch, 1988; Ceccon, 1990; Ewaschuk *et al.,* 2002; Diamantis *et al.,* 2005). Liquid chromatography (HPLC) is of less use (Mathew *et al.,* 1997; Wei *et al.,* 2001). Capillary isotachophoresis and capillary zone electrophoresis are the most widely used electromigration methods (Boček *et al.,* 1978; Buchberger *et al.,* 1997; Dušek *et al.,* 2004). A study carried out by Filipek and Dvorak (2009) comparing GC and capillary isotachophoresis analytical methods in determining VFAs in rumen liquid, yielded comparable results suggesting that any of the two methods can be used for practical diagnosis. However, GC method has an added advantage of clearly differentiating between the "*n"* and "*iso"* forms of butyric and valeric acids into separate peaks in VFAs assay which gives a reflection of the source of the VFA. Furthermore, GC methods are simple, reliable and have been in use for long.

2.9 Stereology as a quantitative tool for structural evaluation

2.9.1 Stereology and its parameters for quantitative study

Stereology is a mathematical tool used to extract information about a three dimensional structure from two dimensional images. In biomedical research the two dimensional images are in the form of thin histological sections prepared for light and/or electron microscopy (Mayhew, 1979; Nyengaard, 1999). Two principles are key in stereological methods to appropriately quantify morphological structures namely unbiased sampling (systematic uniform random sampling) and the use of updated geometric tools (test probes e.g. points, cycloids, lines), which are

superimposed on test structures to give estimates of the parameter being evaluated (Baddeley, 1993; Mandarim-de- Lacerda, 2003; Boyce *et al.,* 2010).

Stereological estimates of a parameter of interest in a structure are usually expressed as densities or ratios, i.e. quantities ‘‘per unit volume of reference space’’. For example volume density of mucosa in the rumen = volume of mucosa per unit rumen volume. The most frequently employed are densities per volume: volume density (Vv), surface density (Sv), length density (Lv), and numerical density (Nv). Densities per area are informative and can also be estimated as area density (AA) and numerical density per area (NA or QA) (Weibel, 1969; Pereira and Mandarim-de- Lacerda 2001; Hsia *et al.,* 2010). The total volume (reference volume) of the organ can be measured by Archimedes’ method (Akosman and Ozdemir, 2010) and Scherle's method (Scherle, 1970) followed by an appropriate stereological method to determine the volume of various subcomponents of the organ (Bolat *et al.,* 2011).

2.9.2 Qualitative studies of the rumen

The mucosal surface of the rumen of sheep is not smooth, but extensively covered by papillae which are of different size, shape and density in the various sacs or compartments. This is similar to all other ruminants except for ruminal pillars which are also papillated though these papillae are few and closely packed (Scott and Gardner, 1973). In the rumen, the shape of papillae varies from short tongue-like forms to large flattened foliate structures. Tongue-shaped papillae occur in regions of the rumen which tend to reach their greatest size and densely packed in the ventral and cranial sacs. The papillae in the dorsal sac are however, more flap-like in appearance and more widely spaced (Dellmann, 1971; Tamate *et al*., 1971; Scott and Gardner, 1973; Berg and

Edvi, 1976; Yamamoto *et al*., 1998; Poonia *et al*., 2011). Areas of the rumen wall with large numbers of papillae absorb more volatile fatty acids than areas with few papillae (Aafjes, 1967).

A study on the forestomach of the Japanese Serow *(Capricornis crispus)* revealed that the mucosal surface of the rumen has ruminal papillae densely distributed everywhere except on the ruminal pillars (Yamamoto *et al.,* 1998). The ruminal papillae are generally of the foliate or filiform type although slight differences may be present. The study further revealed that the ventral sac is sparsely arranged with small filiform papillae (approximately 1.9 mm in height, 0.6 mm in width). Additionally, papillae in the ruminal atrium or cranial sac are well developed (approximately 4.8 mm in height, 1.1 mm in width) and densely distributed. The papillae in the dorsal and caudodorsal blind sacs are of the intermediate type relative to the ruminal atrium and ventral sac. The study also showed that histologically the entire ruminal mucosa is covered with soft keratinized epithelium.

Further histomorphological study of the rumen of sheep confirms that the mucosal surface is populated with varied density, size and shape of papillae. The mucosa is lined by stratified squamous keratinized epithelium, but lamina muscularis mucosa is absent (Poonia *et al*., 2011). However, diet has been shown to have effects on histological structures of ruminant forestomach (Tamate *et al.,* 1963; McGavin and Morrill, 1976; Wang *et al.,* 2009), which may probably affect their optimum function. Nonetheless, there is paucity of quantitative data that depicts these changes in the rumen. There is the need for stereological study to quantitatively evaluate structural components and changes in ruminal tissues, particularly the ruminal absorptive mucosa in an impacted rumen.

2.9.3 Application of stereological methods in quantitative structural analyses

The upsurge in the use of stereological methods to quantitatively assess structural changes in both normal and pathologic tissues gives a better understanding to structure and function of cells, tissues and organs. A recent study successfully used design-based stereology to numerically evaluate cartilage tissue repair (e.g. hyaline, fibrocartilage and fibrous tissue) in goat (Foldager *et al*., 2015). The method highly supplements the existing descriptive semi quantitative histological scoring system for evaluating cartilage repair (O'Driscoll *et al.,* 2001; Roberts *et al.,* 2003; Grogan *et al.,* 2006; Mainil-Varlet *et al.,* 2010), which has poor comparability and reproducibility (de Groot *et al.*, 2005).

Stereology has been applied to estimate cell numbers, total volume, mean volume and mean height of enterochromaffin-like cells in the rat colon (Nyengaard and Alwasel, 2014). Andrade *et al*. (2012) also used stereology to evaluate the volume density of elastic fibres in the glans penis of normal young men which provided useful information in the assessments of patients with erection dysfunction. Stereological methods have also been used in the assessment of neuro-pathological lesions, which provides new strategies for the therapeutic management of afflicted patients (Manaye *et al.,* 2007). Furthermore, stereological methods have been used to effectively quantify the volume densities of the gastrointestinal epithelial cells secretin and gastric inhibitory peptide in prenatal development of the pig small intestines, which has brought better understanding and reduction of postnatal morbidity and mortality (Van Ginneken and Weyns, 2004). Early research by Makanya *et al.* (1995) effectively applied stereological techniques in estimating the functional surface areas of villi and microvilli as well as number of microvilli in

the intestines of three species of bats: *Epomophorus wahlbergi* and *Lisonycteris angolensis* (frugivorous) and *Miniopterus inflatus* (entomophagous).

CHAPTER THREE

3.0 STEREOLOGICAL EVALUATIONS OF RUMINAL TISSUES OF WETHERS IMPLANTED WITH PLASTIC BAGS IN THE RUMEN

3.1 Introduction

The rumen of sheep is the largest of the fore-stomach compartments with numerous mucosal projections known as ruminal papillae. The large sacculated structure of the rumen is divided by a series of pillars into 5 freely communicating yet discrete ruminal sacs; cranial, dorsal, ventral, caudodorsal blind and caudoventral blind sacs (Lentle, 1994). It plays a vital role in the microbial fermentation of forages and other feeds for the production and absorption of volatile fatty acids (VFA), which contribute about 70-80% of the total energy requirement for the animal (Bergman, 1990).

Nevertheless, the optimal function of the rumen may be compromised by ingestion and subsequent persistence of indigestible waste plastic bags in the rumen. Several studies have consistently corroborated findings that scavenging ruminants advertently or accidentally ingest indigestible foreign bodies which consequently leads to rumen impaction. Furthermore in most cases these indigestible foreign bodies recovered from the rumen are mainly plastic bags (Igbokwe *et al*., 2003; Abebe and Nuru, 2011; Abdelaal and El-Maghawry, 2014; Ostyina *et al*., 2015). The ingested indigestible foreign bodies gradually accumulate in the rumen over long periods and eventually are formed into hard compact masses which exert pressure on ruminal tissues. This may cause gross and histological changes in ruminal tissues particularly of the papillae and mucosa which overall may impair ruminal functional efficiency. Papillae vary in size, shape and density throughout the mucosal surface of the rumen (Dobson *et al.,* 1956;

Liebich, 1999) with the highest density in the atrium ruminis, ventral and caudal blind sacs (Berg and Edvi, 1976; Tamate *et al.*, 1971; Dellmann, 1971).

Stereology is a tool that has been extensively used in morphological studies to evaluate quantitative structural changes in organs, tissues and cells and has given a better understanding to these morphological changes. Hence the need to apply this tool to quantitatively assess structural changes in ruminal tissues of wethers whose rumen were experimentally implanted with plastic bags.

3.2 Materials and methods

3.2.1 Experimental animals and acclimatization

Thirty two (32) healthy castrated male sheep (wethers) aged between 12-15 months and weighing between 22-35 kg were purchased from Gicheha farm Nairobi-Kenya. All wethers were born and raised within the farm. The farm was chosen because the pastures were free of any pollution with plastic bags. The animals were clinically examined by a Veterinarian to ensure they were in good health and without any condition that would interfere with the experimental results. The wethers were transported to the Department of Clinical Studies, Faculty of Veterinary Medicine, University of Nairobi taking into consideration the requirements of the Animal Disease Act CAP 364.

They were housed in stalls at the Large Animal Unit of the Department for 5 weeks prior to commencement of the experiments, during which they acclimatized. During the acclimatization

period all wethers were de-wormed, randomly placed into their experimental groups, given identification by ear-tagging them, regularly restrained during which rectal temperature, pulse rate, heart rate and ruminal motility were taken to monitor their health as well as get them adapted to handling for the impending data and sample collection.

3.2.2 Experimental design

3.2.2.1 Pilot study

Two of the 32 wethers were used for a pilot study to give guidance on the approximate quantities of plastic bags to implant into the rumen for the actual experiment. The 2 wethers were ear-tagged as Pilot 1 and Pilot 2 respectively. Implanting of plastic bags in the rumen was done through rumenotomy. In Pilot 1, 200 pieces of plastic bags weighing 200g were implanted while in Pilot 2, 100 pieces weighing 100g were implanted. The wethers were housed together and observed for a period of 4 weeks post-implantation. During this period weight was taken and recorded once per week, rumen motility, rectal temperature, pulse rate and heart rate were also recorded twice weekly. Other parameters such as demeanor, appetite, posture, defecation, were also observed and noted. The animals were photographed to record their condition. They were fed with Rhodes grass hay, commercially produced concentrate meal (UNGA AFYA Meal®, UNGA Farm Care Ltd, Nairobi-Kenya), supplemented with mineral lick and provided with water *ad libitum*.

3.2.2.2 Experimental phases

The experiments were divided into two phases. Phase I experiments were terminated at 4 weeks after implantation of the plastic bags in the rumen (post-implantation) and Phase II experiments were terminated at 8 weeks post-implantation. Therefore the experiments had two endpoints, 4

and 8 weeks post-implantation respectively. At the endpoint for each phase, the wethers were euthanized to collect ruminal tissue for histological processing. A total of 30 wethers were used in two phases of which 15 wethers (n = 15) were assigned randomly to each phase. The 15 wethers in each phase were further divided into 3 groups of 5 each.

3.2.2.2.1 Phase I experimental wethers

The 15 wethers in Phase I were randomly divided into 3 experimental groups of 5 wethers each. Group 1 coded PPI (n = 5) was the test group whose rumen were implanted with plastic bags through rumenotomy. Group 2 coded NPPI (n = 5) was positive control group in which rumenotomy was performed but no plastic bags were implanted in the rumen. Group 3 coded CPI (n = 5), was negative control group in which neither rumenotomy nor implantation of plastic bags into the rumen was done. The wethers in each group were housed together for 4 weeks. All groups of wethers were fed with Rhodes grass, commercially produced concentrates (UNGA AFYA Meal®, UNGA Farm Care Ltd, Nairobi, Kenya), supplemented with mineral lick and provided with water *ad libitum*. The body weights of all wethers in each group were taken and recorded prior to beginning of the experiment and thereafter taken once per week for the 4-week duration.

3.2.2.2.2 Phase II experimental wethers

The 15 wethers in Phase II were randomly divided into 3 experimental groups of 5 wethers each. Group 1 coded PPII (n = 5) was the test group whose rumen were implanted with plastic bags through rumenotomy. Group 2 coded NPPII (n = 5) was positive control group in which rumenotomy was performed but no plastic bags were implanted in the rumen. Group 3 coded CPII (n = 5), was negative control group in which neither rumenotomy nor implantation of plastic bags into the rumen was done. The wethers in each group were housed together for 8

weeks. All groups of wethers were fed with Rhodes grass, commercially produced concentrates (UNGA AFYA Meal®, UNGA Farm Care Ltd, Nairobi, Kenya), supplemented with mineral lick and provided with water *ad libitum*. The body weights of all wethers in each group were taken and recorded prior to beginning of the experiment and thereafter taken once per week for the 8-week duration.

3.2.3 Rumenotomy and plastic bag implantation

3.2.3.1 Implanting plastic bags in the rumen of test groups Phase I and Phase II

The plastic bags that were implanted into the rumen of PPI and PPII groups of wethers were soft, thin, transparent non-perforated type (Malaika Poly Bags®, KEBS producers, Nairobi, Kenya) each measuring 215 mm × 360 mm and commonly used for packaging foods. From the outcome of the pilot study, it was decided that 150 pieces of these plastic bags weighing166g would be implanted in each wether of the test groups. The 150 plastic bags were removed from the packaging plastic bag packet, rolled and compacted length-wise. The whole roll of all the 150 pieces was cut transversely into two equal halves (Fig. 3.1) so that once implanted into the rumen, they were free to separate into individual pieces. Both halves were implanted into the rumen of each wether in PPI and PPII groups through rumenotomy. Rumenotomy was performed using standard procedure as described by Hendrickson (2007).

Post-operative penicillin-streptomycin (PenStrep® Norbrook, Ireland) was administered for 5 consecutive days to prevent possible infection and 20% phenylbutazone (Phenylbutazone® Agrar, Holland BV) was administered every alternate day for 1 week to manage pain. Aerosol Oxytetracycline HCL (Alamycin®, Norbrook, Kenya) was applied on the skin wound daily for

14 days. Wethers were given light diet of concentrate and hay for 3 days after the surgery then thereafter fed *ad libitum*. Skin sutures were removed on day 14 post-surgery.

Figure 3.1: Plastic bags implanted into the rumen of wethers in groups PPI and PPII of Phase I and Phase II respectively. A packet of 100 pieces of plastic bags is shown within the outer packaging packet (**A**). A total of 150 pieces of plastic bags with the outer packet removed (**B**). The 150 pieces rolled and compacted together (**C**).The compacted roll of 150 pieces cut into two equal halves (**D**) both of which were implanted in the rumen of each test group wether.

Key:
PPI = test group of wethers in Phase I experimentation whose rumen were implanted with plastic bags through rumenotomy
PPII = test group of wethers in Phase II experimentation whose rumen were implanted with plastic bags through rumenotomy

3.2.4 Measurement of body weights

Measurements of body weights (BW) of wethers in phase I and phase II groups were done using a 50kg capacity Salter Spring hanging type scale. Each wether was weighed while supported by a sling hooked to the weighing scale suspended to hang from an overhead metal bar. The animals were weighed in the mornings before feeding. Just before implantation of plastic bags in the rumen of each wether, the body weights were recorded as baseline values. After implantation of plastic bags, the body weight of each wether was taken at the beginning of each week up to the 4^{th} week for phase I and 8^{th} week for phase II. The latter measurements were post-implantation BW measurements.

3.2.5 Experimental endpoint-harvesting of tissues for macroscopic, histological and stereological analysis

At the completion of the 4 and 8 week post-implantation endpoints respectively, each wether was humanely euthanized with an overdose of 20% sodium pentobarbital given intravenously. The abdomen was immediately opened through a penetrating full-length ventral midline incision and the pelvis was also opened by cutting through the pubis to expose the terminal part of the alimentary tract. The gastrointestinal tract including the fore-stomachs were removed from abdominal cavity within a short time of opening the abdomen by severing the oesophagus several centimeters cranial to the diaphragm and the terminal part of rectum.

3.2.5.1 Harvesting of rumen tissues

The fore-stomach was freed from intestines at the pyloro-duodenal junction and from the oesophagus about 5 cm from the cardia. The weight of the whole fore-stomach (rumen, reticulum, omasum and abomasum) with its content was measured and recorded. All omental

and fibrous attachments around the cranial, caudal, left and right longitudinal grooves, dorsal and ventral coronary grooves of the rumen were also detached to clearly delineate all grooves. The whole structure was photographed.

The fore-stomach was placed on a dissecting table with the visceral side facing up. A long incision to open the rumen was made into the dorsal sac beginning from the caudal end of the cranial groove just dorsal to the left longitudinal groove and continued to the cranial end of the caudal groove. The rumen with contents was examined, photographed and emptied. While all parts of the fore-stomach were still attached together, incisions were made into the reticulum, omasum and abomasum to open each of them and evacuate them of their contents. Each of the fore-stomach compartments was washed thoroughly in clean water initially, followed by washing in phosphate buffered saline (pH 7.2-7.4) and dried gently with gauze towels. The rumen was separated from the rest of the fore-stomach compartments by cutting through the rumeno-reticular junction. It was weighed and photographed with mucosal surface facing upwards. The 5 different sacs of the rumen were dissected to separate them from each other using the procedure described by McGavin and Morrill (1976) with modification (Fig 3.2). The sacs were: the cranial sac or atrium ruminis (AR), dorsal sac (DS), ventral sac (VS), caudodorsal blind sac (CDB) and caudoventral blind sac (CVB). The cranial sac (AR) was separated from the dorsal sac by cutting lateral to the cranial coronary pillar. The CDB and CVB were dissected by cutting around the periphery cranial to the dorsal and ventral coronary pillars leaving the caudal pillar attached to the ventral sac. The CDB and CVB with their respective coronary pillars attached allowed orientation into their correct anatomical position. The VS was separated from the DS by cutting just dorsal to the right longitudinal and cranial pillars. The VS was identified with the right and left longitudinal pillars as well as cranial and caudal pillars which guided correct anatomic

orientation. The rumen sacs were post-fixed in 10% Neutral buffered formalin (NBF) fixative in individually labelled containers for ease of identification.

3.2.5.2 Measurement of reference volume and macroscopic surface area of ruminal sacs

The reference volume (Vref) of each ruminal sac: AR, DS, VS CDB and CVB fixed in 10 % NBF solution was determined using the method described by Scherle (1970). In this method, a 500 ml pyrex beaker was partially filled with 10% NBF (fixative) solution and placed on a digital weighing scale (Mettler® PM4600 DeltaRange, Switzerland). A thin metal rod clamped to a laboratory stand was submerged in the fluid and weighing balance turned to zero. Excess fluid on the surface of post-fixed ruminal sac was wiped off then hooked under the metal rod and completely submerged in the fluid without tissue touching the sides of the beaker. The weight recorded in grams on the electronic weighing balance corresponded to the reference volume in millilitres or cubic centimetres of the specific rumen sac such as the reference volume of cranial sac [$Vref_{(AR)}$].

Thus reference volume of each sac was obtained as:

$$\mathbf{Vref_{sac}(cm^3) = Weight_{sac}(g)}$$

Where;

$Vref_{sac}$ = the reference volume of the ruminal sac in cubic centimeters

$Weight_{sac}$ = weight of the ruminal sac in grams

The macroscopic surface area of each ruminal sac was then estimated using the point-associated counting method. In brief, to estimate the area of each ruminal sac a transparent counting grid with test points (+) printed on it which had a value representative of the area associated with a

test point, was randomly, yet completely superimposed on the mucosal surface of each ruminal sac. The number of test points hitting the surface of the ruminal sac was counted. The total area of each ruminal sac at the macroscopic level was then estimated by multiplying the total number of test points counted on each ruminal sac by the area associated with the test point on the counting grid. The total surface area of the entire rumen for each animal was obtained by adding the estimated values of each ruminal sac.

Thus the macroscopic surface area of each ruminal sac was obtained as;

$$\mathbf{S_m} = \sum \mathbf{P} \times \mathbf{a}(\mathbf{p})$$

Where $\mathbf{S_m}$ = Surface area of ruminal sac at the macroscopic level

$\sum \mathbf{P}$ = total number of test points counted

$\boldsymbol{a}(\mathbf{p})$ = area associated with a test point

3.2.5.3 Sampling of the ruminal sacs for tissue processing and histological evaluation

Each sac of the rumen namely: Atrium ruminis (AR), Dorsal sac (DS), Ventral sac (VS), Caudodorsal blind sac (CDB) and Caudoventral blind sac (CVB) were sampled in a systematic uniform random manner as the example described below for the dorsal sac . The rest of the sacs were sampled in a similar procedure.

Dorsal sac:

The dorsal sac (DS) was placed on a dissection table with the mucosal surface facing up. It was cut serially in a vertical plane into long slices of tissue at intervals of 5 mm apart, in which the first cut was randomly made between 1-5 mm from the periphery of the tissue. The vertical slices of the sac were further cut serially again in a horizontal plane at intervals of 3 mm with the first cut starting between 1-3 mm to ultimately end up with smaller rectangular slices of the dorsal sac

(Fig 3.3). From the total number of the rectangular blocks of tissues obtained, 5 were selected for tissue processing through a systematic sampling pattern. The first block of tissue was randomly picked from among the first five blocks when a concealed number between 1 and 5 was selected. Thus the selected number determined the start position for picking the first block. Subsequently the 4 additional tissue blocks were selected from the remaining lot by picking every 5^{th} on the serial count after picking the first block. Counting every fifth block of tissue was done serially through each row alternating directions between left and right for the successive rows. For example row number one was sampled from left to the right, row number two from right to the left and the direction continued alternately through the rows until the required number of tissue blocks was reached. Each of the rumen sacs was sliced and sampled as described for the dorsal sac. Therefore, each rumen had a total of 25 tissue blocks.

The 5 blocks of tissue from each ruminal sac per wether were placed as a group in individually labelled appropriate containers with 10% NBF solution and stored until processing time.

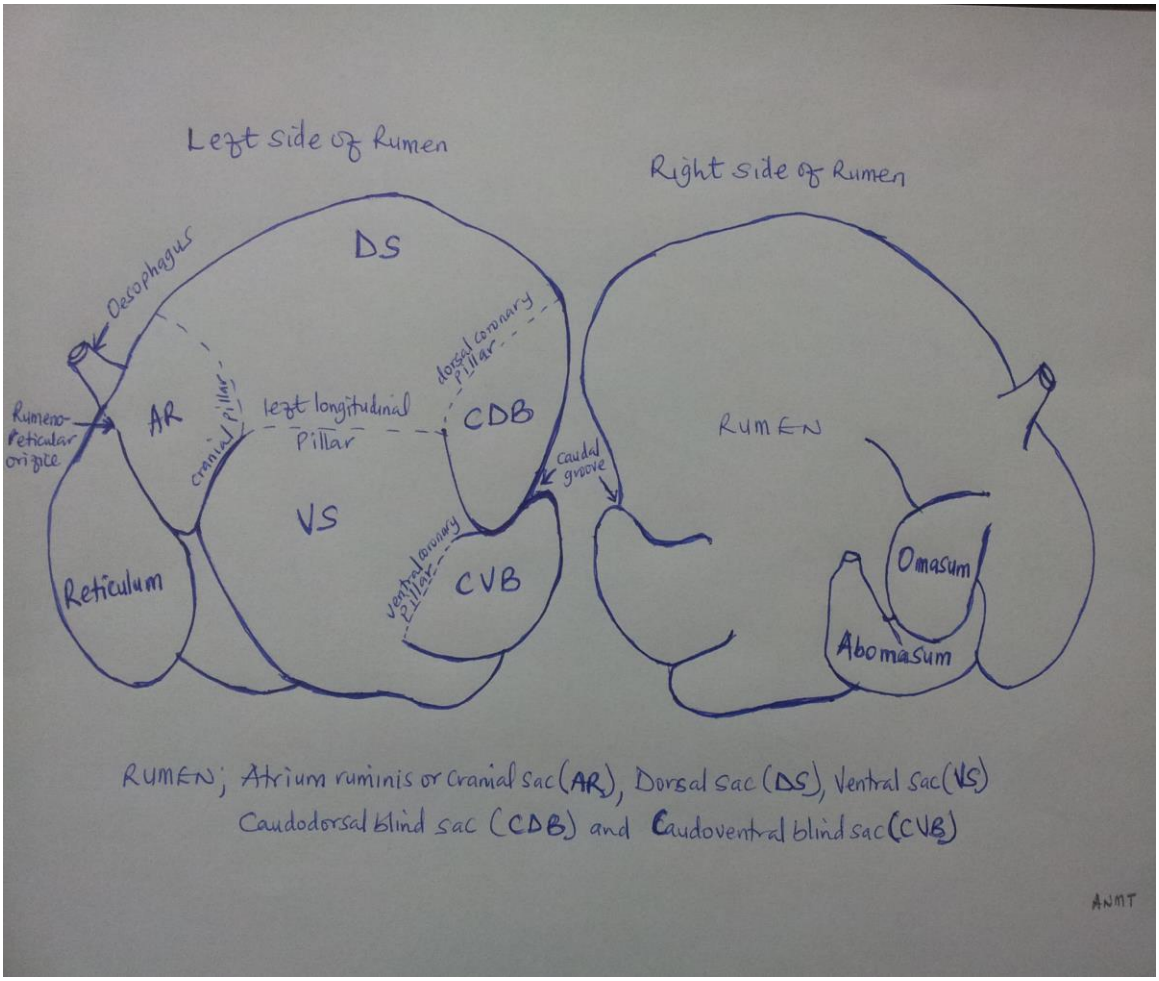


Figure 3.2: Sketch of fore-stomach showing the five ruminal sacs of the rumen clearly demarcated from each other with dotted lines along their respective ruminal pillars. The ruminal sacs are abbreviated as indicated; atrium ruminis or cranial sac (AR), dorsal sac (DS), ventral sac (VS), caudodorsal blind sac (CDB) and caudoventral blind sac (CVB).

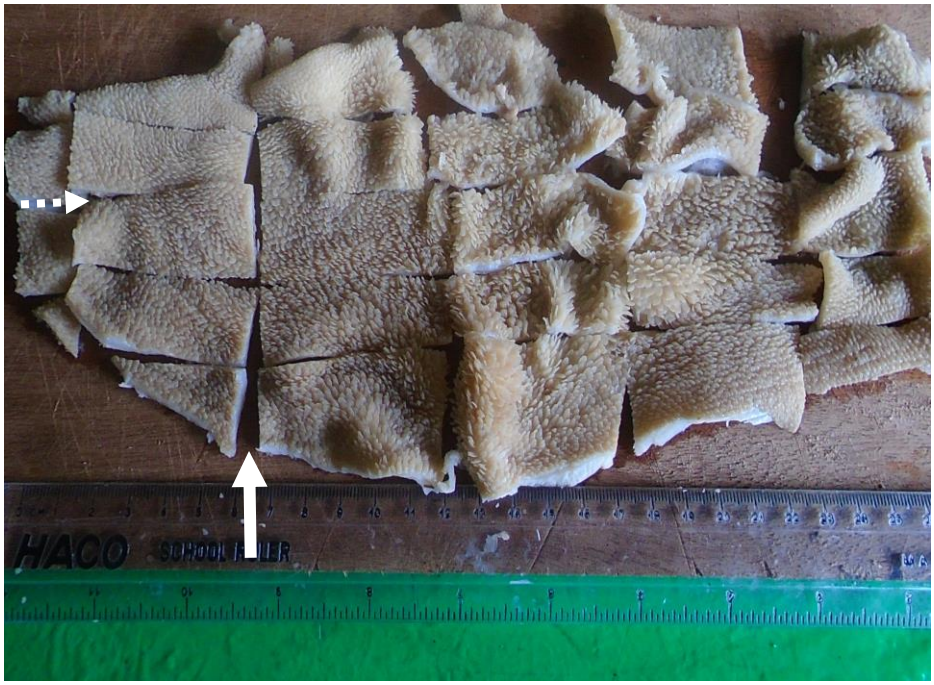


Figure 3.3: A ruminal sac cut into rows of large serial slices in a vertical (bold arrow) and a horizontal plane (dotted arrow).

3.2.5.4 Tissue processing for light microscopy

All the fixed blocks of ruminal tissues were trimmed to thin pieces of 2 mm thickness, which were processed following routine standard histological procedures (Smith and Bruton, 1977). In summary, the 5 tissue blocks from each ruminal sac clearly labelled were washed overnight under running water to initiate tissue processing. Dehydration and clearing steps of tissue processing were accomplished using an automated LM tissue processor (Shandon-Elliot Duplex Processor®, Shandon Scientific Co. Ltd, England). The tissues were embedded in paraffin wax in such a way as to make it possible to obtain vertical section profiles for histological and stereological evaluation as described by Baddeley *et al.* (1986). This was done by rotating the tissue blocks about their vertical axis before embedding in paraffin. Tissue sections 5-7 microns thick were cut with a microtome. For each block of the rumen sac 3 sections were obtained at a regular interval of 100microns. A total of 15 tissue sections per ruminal sac were collected, mounted onto microscope slides, appropriately labelled and processed for staining. The sections were stained with Hematoxylin-Eosin (H&E) following standard histological procedures (Smith and Bruton, 1977). By use of light microscopy, photomicrographs were obtained for histological evaluation and stereological analysis.

3.2.5.5 Photomicrographs of tissue sections and histological evaluations

Photomicrographs of H&E stained tissue sections of the rumen were acquired and processed using Leica Application Suite LAS EN software (version 1.8.0, Switzerland) installed in a computer. The images of tissue sections were obtained at a lower magnification of ×4 objective lens of Leica microscope which was coupled to a Leica camera connected to the computer. The images were then acquired as photomicrographs in jpeg format, processed by labelling

appropriately and stored into a folder on a laptop computer for stereological analysis. A total of 8 processed photomicrographs were obtained for each ruminal sac for histological examination as well as estimation of volume and surface parameters of the rumen. Photomicrographs of each of the five ruminal sacs were compared between experimental groups of wethers in Phase I and Phase II.

3.2.6 Estimation of volume and surface parameters of ruminal sacs

Photomicrographs of the vertical stained sections of ruminal sacs of wethers were used to estimate volume densities of tunica mucosa, tunica submucosa, inner and outer layer of tunica muscularis using a point-count stereological grid. The surface density of the ruminal mucosa was estimated using a cycloid test system. These test systems/grid and stereological estimates were obtained using STEPanizer® software developed by Tschanz *et al.* (2011) and the procedure for counting individual parameters were followed as described in the flow chart of the manufacturers manual (Appendix 1).

From outcome of the pilot study, 8 photomicrographs or images per ruminal sac of every wether were used to quantify volume and surface parameters. This gave 100–200 counts per estimate for surface density and 200–400 counts per estimate for volume density (Gundersen, 1988). Thus a total of forty, two-dimensional histological images representing the 5 ruminal sacs per animal were used for stereological estimations. Briefly, an image file of a ruminal sac was selected and displayed in the "*counting window"* of STEPanizer stereological tool installed in a computer and the appropriate test system was superimposed on the scaled image. A region of interest in the

two-dimensional histological image was counted by hitting a number on the numerical pad of the keyboard that had been configured to that particular region.

For surface density estimates a cycloid test system was superimposed on the projected image of ruminal sac with the vertical axis of the tissue and lattice of test system running parallel (Baddeley *et al.,* 1986). The vertical axis of the tissue was identified as the long axis of the ruminal papillae. The number of intersections (crossings) of the cycloid grid with the boundary of the papillary surface of the ruminal sac was counted (Fig. 3.4). This was done for all 8 images per rumen sac of each animal. Values were calculated and expressed as mean values per experimental group.

"Surface Density" of each ruminal sac was calculated as follows:

$$\mathbf{Sv = 2 * \frac{\sum I}{l/p * \sum P}}$$

Where;

Sv = Surface density of the ruminal sac
∑I = the total number of intersections between the cycloid test lines and the boundary of the papillae surface for the ruminal sac
l/p = the test line length per point
∑P = the total number of points hitting the ruminal surface

"Surface Area" of each ruminal sac was calculated as follows:

$$\mathbf{Ssac = Sv * Vref}$$

Where;

Ssac = Surface area of the ruminal sac
Sv = Surface density of the ruminal sac
Vref = Reference volume of the ruminal sac

"*Total surface Area*" *of the entire rumen per animal was calculated as follows:*

$$\mathbf{S_{Tot} = S_{AR} + S_{DS} + S_{VS} + S_{CDB} + S_{CVB}}$$

Where

S_{Tot} = Total surface area of the entire rumen per animal
S_{AR} = surface area of cranial sac of rumen
S_{DS} = surface area of dorsal sac of rumen
S_{VS} = surface area of ventral sac of rumen
S_{CDB} = surface area of caudodorsal blind sac of rumen
S_{CVB} = surface area of caudoventral blind sac of rumen

Volume density of histological layers in the wall of ruminal sac was estimated using point-count test grid of STEPanizer that was superimposed on the projected image (Fig. 3.5). Each point falling in the particular layer was counted using the numerical pad on the computer keyboard to generate individual volume fraction or density (Vv) in the rumen wall. The Vv was determined by dividing the number of points falling in the particular layer in the rumen wall by the number of points falling in the entire rumen wall (Gundersen *et al.,* 1981) and expressed as percentage.

Volume density was calculated as follows:

$$\mathbf{Vv(layer) = \frac{\sum Pt(layer)}{\sum Pt\ (ref\ space)} * 100\%}$$

Where

Vv(layer) = volume density of tissue in the wall of the ruminal sac
$\sum$Pt(layer) = total number of points falling in the tissue layer in the rumen wall
$\sum$Pt (ref space) = total number of points falling in the rumen wall

Absolute volume of tissue in the wall of the ruminal sac was estimated as follows:

$$\mathbf{Vsac = Vv * Vref}$$

Where;

Vsac = absolute volume of tissue (e.g. mucosa) in the ruminal sac
Vv = volume density of tissue (e.g. mucosa) in the wall of ruminal sac
Vref = reference volume of ruminal sac

Total absolute volume of tissue in an entire rumen per animal calculated as follows:

$$\mathbf{V_{Tot}(mucosa) = V_{AR} + V_{DS} + V_{VS} + V_{CDB} + V_{CVB}}$$

Where

V_{Tot} = Total absolute volume of tissue (e.g. mucosa) in the rumen
V_{AR} = absolute volume of tissue in the cranial sac
V_{DS} = absolute volume of tissue in the dorsal sac
V_{VS} = absolute volume of tissue in the ventral sac
V_{CDB} = absolute volume of tissue in the caudo-dorsal blind sac
V_{CVB} = absolute volume of tissue in the caudo-ventral blind sac

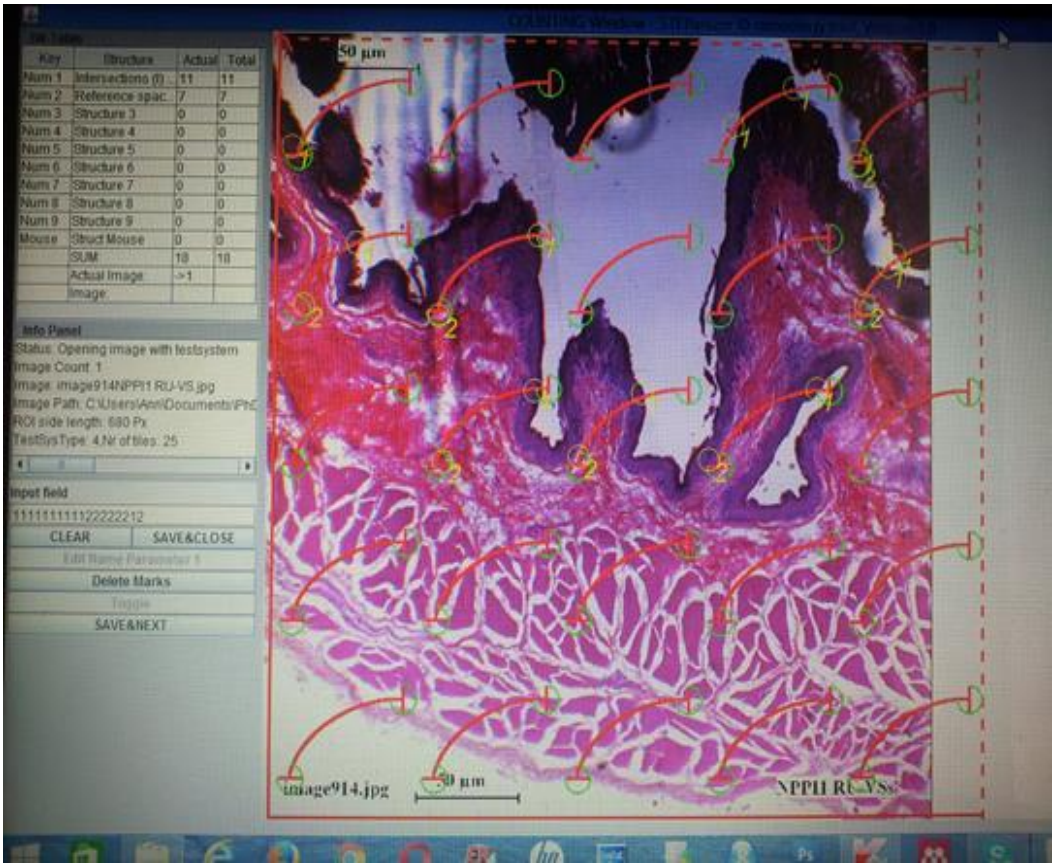


Figure 3.4: Counting Window of STEPanzier® sterological tool with a projected LM image of a ruminal sac stained with H&E. Cycloid of 25 arcs (red arcs with green points) superimposed on image counting frame in red (solid and dashed red lines). Intersections of cycloid arc with the papillary surface (projections) marked as 1 (yellow circle) correspond to counting points for surface density estimates. The 'Hit−Table' on top left shows the coupling of a structure parameter with the corresponding numerical keys 1-2 and already counted hits.

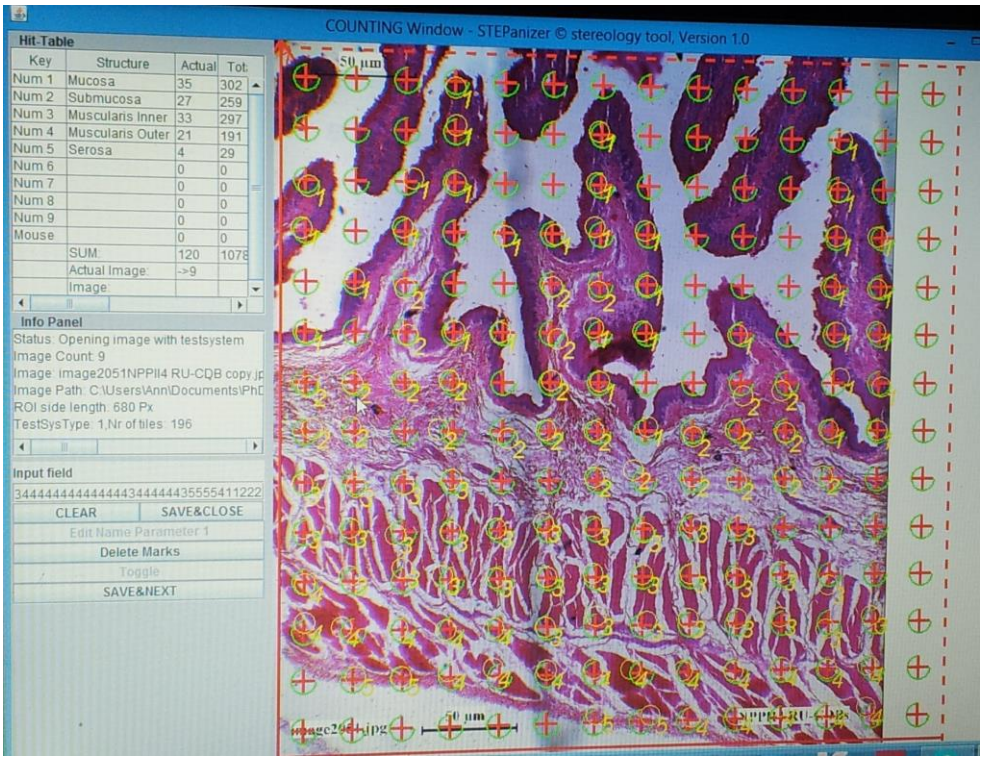


Figure 3.5: Counting Window of STEPanzier® sterological tool with a projected LM image of a ruminal sac stained with H&E. A test grid (red crosses encircled green) superimposed on image for stereological volume estimates. Points on structures are recorded by typing the corresponding numeric key (1-5). Recorded hits are displayed in the 'Hit-Table' on top left.

3.3 Data management and statistical analysis

The raw data collected for all parameters were verified, validated and entered into Microsoft Office 2010 excel spread sheet. The data sets were coded with letters and numbers representing each sample in an experimental group. Data were managed in Excel 2010 and calculation values obtained for each data set were expressed as mean ± S.E.M. The data obtained were imported into GraphPad Prism software version 6.0 (GraphPad Prism Statistical Software, Inc. California, USA) for graphical presentation and statistical analysis. One-way and Two-way Analysis of variance (ANOVA) with Tukey's Multiple Comparison (Post hoc) Test were performed using GraphPad Prism software (version 6.0) to compute associations and comparisons between the different parameters and experimental groups. Comparisons of the means were considered significant at the level of $P < 0.05$.

3.4 Ethical approval

The treatment of animals and experimental set up of the present study was approved by the Biosafety, Animal use and Ethics Committee of the Faculty of Veterinary Medicine, University of Nairobi, Kenya as per the attached letter (Appendix 2).

3.5 Results

3.5.1 Measurements of weekly body weights of wethers in Phase I and Phase II

The results of mean body weights of 3 groups of wethers in Phase I and Phase II experiments measured weekly are summarized and presented as tables and figures in sections 3.5.1.1 and 3.5.1.2 respectively.

3.5.1.1 Effect of rumen impaction with plastic bags on body weight of wethers in Phase I

The effect of impaction of the rumen with plastic bags on body weight of wethers measured weekly over 4 weeks post-implantation is summarized as mean ± SEM and presented in Figure 3.6. The differences in mean body weights between the 3 experimental groups over the 4 weeks period are shown in Table 3.1. There was a consistent decrease in the weekly mean body weight of wethers with plastic bags in the rumen (PPI) over the 4 week post-implantation period (Fig. 3.6). After 2 weeks post-impaction group PPI had lost 11.6% of its weight from baseline (Table 3.1). At the end of experimentation the reduction in mean body weights of the test group (PPI) was 10.1% compared to baseline value (Table 3.1). Wethers which had rumenotomy but no plastic bags implanted in the rumen (NPPI) showed a decline of 4.1% in mean body weights from baseline values at week 2 post-implantation, but the mean body weights increased thereafter.

At the experimental endpoint group NPPI lost 2.3% of its mean body weight from baseline value (Table 3.1). Wethers which had neither rumenotomy done, nor plastic bags implanted in the rumen (CPI) gained weight over the 4 weeks period, gaining up to 4.3% of their initial mean body weights at the end of experiment. Using the Tukey's multiple comparison test of two-way ANOVA, it was found that at 4 weeks post-implantation, there were significantly lower mean body weights in the test group of wethers (PPI) compared to the negative control group of

wethers (CPI) ($p = 0.0298$). Furthermore, at 4 weeks post-implantation there were also significantly lower mean body weights in the test (PPI) compared to the positive control group wethers (NPPI) ($p = 0.0466$) (Table 3.1).

Table 3.1: Weekly mean body weights of three groups of wethers with or without rumen impaction with plastic bags during the 4-week post-impaction period of Phase I experiment

Time (Weeks)	**Weekly mean (± SE) body weights of wethers in kg**			***P* value**
	PPI (n = 5)	**NPPI (n = 5)**	**CPI (n = 5)**	
0	26.1 ± 0.9	28.2± 1.6	26.7 ± 0.9	0.9293
1	24.7 ± 0.9	27.4 ± 1.6	26.8 ± 0.9	0.4134[a]
2	23.1 ± 0.8	27.0 ± 1.6	28.0 ± 0.8	0.0114[a]
3	24.0 ± 0.6	26.5 ± 1.7	27.7 ± 0.6	0.0709
4	23.5 ± 0.6	27.5 ± 1.9	27.8 ± 0.7	0.0298[a] 0.0466[b]

Data are expressed as means. Significance at $p < 0.05$

[a]*P*-value = test group PPI compared with negative control group CPI

[b]*P*-value = test group PPI compared with positive control group NPPI

Key:

PPI = test group of wethers whose rumen were implanted with plastic bags through rumenotomy

NPPI = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen

CPI = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

kg = kilogram

n = number of wethers in the group

± SE = plus or minus standard error of mean

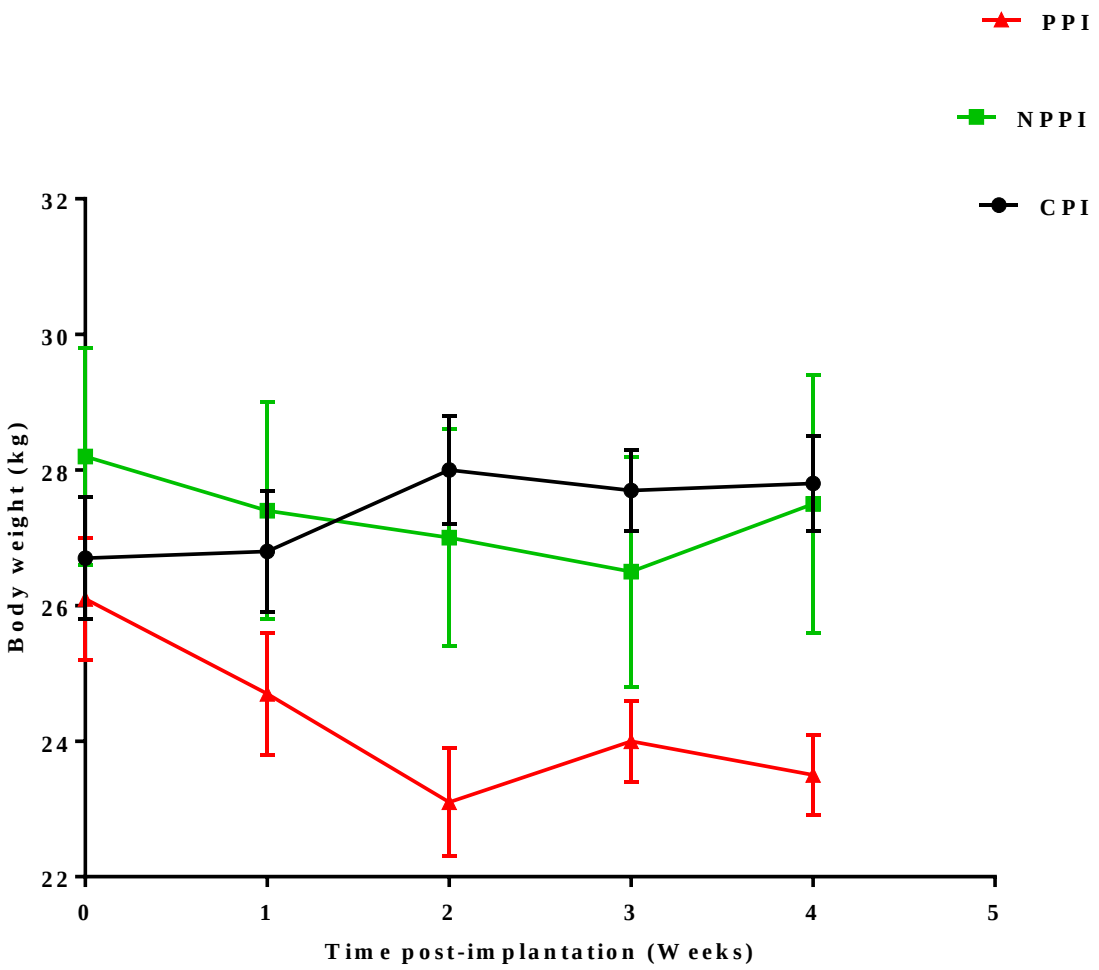


Figure 3.6: Weekly mean body weight fluctuations in three groups of wethers during the 4 weeks of Phase I experiment. The results showed a significant loss in body weight of PPI

Key:
PPI = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPI = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen
CPI = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.
kg = kilogram

3.5.1.2 Effect of plastic bags implanted in the rumen on body weights of wethers in Phase II

The effect of rumen impaction with plastic bags on the body weight of wethers taken weekly over 8 weeks after implantation are summarized as mean ± SEM and presented in Figure 3.7. The differences in mean body weights between the 3 groups over 8 week period are also shown in table 3.2. Consistently, the mean body weight of wethers implanted with plastic bags in the rumen (PPII) decreased over the 8 weeks post-implantation period. By week 2 group PPII had lost 11.0% of its mean body weight compared to the baseline weight and this weight loss continued over the remaining 6 weeks period (Fig. 3.7). By the end of experimentation, in the test group PPII mean body weights had decreased by 13.1% from the baseline weights. Similarly, wethers which had no plastic bags in their rumen but underwent rumenotomy (NPPII) at week 2 showed a decrease in mean body weight of 6.1% compared to baseline weights. Nonetheless, their mean body weight increased over the remaining 6 weeks and at endpoint they were 3.3% heavier than the baseline weights (Fig. 3.7). Wethers which neither had plastic bags in their rumens nor had rumenotomy (CPII) showed a consistent increase in mean body weight over the entire 8 weeks period indicating an increase of 6.5% at the end of experiment compared to baseline weights (Fig.3.7). Analysis using Tukey's multiple comparison test of two-way ANOVA revealed that at the end of 8 weeks post-implantation there was a significant loss in body weight of wethers with impacted rumen (PPII) compared to those of the negative control group (CPII) ($p = 0.0277$) (Table 3.2).

Table 3.2: Weekly mean body weights in three groups of wethers with or without rumen impaction with plastic bags during the 8-week post-impaction period of Phase II experiment

Time (Weeks)	**Weekly mean (± SE) body weight of wethers in kg**			***P* value**
	PPII (n = 5)	**NPPII (n = 5)**	**CPII (n = 5)**	
0	28.8 ± 0.6	27.7±0.8	29.0 ± 2.0	0.9943
1	26.8 ± 1.3	26.7 ± 0.2	29.4 ± 1.9	0.3824
2	25.6 ± 1.1	26.0 ± 0.6	29.6 ± 1.9	0.1068
3	25.6 ± 1.1	26.8 ± 1.0	29.3 ± 1.8	0.1463
4	24.4 ± 1.4	26.6 ± 1.1	29.6 ± 2.0	0.0245[a]
5	25.9 ± 1.1 (n = 4)	26.2 ± 1.0	30.0 ± 1.8	0.1233
6	23.4 ± 2.1 (n = 4)	27.7 ± 0.8	30.2 ± 1.9	0.0041[a]
7	24.5 ± 1.7 (n = 3)	27.8 ± 1.4	30.5 ± 1.8	0.0247[a]
8	25.0 ± 1.4 (n = 3)	28.6 ± 0.7	30.9 ± 1.8	0.0277[a]

Data expressed as mean. Significance at $p < 0.05$

[a]*P*-value = test group PPII compared with negative control group CPII

Key:

PPII = test group of wethers whose rumen were implanted with plastic bags through rumenotomy

NPPII = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen

CPII = negative control group of wethers that had neither rumenotomy done nor plastic bags implanted in their rumen.

kg = kilogram

n = number of wethers in the group

± SE = plus or minus standard error of mean

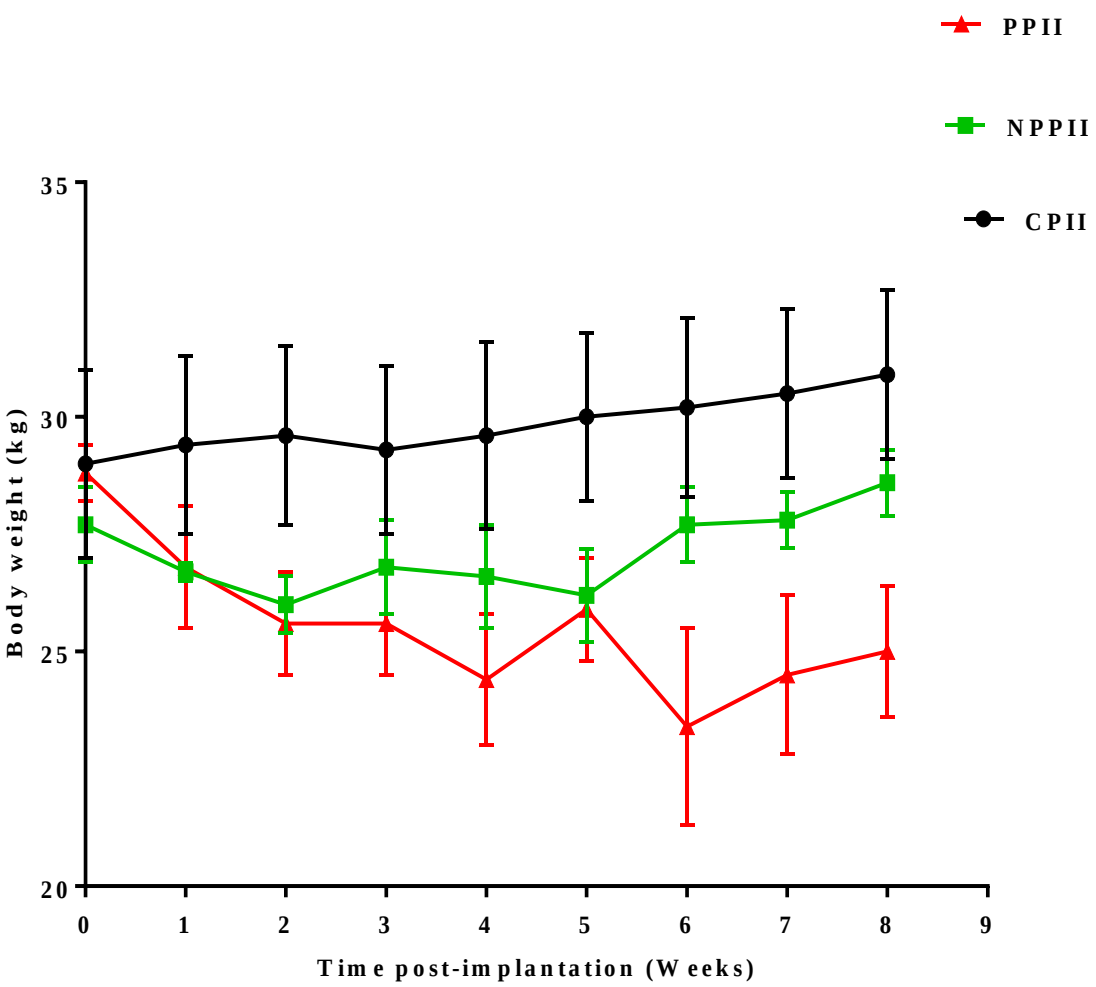


Figure 3.7: Changes in weekly mean body weights in three groups of wethers during the 8 weeks post-impaction period of Phase II experiments. The results showed a significant loss in body weights of PPII.
Key:
PPII = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPII = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen
CPII = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.
kg = kilogram

3.5.2 Gross observations of rumen after the sacrifice of wethers at experimental endpoint

At the end of the 4-week or 8-week experimentation, the rumen from wethers impacted with plastic bags showed dry ruminal content with the presence of the plastic bags intertwined and intermingled with scanty ingesta blocking the rumino-reticular orifice. The rumen from the impacted wethers appeared pale in colour and the ruminal wall flabby and overstretched (Fig. 3.8). On the other hand the opened rumen from the negative and positive control groups revealed large amounts of ingesta moistened with ruminal fluid (Fig. 3.8).

Gross observation of the emptied and washed rumen from wethers which were subjected to impaction in Phase I experiment showed discolouration and loss of papillae on the mucosal surface with a thin overstretched wall (Fig. 3.9). This observation was more pronounced in rumen from impacted wethers in Phase II experiment (Fig. 3.10). However, rumen from the negative and positive control groups in both Phase I and Phase II experiments were healthy with numerous papillae on the mucosal surface with a thick firm muscular ruminal wall and normal colouration of mucosa (Fig. 3.9 and Fig. 3.10).

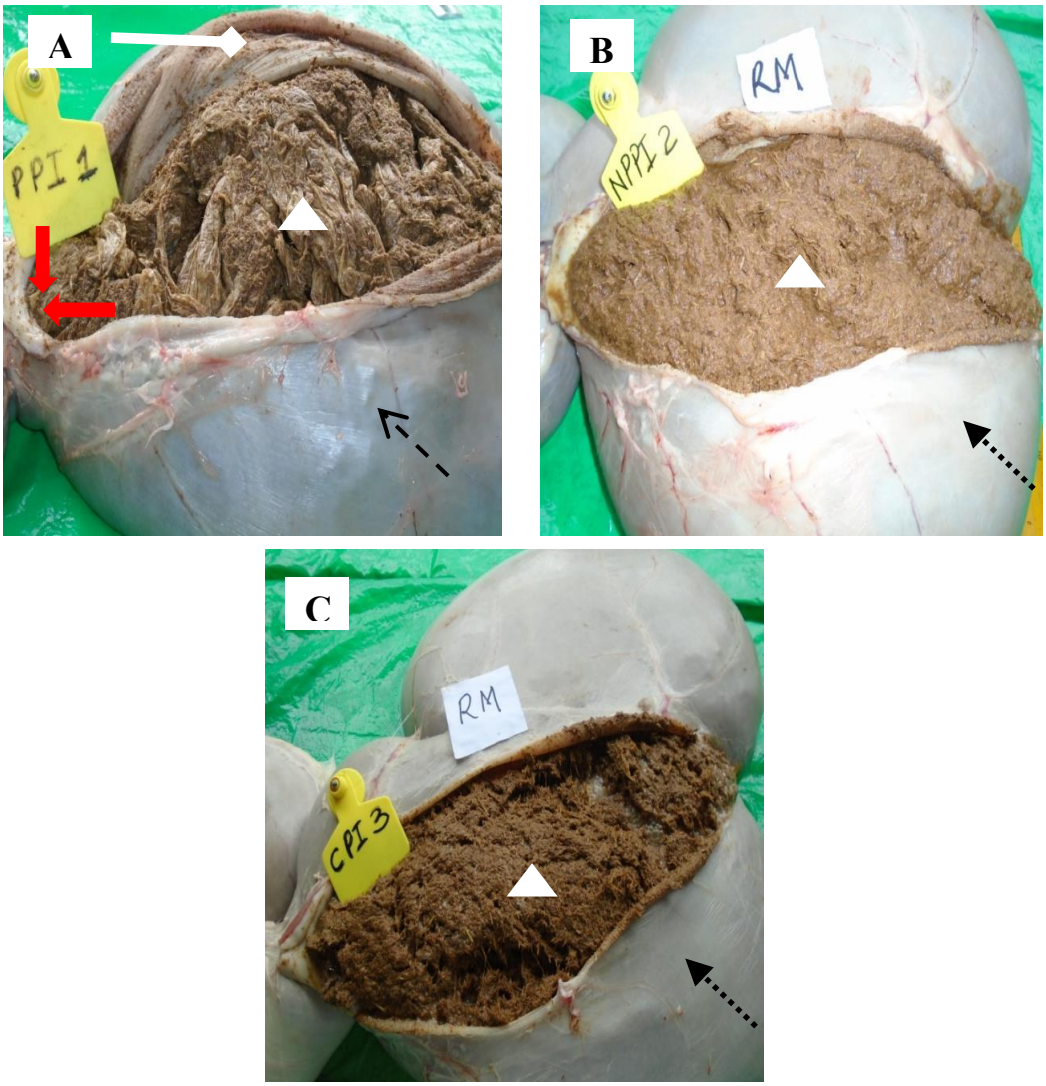


Figure 3.8: Open rumen from wethers in the three groups at the 4 week endpoint of Phase I experiments. (**A**) rumen from the group implanted with plastic bags (PPI) showing dry contents inclusive of plastic bags and scanty ingesta (*white arrow head*), overstretched flabby rumen surface (*white diamond arrow*), plastic bags blocking rumeno-reticular orifice (*red block arrows*), thin ruminal wall (*black dash arrow*), (**B**) rumen from positive control wether (NPPI) with normal ruminal wall (*black dotted arrow*) and large amount of ingesta (*white arrow head*), (**C**) rumen from negative control wether (CPI) with normal ruminal wall (*black dotted arrow*) and large amount of ingesta (*white arrow head*).

Figure 3.9: Representative emptied and cleaned rumen from each of the three groups of wethers at the 4-week endpoint of Phase I experiments showing the effect of impaction with plastic bags on the rumen wall. (**A**) Loss of papillae (*white arrow head*) and thin stretched ruminal wall and pale mucosa (white dotted *arrow*) in the rumen that was implanted with plastic bags (PPI), (**B**) rumen from positive control group (NPPI) showing numerous healthy papillae (*white arrow head*) and thick firm muscular ruminal wall with normal coloured ruminal mucosa (*white bold arrow*), (**C**) rumen from negative control group (CPI) showing numerous healthy papillae (*white arrow head*) and thick firm muscular wall and normal coloured ruminal mucosa (*white bold arrow*).

Figure 3.10: Representative emptied and cleaned rumen from each of the three groups of wethers at the 8 week endpoint of Phase II experiment showing the effect of impaction with plastic bags on the rumen wall. (**A**) rumen from wether with impacted rumen (PPII) showing severe loss of papillae (*white arrow head*), thin over stretched transparent wall and pale ruminal mucosa (*white dash diamond arrow*), (**B**) rumen from positive control group (NPPII) showing numerous healthy papillae (*white arrow head*) and thick firm muscular ruminal wall and normal coloured ruminal mucosa (*white block arrow*), (**C**) rumen from negative control group (CPII) showing numerous healthy papillae (*white arrow head*) and thick firm muscular wall and normal coloured ruminal mucosa (*white block arrow*).

3.5.3 Macroscopic measurements of the surface area of the whole rumen of the three experimental groups of wethers at the end of 4 weeks in Phase I and 8 weeks in Phase II

Four weeks after implanting plastic bags into the rumen of wethers (PPI) during Phase I experiments, the total mean surface area of the entire rumen was 1149.30 ± 43.80cm^2 compared to 1074.15 ± 43.59cm^2 and 1084.50 ± 47.98cm^2 in the positive control group (NPPI) and negative control group (CPI) respectively. But the differences were not significant. Eight weeks after implanting plastic bags into the rumen of group PPII, the total mean macroscopic surface area of the entire rumen was 1254.15 ± 44.55cm^2. This was significantly higher by 20% than the 1038.15 ± 34.86cm^2 ($p = 0.0036$) in the positive control group (NPPII) and by 15% than the 1091.25 ± 29.48cm^2 ($p = 0.0221$) in the negative control (CPII).

3.5.4 Histological examination of ruminal sacs of the three groups of wethers in Phase I and Phase II of the experiments

Photomicrographs of ruminal tissue sections cut from ruminal sacs depicting the effects of implanted plastic bags in the 3 groups of wethers at the 4 and 8 week post-implantation endpoints are presented in Figures 3.11-3.16.

All the rumen tissues from the groups implanted with plastic bags (PPI and PPII) had reduced density of ruminal papillae at the 4 week and 8 week post-implantation experimental endpoints respectively (Fig. 3.11, Fig. 3.14). The papillae were short and heavily bent over in the tissue sections of the ruminal sacs from wethers in Phase I experiment (Fig. 3.11). Photomicrographs of ruminal tissue sections from wethers in Phase II experiments, revealed the loss or absence of papillae in the cranial sac (Fig. 3.14). At the end of 8 weeks post-implantation the ruminal papillae of wethers with rumen impaction (PPII) were short and had enlarged base and thin

epithelia. Some papillae were abnormally thin with constrictions at their bases. Additionally, the inner layer of tunica muscularis appeared thicker (Fig. 3.14). All the rumen from the two control groups of wethers in Phase I (NPPI and CPI) (Fig. 3.12, Fig. 3.13) and Phase II (NPPII and CPII) (Fig. 3.15, Fig. 3.16) had numerous normal healthy papillae as well as normal ruminal walls at the end of their respective experimental periods.

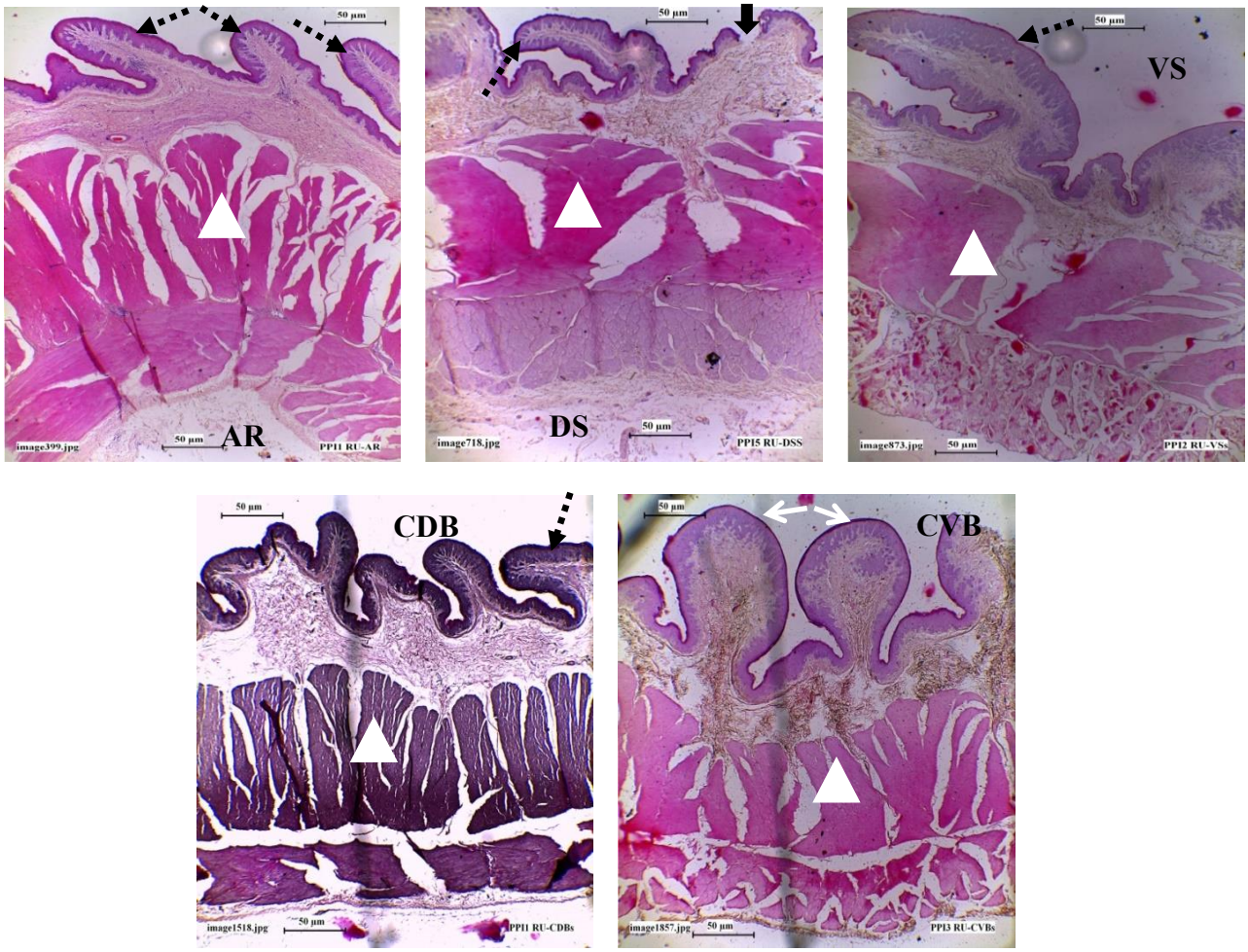


Figure 3.11: Photomicrographs of rumen tissue sections harvested at the 4-week endpoint of Phase I experiments from wethers whose rumen were implanted with plastic bags (PPI). The photomicrographs represent Cranial sac (AR), dorsal sac (DS), ventral sac (VS) and caudodorsal blind sac (CDB), which show short slender and bent over papillae (*black dotted arrow*) and stunted papillae in caudoventral blind sac (CVB) (*white solid open arrow*), and loss of epithelium in DS (*black bold arrow*). The ruminal sacs showed an enlargement of the inner tunica muscularis (*white arrow head*) (scale bar 50µm, H&E, magnification ×4).

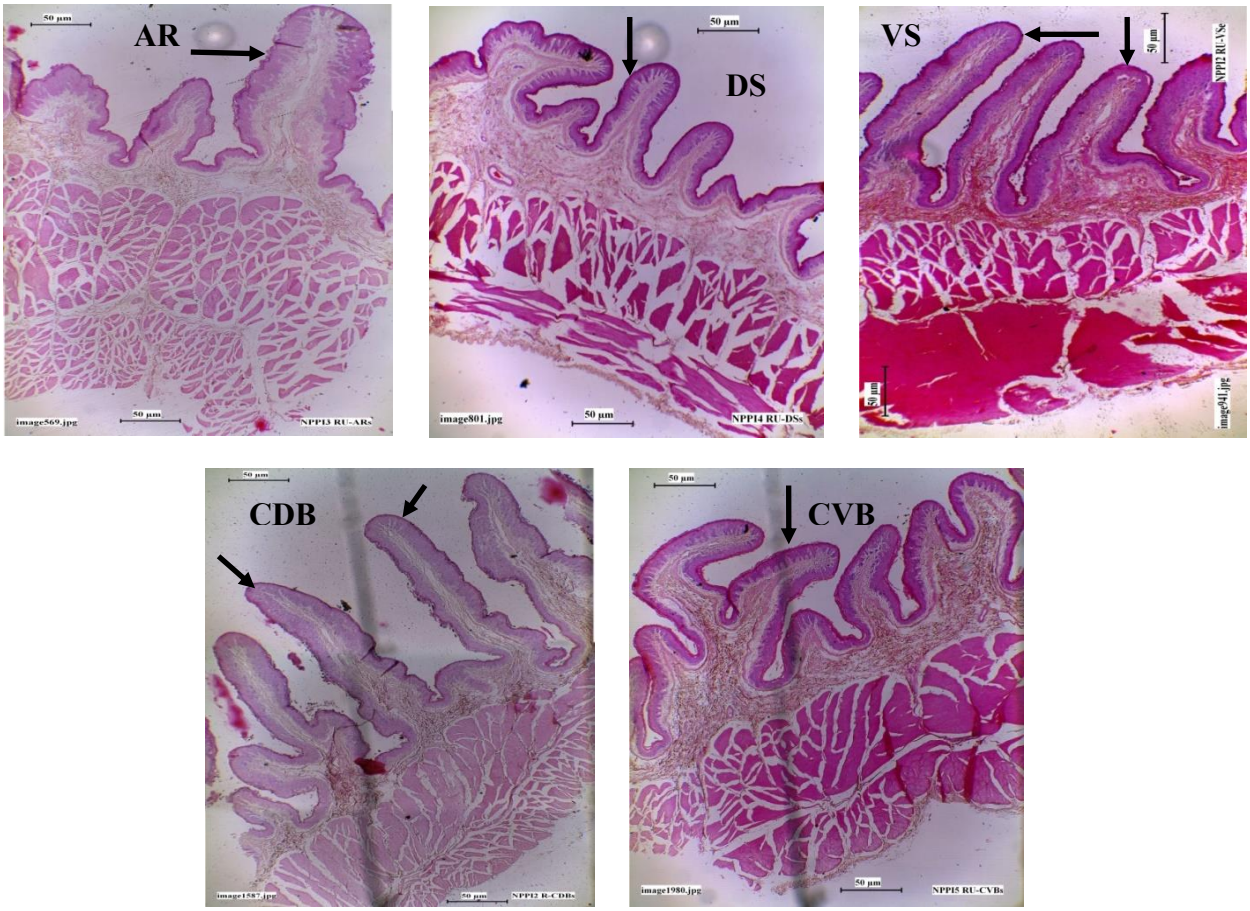


Figure 3.12: Photomicrographs of ruminal tissue sections harvested at 4-week endpoint of Phase I experiments from all the ruminal sacs in wethers whose rumen were not implanted with plastic bags but underwent rumenotomy (NPPI). Photomicrographs represents the Cranial sac (AR), Dorsal sac (DS), Ventral sac (VS), caudodorsal blind sac (CDB) and caudoventral blind sac (CVB). Normal tissues layers can be seen with elongated papillae with variable sizes on mucosal surface of the sacs (*black block arrows*). (scale bar 50µm, H&E, magnification ×4).

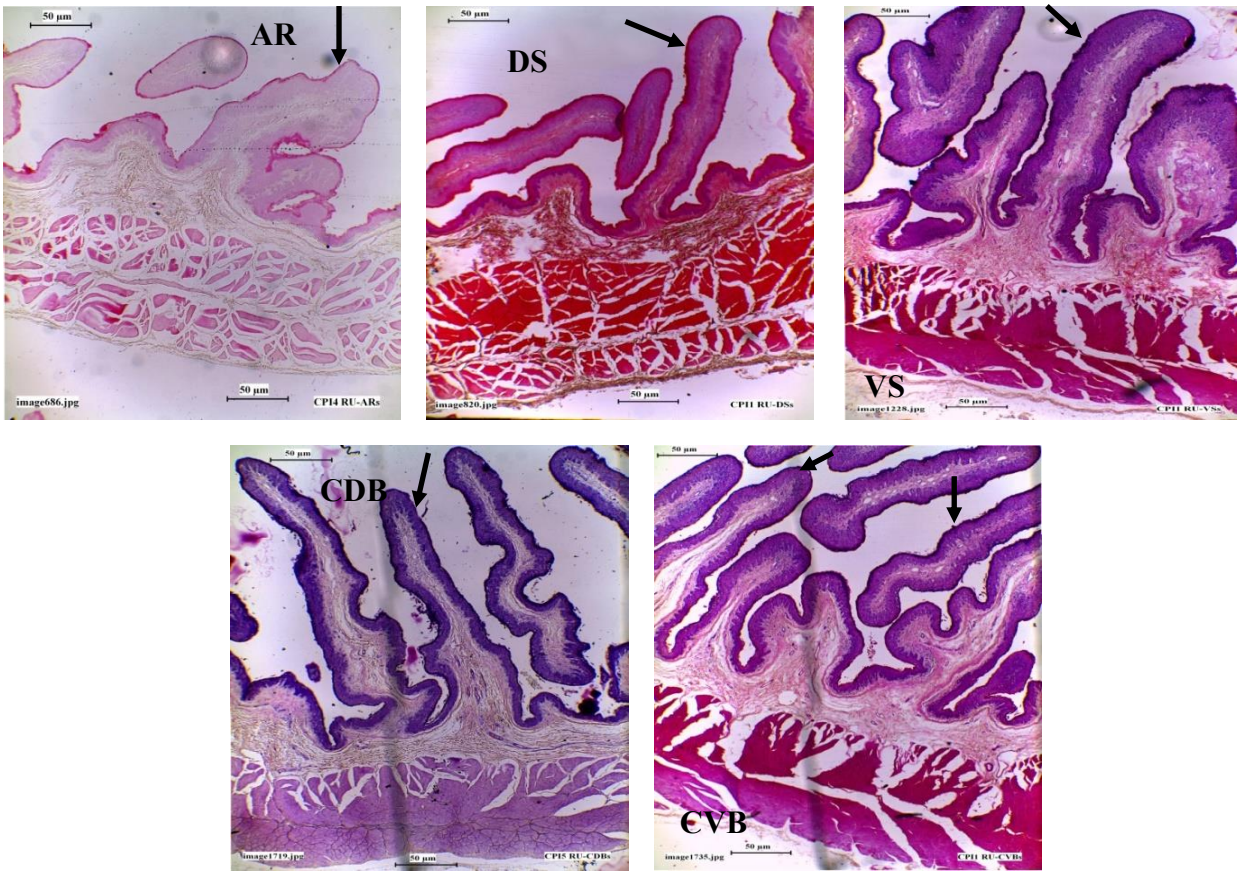


Figure 3.13: Photomicrographs of ruminal tissue sections harvested at 4-week endpoint of Phase I experiment from all the ruminal sacs in wethers whose rumen were neither implanted with plastic bags nor underwent rumenotomy (CPI). Photomicrographs represents the Cranial sac (AR), Dorsal sac (DS), Ventral sac (VS), caudodorsal blind sac (CDB) and caudoventral blind sac (CVB). Normal tissues layers can be seen with elongated papillae with variable sizes on mucosal surface of the sacs (*black block arrows*). (scale bar 50µm, H&E, magnification ×4).

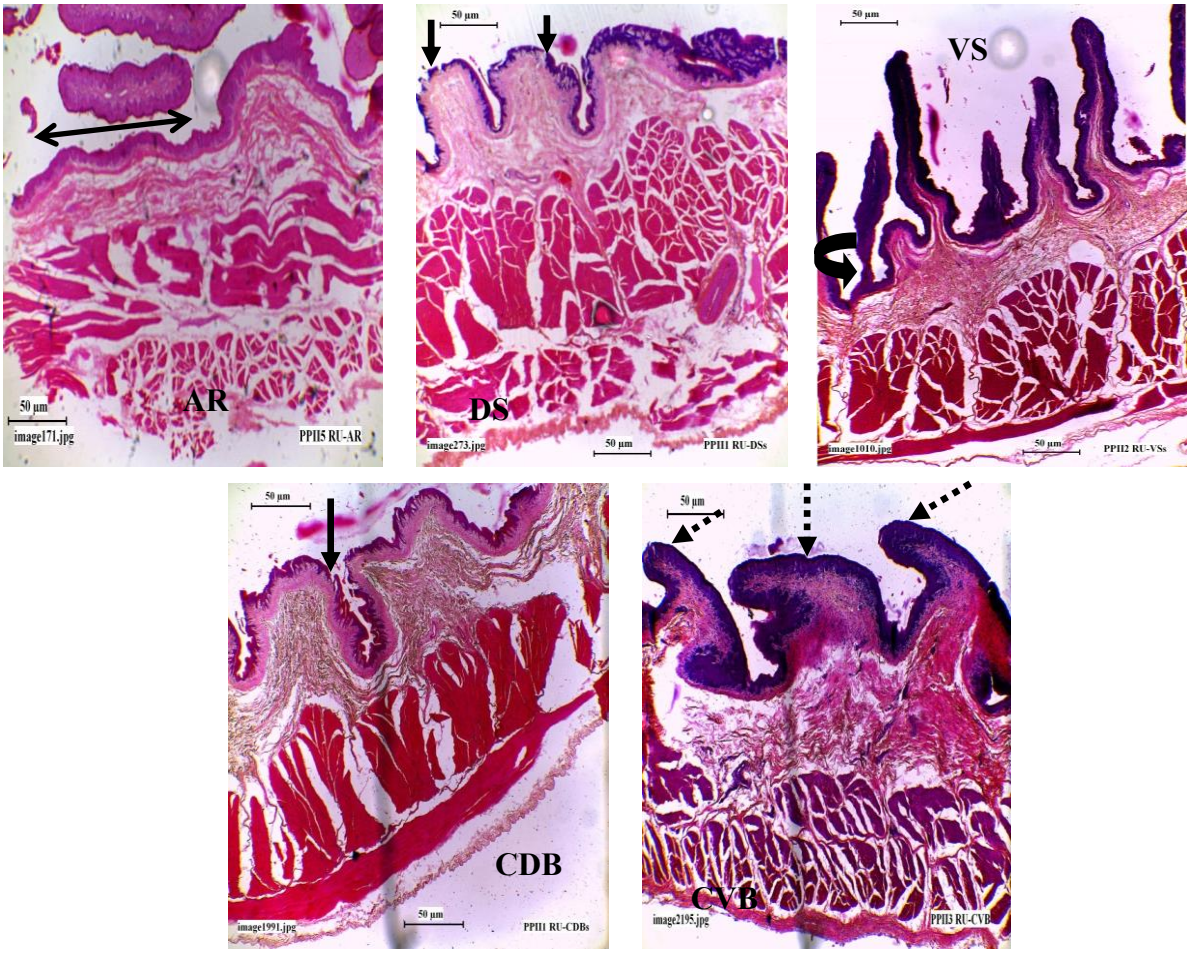


Figure 3.14: Photomicrographs of rumen tissue sections harvested at the 8-week endpoint of Phase II experiments from wethers whose rumen were implanted with plastic bags (PPII). The photomicrographs represent cranial sac (AR) showing loss of papillae (*black double headed arrow*), dorsal sac (DS) and caudodorsal blind sac (CDB) showing stunted papillae with broad base and thin and eroded epithelium (*black block arrows*), ventral sac (VS) showing slender and constricted papillae (*black curved arrow*) and caudoventral blind sac (CVB) showing stunted flattened papillae (*black dotted arrow*) (scale bar 50µm H&E, magnification ×4).

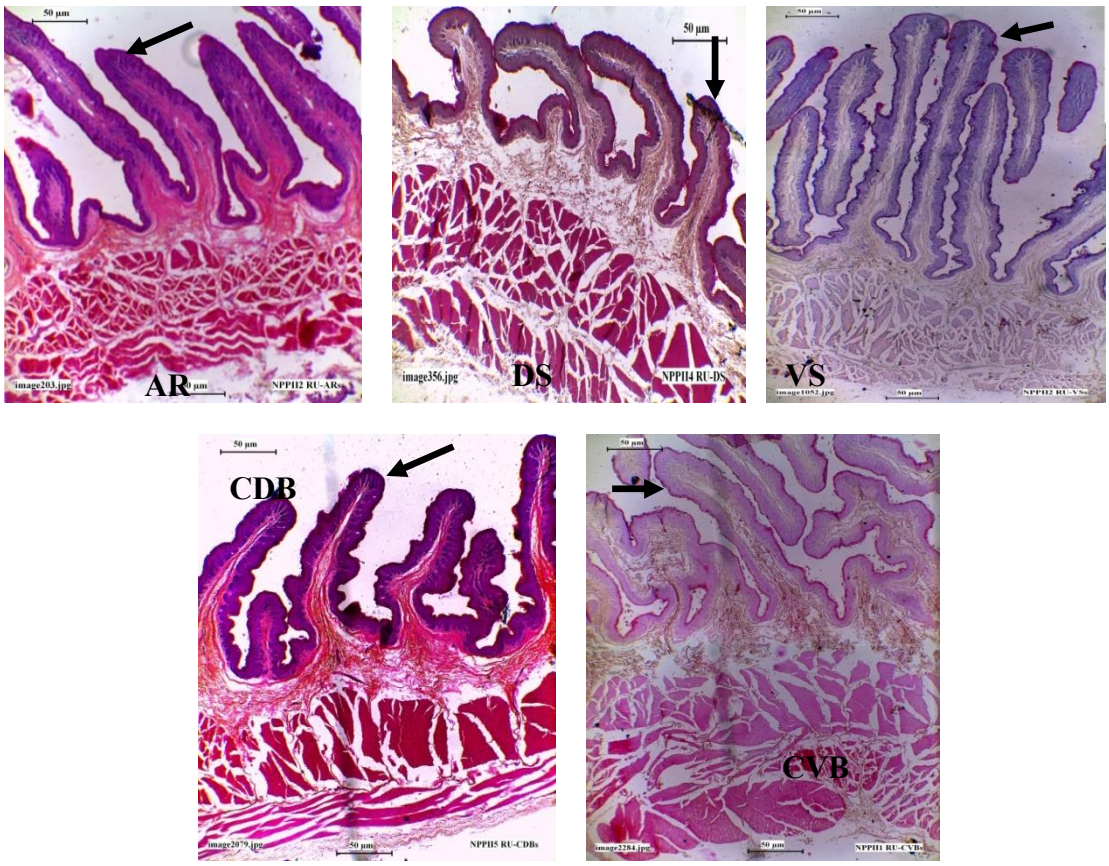


Figure 3.15: Photomicrographs of ruminal tissue sections harvested at the 8-week endpoint of Phase II experiments from all the ruminal sacs in wethers whose rumen were not implanted with plastic bags but underwent rumenotomy (NPPII). Photomicrographs represents the Cranial sac (AR), Dorsal sac (DS), Ventral sac (VS), caudodorsal blind sac (CDB) and caudoventral blind sac (CVB). Normal tissues layers can be seen with elongated papillae with variable sizes on mucosal surface of the sacs (*black block arrows*) (scale bar 50µm, H&E, magnification ×4).

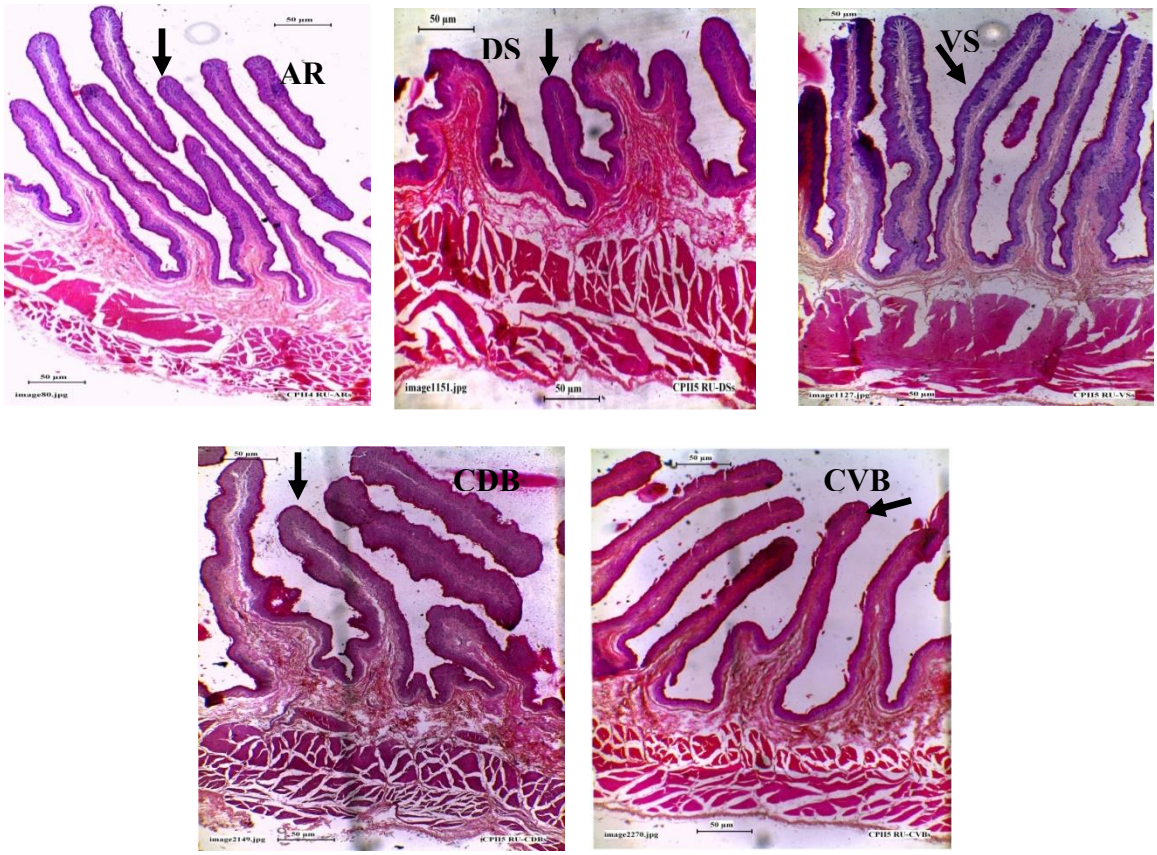


Figure 3.16: Photomicrographs of ruminal tissue sections harvested at the 8-week endpoint of Phase II experiments from all the ruminal sacs in wethers whose rumen were neither implanted with plastic bags nor underwent rumenotomy (CPII). Photomicrographs represents the Cranial sac (AR), Dorsal sac (DS), Ventral sac (VS), caudodorsal blind sac (CDB) and caudoventral blind sac (CVB). Normal tissues layers can be seen with elongated papillae with variable sizes on mucosal surface of the sacs (*black block arrows*). (scale bar 50µm, H&E, magnification ×4).

3.5.5 Stereological analysis of structural changes in the rumen implanted with plastic bags compared to the controls in Phase I experiments

The quantitative analysis of the effects of presence of plastic bags in the rumen on surface density, surface area, volume density and absolute volume of ruminal sacs in the 3 groups of wethers at the end of Phase I experiments are presented in the sections below. This includes the total mean surface area and total mean absolute volume of the entire rumen.

3.5.5.1 Mean surface density and mean surface area estimates of ruminal sacs in Phase I

A summary of quantitative estimates of the effects of rumen impaction with plastic bags on surface density and surface area of each of the five rumen sacs is presented in Tables 3.3 and 3.4 as well as Figures 3.17 and 3.18. After 4 weeks, the ruminal sacs of the group implanted with plastic bags (PPI) had significant decrease ($p < 0.05$) in the values of mean surface density (Sv) compared to the rumen sacs from the positive control group (NPPI) and negative control group (CPI). There was a 30% reduction in mean surface density of the cranial ruminal sac (AR) in wethers whose rumen were impacted with plastic bags (PPI) compared with the negative control group (CPI) and 25.5% reduction compared with the positive control (NPPI) group. These differences in mean surface densities between PPI and CPI as well as between PPI and NPPI were all significant ($p < 0.0001$). In comparison to the NPPI group, the mean surface densities (Sv) in PPI groups decreased significantly for dorsal sac (DS) by 17% ($p = 0.0145$), ventral sac (VS) by 19% ($p = 0.0014$), caudodorsal blind sac (CDB) by 21% ($p = 0.0005$) and caudoventral blind sac (CVB) by 25% ($p < 0.0001$). Similarly, in comparison to CPI group, the mean surface densities (Sv) in PPI groups decreased significantly for dorsal sac (DS) by 19% ($p = 0.0028$),

ventral sac (VS) by 20% ($p = 0.0006$), caudodorsal blind sac (CDB) by 21% ($p = 0.0006$) and caudoventral blind sac (CVB) by 25% ($p < 0.0001$) (Table 3.3).

The mean surface area of the different ruminal sacs had variations between the test group (PPI) and control groups (CPI and NPPI). The test group had a reduction in mean surface area of all the ruminal sacs in which significant differences ($p < 0.05$) were found only in the cranial (AR) and caudoventral blind (CVB) sacs when compared with control groups. The mean surface area of the cranial ruminal sac (AR) of the test group (PPI) decreased by 34% compared with either the negative or positive control groups (CPI, NPPI) after 4 weeks of rumen impaction. Using Tukey's multiple comparison test of two-way ANOVA indicated that there were significant differences in the mean surface area of the cranial sac (AR) between the test group (PPI) and the negative control group (CPI) ($p = 0.0163$) and also positive control (NPPI) ($p = 0.0161$). The mean surface area of the caudoventral blind sac (CVB) of the test group PPI was significantly lower by 44% compared with the negative control group CPI ($p = 0.0170$) and significantly lower by 39% compared with the positive control group NPPI ($p = 0.0345$). The mean surface areas of the dorsal, ventral and caudodorsal blind sacs had no significant difference between the test and the control groups.

Table 3.3: The mean surface densities of ruminal sacs in three groups of wethers with or without rumen impaction with plastic bags at 4-week endpoint of Phase I experiment

Rumen compartments	**Mean surface density (cm^{-1}) of ruminal sacs (Mean ± SE)**			***P* Value**
	PPI (n = 5)	**NPPI (n = 5)**	**CPI (n = 5)**	
Cranial sac (AR)	6.197 ± 0.049	8.323 ± 0.184	8.811 ± 0.267	< 0.0001[a] < 0.0001[b]
Dorsal sac (DS)	6.079 ± 0.291	7.287 ± 0.306	7.529 ± 0.319	0.0028[a] 0.0145[b]
Ventral sac (VS)	6.550 ± 0.202	8.088 ± 0.354	8.211 ± 0.390	0.0006[a] 0.0014[b]
Caudodorsal blind sac (CDB)	6.392 ± 0.246	8.056 ± 0.437	8.054 ± 0.261	0.0006[a] 0.0005[b]
Caudoventral blind sac (CVB)	6.168 ± 0.219	8.250 ± 0.322	8.200 ± 0.365	< 0.0001[a] < 0.0001[b]

Data expressed as means. Significance at $p < 0.05$

[a]*P*-value = test group PPI compared with negative control group CPI

[b]*P*-value = test group PPI compared with positive control group NPPI

Key:

PPI = test group of wethers whose rumen were implanted with plastic bags through rumenotomy

NPPI = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen

CPI = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

cm^{-1} = per centimeter

n = number of wethers in the group

± SE = plus or minus standard error of mean

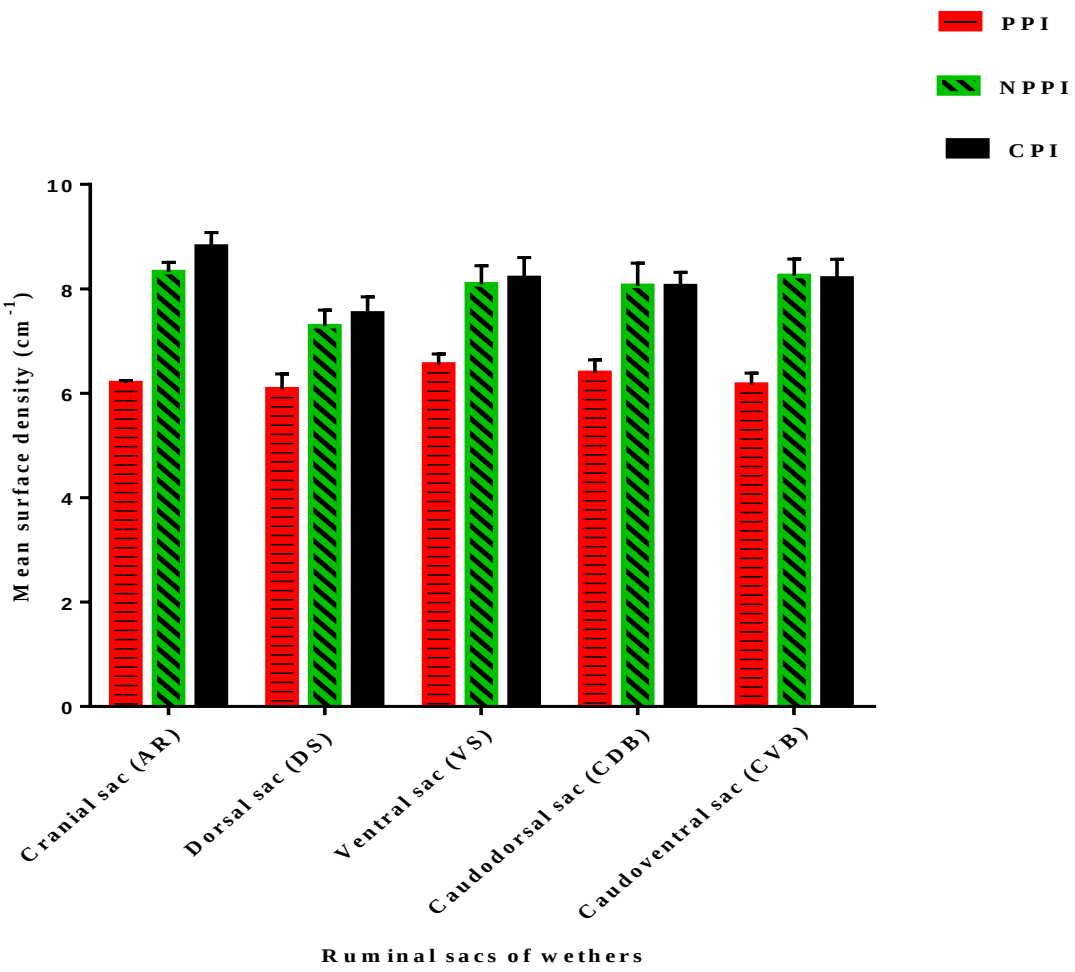


Figure 3.17: A comparison of mean surface densities of ruminal sacs from three groups of wethers at the 4-week endpoint of Phase I experiment. The results showed significant loss in surface densities of ruminal sacs in the test group.

Key:
PPI = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPI = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen
CPI = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

Table 3.4: Mean surface area of ruminal sacs of three groups of wethers with or without rumen impaction with plastic bags at the end of 4-week post-impaction period in Phase I experiment

Rumen compartments	**Mean surface area (m^2) of ruminal sacs (Mean ± SE)**			***P* Value**
	PPI (n = 5)	**NPPI (n = 5)**	**CPI (n = 5)**	
Cranial sac (AR)	0.057 ± 0.004	0.088 ± 0.003	0.088 ± 0.004	0.0163[a] 0.0161[b]
Dorsal sac (DS)	0.088 ± 0.011	0.097 ± 0.010	0.096 ± 0.010	0.7758 0.6751
Ventral sac (VS)	0.165 ± 0.012	0.182 ± 0.010	0.173 ± 0.009	0.7431 0.2465
Caudodorsal blind sac (CDB)	0.012 ± 0.002	0.022 ± 0.002	0.022 ± 0.004	0.7002 0.7079
Caudoventral blind sac (CVB)	0.045 ± 0.006	0.074 ± 0.006	0.080 ± 0.009	0.0170[a] 0.0345[b]

Data expressed as means. Significance at $p < 0.05$

[a]*P*-value = test group PPI compared with negative control group CPI

[b]*P*-value = test group PPI compared with positive control group NPPI

Key:

PPI = test group of wethers whose rumen were implanted with plastic bags through rumenotomy

NPPI = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen

CPI = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

m^2 = squared meters

n = number of wethers in the group

± SE = plus or minus standard error of mean

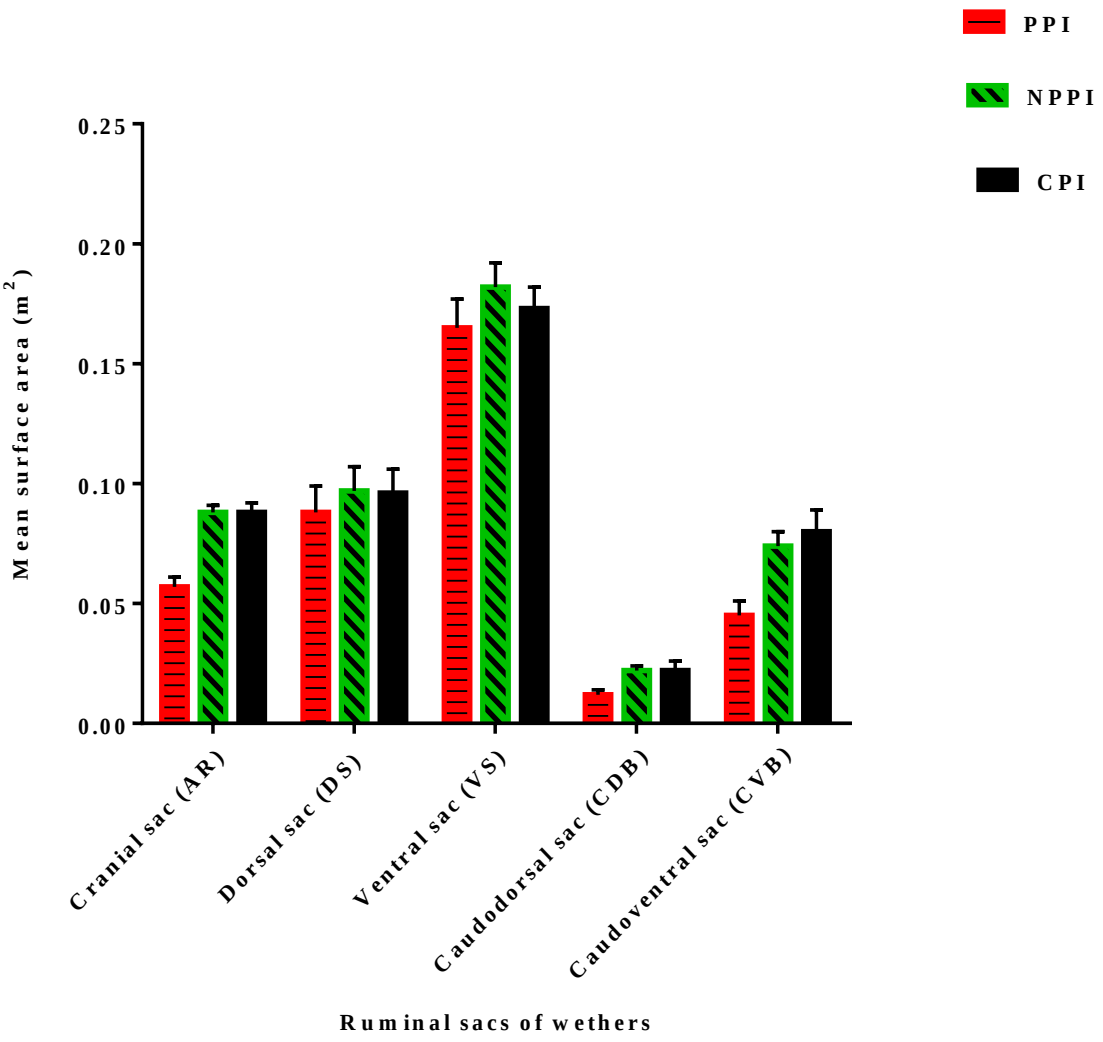


Figure3.18: A comparison of the mean surface areas of different ruminal sacs from three groups of wethers at the 4-week endpoint of Phase I experiment. The results showed significant loss in surface area of AR and CVB.

Key:
PPI = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPI = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen
CPI = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

3.5.5.2 Mean volume density and absolute volume of tissues in the ruminal sacs at the 4-week endpoint of Phase I experiments

Quantitative analysis of mean volume density (Vv) and mean absolute volume measurements of mucosa, submucosa, muscularis interna and muscularis externa of the ruminal sacs 4 weeks after implanting plastic bags in the rumen of wethers are presented in Tables 3.5 and 3.6. The comparative mean volume densities and mean absolute volumes of the ruminal sacs between the test group (PPI) and the positive control group (NPPI), as well as negative control group (CPI) are also presented in Figures 3.19 and 3.20.

The results revealed differences in mean volume densities (Vv) of mucosa in the cranial, dorsal, ventral, caudodorsal and caudoventral ruminal sacs between wethers whose rumen were implanted with plastic bags (PPI) and those in the positive (NPPI) and negative control (CPI) groups. At the end of 4 weeks post-implantation the results revealed that the mean volume density (Vv) of mucosa in the cranial, dorsal and ventral sacs of wethers whose rumen were implanted with plastic bags (PPI), diminished significantly by about 11% ($p < 0.0001$), 9% ($p < 0.0001$) and 6% ($p = 0.0159$) respectively compared with either positive (NPPI) or negative (CPI) control groups (Table 3.5). The mean volume density of mucosa in the wall of the caudo-dorsal and ventral blind sacs of the test group (PPI) decreased by 8% ($p = 0.0239$) and 10% ($p < 0.0001$) respectively when compared with the positive control group NPPI. The mean volume densities of muscularis interna in the cranial and dorsal ruminal sacs of the test group PPI increased significantly by 7% ($p = 0.0084$) and 7% ($p < 0.0001$) respectively compared with that from the positive control group NPPI. Group PPI showed an increase of 8% ($p = 0.0154$) and 9% ($p = 0.0007$) in mean volume densities of submucosa in the wall of the caudodorsal and

caudoventral blind sacs respectively when compared with those from the positive control group NPPI (Table 3.5).

Results obtained for mean absolute volume of mucosa in the wall of the ruminal sacs revealed significant differences ($p < 0.05$) in the cranial, dorsal, caudodorsal and caudoventral sacs between the test group (PPI) and control groups. Mean absolute volume of mucosa in the cranial sac of PPI wethers decrease significantly ($p = 0.0002$) by 45% compared to negative control group CPI and also decreased significantly ($p < 0.0001$) by 47% compared to the positive control group NPPI.

Tukey's multiple comparison test of two-way ANOVA also revealed that the 33% difference in mean absolute volume of mucosa in the dorsal sac between test wethers (PPI) and negative control group (CPI) was significant ($p = 0.0449$) as well as the 40% difference between group PPI and positive control group (NPPI) ($p = 0.0280$). The mean absolute volume of mucosa in the caudodorsal blind sac of test group PPI was lower by 50% ($p = 0.0011$) compared with negative control group CPI. In the caudoventral blind sac the decrease was more than 40% as compared with each of the control groups (CPI, NPPI) and this was also significant ($p = 0.0015$).

The mean absolute volume of muscularis interna in the cranial and dorsal sacs between the test group PPI and each of the control groups (NPPI and CPI) were different (Table 3.6). In the cranial sac the muscularis interna of test group PPI was 24% ($p = 0.0469$) more than that of group CPI. In the dorsal sac group PPI had 33% ($p = 0.0064$) more mean absolute volume of muscularis mucosa than either control groups (NPPI, CPI).

Table 3.5: Mean volume densities of tissues in the ruminal sacs of three groups of wethers with or without rumen impaction with plastic bags after 4-week Phase I experimentation

Histological layers in ruminal sacs	Mean volume density (%) of tissues (Mean ± SE)			*P* Value
	PPI (n = 5)	NPPI (n = 5)	CPI (n = 5)	
Cranial sac (AR)				
Mucosa	17.2 ± 1.7	28.8 ± 1.9	29.0 ±1.2	< 0.0001[a] < 0.0001[b]
Submucosa	16.8 ± 1.6	14.8 ± 1.0	14.9 ± 0.9	0.6482
Muscularis interna	39.9 ± 3.5	32.2 ± 1.1	29.6 ± 0.8	0.0003[a] 0.0084[b]
Muscularis externa	23.5 ± 0.8	22.8 ± 1.7	24.8 ± 0.5	0.9478
Dorsal sac (DS)				
Mucosa	12.6 ± 0.8	23.1 ± 1.2	21.9 ± 0.9	< 0.0001[a] < 0.0001[b]
Submucosa	19.7 ± 0.8	17.2 ± 0.8	18.9 ± 0.5	0.2382
Muscularis interna	41.1 ± 0.3	33.6 ± 1.0	35.1 ± 1.8	0.0008[a] < 0.0001[b]
Muscularis externa	24.5 ± 0.2	24.3 ± 1.6	22.4 ± 1.6	0.9905
Ventral sac (VS)				
Mucosa	23.4 ± 1.9	29.8 ± 1.5	28.8 ± 0.8	0.0484[a] 0.0159[b]
Submucosa	20.5 ± 1.5	17.6 ± 2.0	17.5 ± 2.1	0.3985
Muscularis interna	30.3 ± 1.9	30.9 ± 1.5	30.7 ± 1.7	0.9606
Muscularis externa	24.0 ± 1.2	19.9 ± 1.0	21.1 ± 1.1	0.1654
Caudo-dorsal blind sac (CDB)				
Mucosa	18.5 ± 1.9	26.5 ± 2.1	29.9 ± 1.4	0.0009[a] 0.0239[b]
Submucosa	26.5 ± 3.5	18.0 ± 1.6	17.8 ± 1.5	0.0129[a] 0.0154[b]
Muscularis interna	34.8± 2.7	33.3 ± 1.5	30.6 ± 1.5	0.8665
Muscularis externa	17.6 ± 2.1	20.2 ± 1.5	18.9 ± 2.5	0.6520
Caudo-ventral blind sac(CVB)				
Mucosa	20.5 ± 2.0	30.3 ± 1.6	30.8 ± 0.8	< 0.0001[a] < 0.0001[b]
Submucosa	25.1 ± 0.9	17.5 ± 0.8	17.3 ± 1.7	0.0005[a] 0.0007[b]
Muscularis interna	32.6 ± 1.8	31.2 ± 0.9	30.5 ± 1.8	0.7485
Muscularis externa	19.9 ± 1.3	19.0 ± 1.1	19.0 ± 0.7	0.8867

Values are presented as plus or minus standard error of mean (Mean ± SE)
Significant at $p < 0.05$. [a]P-value = test group PPI compared with negative control group CPI
[b]P-value = test group PPI compared with positive control group NPPI
NB: Serosa values per ruminal sac not included because they were negligible.
Key: **PPI** = wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPI** = wethers that had rumenotomy performed but no plastic bags implanted, **CPI** = wethers that had neither rumenotomy done nor plastic bags implanted, **n** = number of wethers in the group.

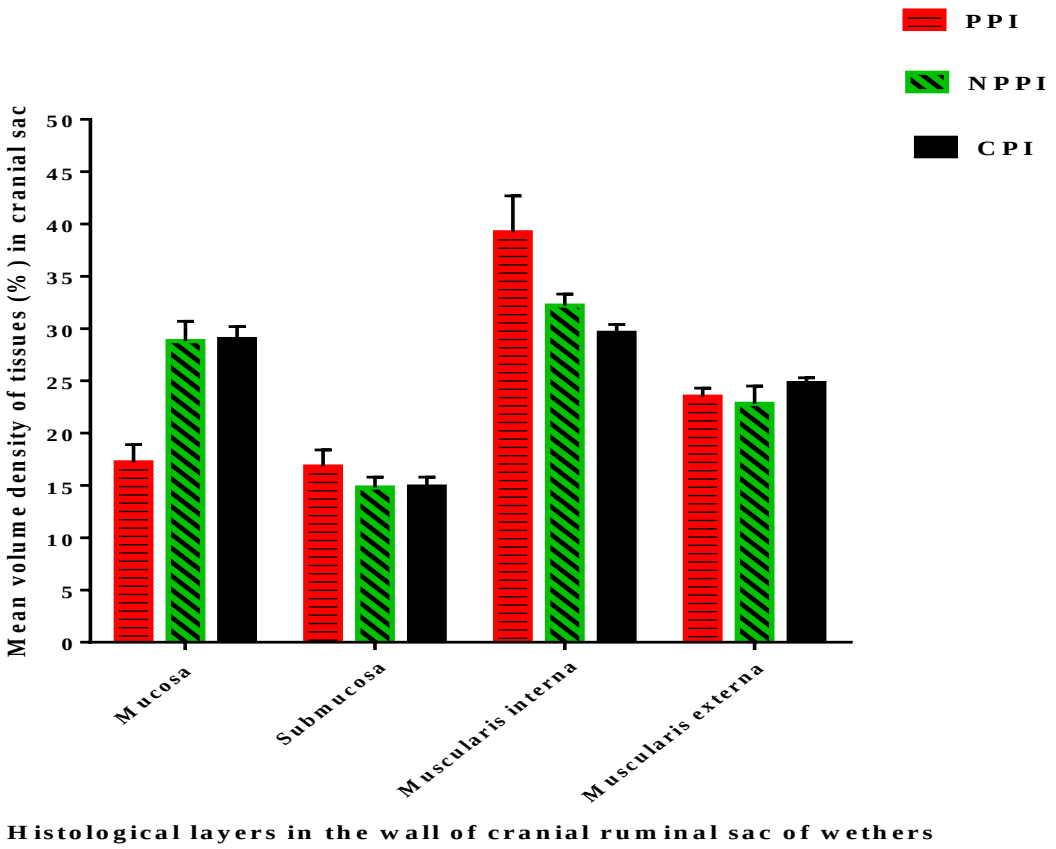


Figure 3.19a: Comparison of percentage mean volume densities of tissues in the cranial ruminal sacs of three groups of wethers at the 4-week end point in Phase I experimentation. Results showed reduction in volume densities of mucosal tisues in the cranial sac of PPI

Key:
PPI = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPI = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen
CPI = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

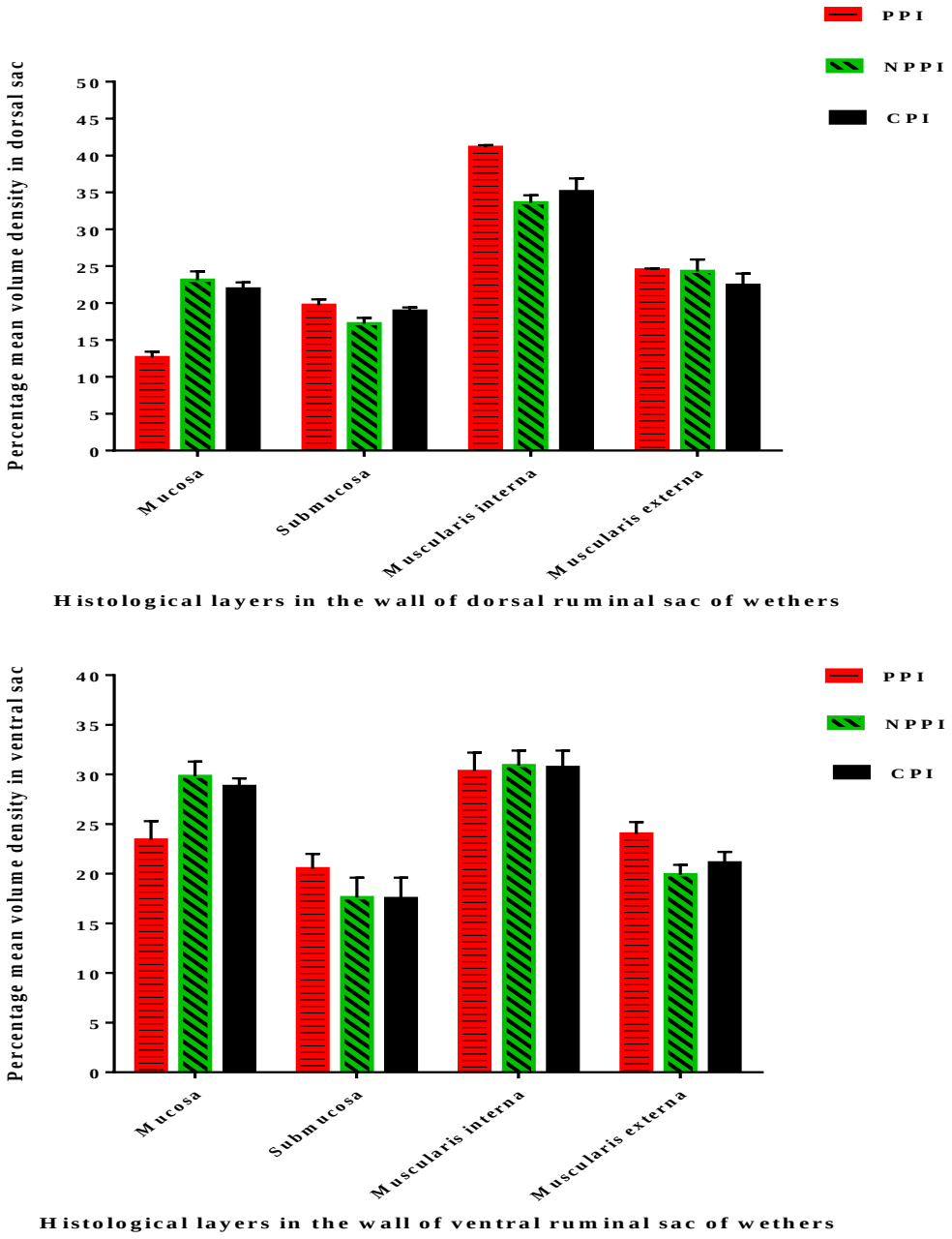
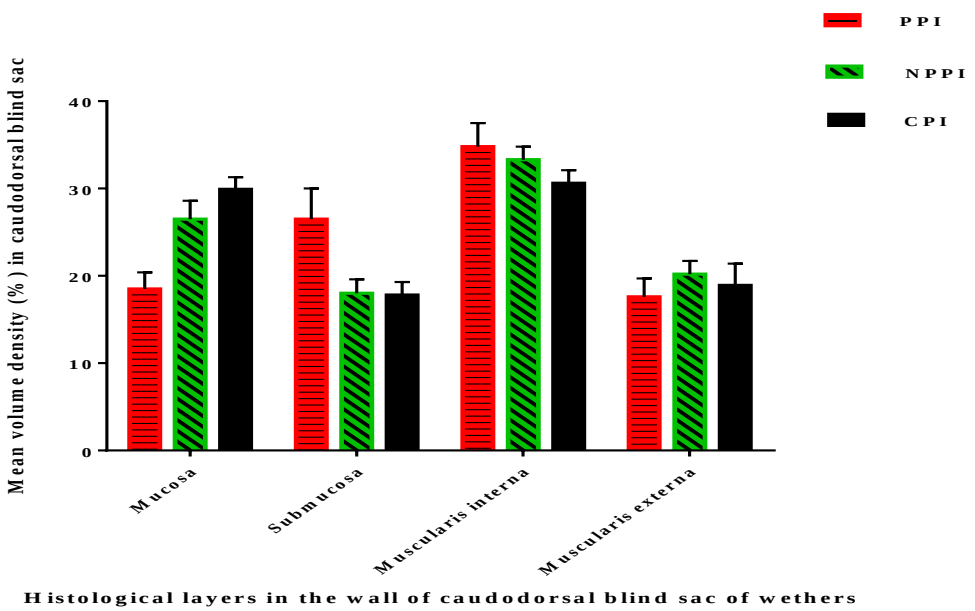


Figure 3.19b: Mean volume densities of tissues in the dorsal and ventral ruminal sacs of three groups of wethers at 4-week endpoint of Phase I experimentation. The results showed decrease in volume densities of tissues in dorsal and ventral sacs of PPI.
Key: **PPI** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPI** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPI** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.



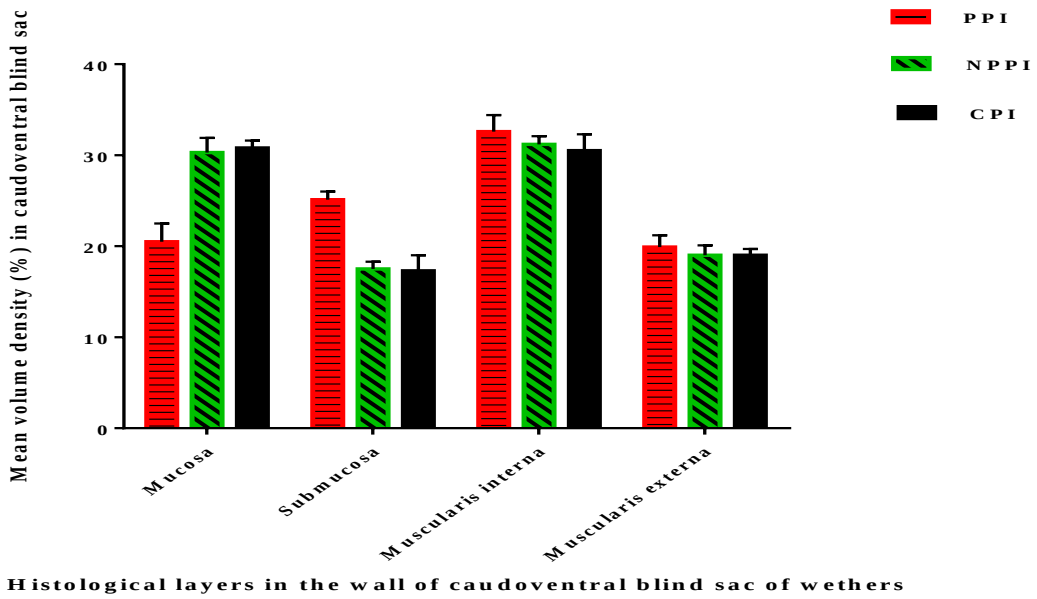


Figure 3.19c: Mean volume densities of tissues in the caudodorsal blind and caudoventral blind sacs of three groups of wethers at 4-week endpoint of Phase I experiment. The result showed decreased volume densities in the caudodorsal and caudoventral sacs of PPI.
Key: **PPI** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPI** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPI** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

Table 3.6: Comparison of mean absolute volume of tissues in the ruminal sacs in three groups of wethers with or without rumen impaction with plastic bags at 4-week Phase I experimentation

Histological layers in ruminal sacs	**Mean absolute volume (cm^3) of tissues in ruminal sacs (Mean ± SE)**			***P* Value**
	PPI (n = 5)	**NPPI (n = 5)**	**CPI (n = 5)**	
Cranial sac (AR)				
Mucosa	15.96 ± 2.09	30.26 ± 1.66	28.79 ± 1.39	0.0002[a] < 0.0001[b]
Submucosa	15.56 ± 1.89	15.70 ± 1.40	14.79 ± 0.90	0.9993
Muscularis interna	36.40 ± 4.24	34.14 ± 2.62	29.31 ± 0.76	0.0469[a]
Muscularis externa	21.76 ± 1.58	24.16 ± 2.38	24.66 ± 1.25	0.6883
Dorsal sac (DS)				
Mucosa	18.60 ± 2.76	30.94 ± 3.72	27.66 ± 2.69	0.0449[a] 0.0280[b]
Submucosa	27.97 ± 1.91	22.82 ± 2.26	23.86 ± 2.34	0.5123
Muscularis interna	59.09 ± 5.53	44.27 ± 2.10	44.17 ± 3.77	0.0064[a] 0.0068[b]
Muscularis externa	35.33 ± 3.56	32.18 ± 2.97	28.36 ± 3.11	0.7766
Ventral sac (VS)				
Mucosa	59.23 ± 6.80	67.19 ± 4.23	61.42 ± 5.25	0.4767
Submucosa	51.27 ± 4.36	39.48 ± 4.21	36.97 ± 4.70	0.2036
Muscularis interna	76.15 ± 6.74	69.50 ± 3.45	65.36 ± 6.60	0.5946
Muscularis externa	59.65 ± 0.98	44.76 ± 2.25	44.89 ± 4.51	0.0834
Caudodorsal blind sac (CDB)				
Mucosa	3.57 ± 0.63	7.06 ± 0.51	8.01 ± 1.01	0.0011[a] 0.0120[b]
Submucosa	5.39 ± 1.45	4.86 ± 0.51	5.03 ± 1.11	0.8927
Muscularis interna	6.63 ± 0.79	8.98 ± 0.66	8.16 ± 0.93	0.1197
Muscularis externa	3.28 ± 0.40	5.41 ± 0.45	5.03 ± 0.78	0.1721
Caudoventral blind sac (CVB)				
Mucosa	14.94 ± 2.80	27.47 ± 3.06	29.59 ± 1.44	0.0002[a] 0.0015[b]
Submucosa	18.08 ± 2.39	15.74 ± 1.27	16.74 ± 2.02	0.7679
Muscularis interna	23.86 ± 3.87	27.94 ± 1.70	29.68 ± 3.04	0.4528
Muscularis externa	14.20 ± 1.95	17.23 ± 1.85	18.48 ± 1.77	0.6435

Values are presented as plus or minus standard error of mean (Mean ± SE) in centimeters cube (cm^3). Significant at $p < 0.05$. [a]P-value = test group PPI compared with negative control group CPI. [b]P-value = test group PPI compared with positive control group NPPI

NB: Serosa values per ruminal sac not included because they were negligible.

Key: **PPI** = wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPI** = wethers that had rumenotomy performed but no plastic bags implanted, **CPI** = wethers that had neither rumenotomy done nor plastic bags implanted, **n** = number of wethers in the group.

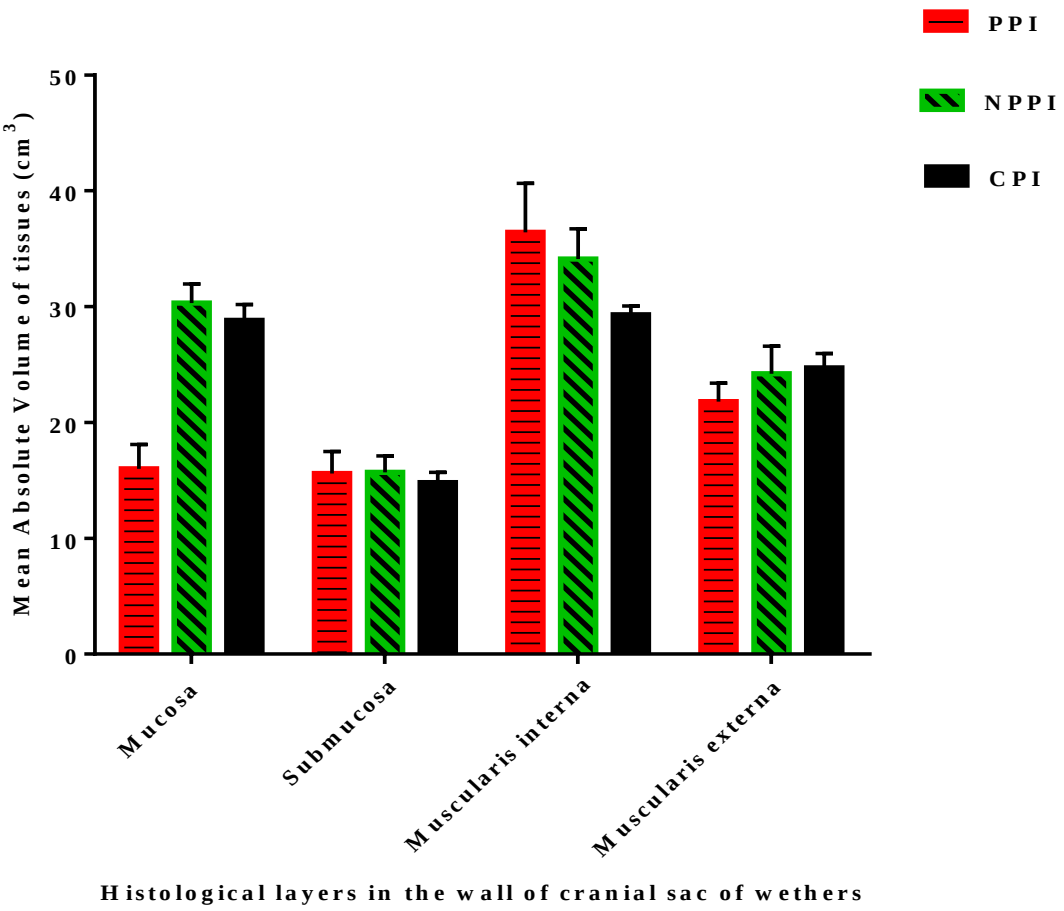
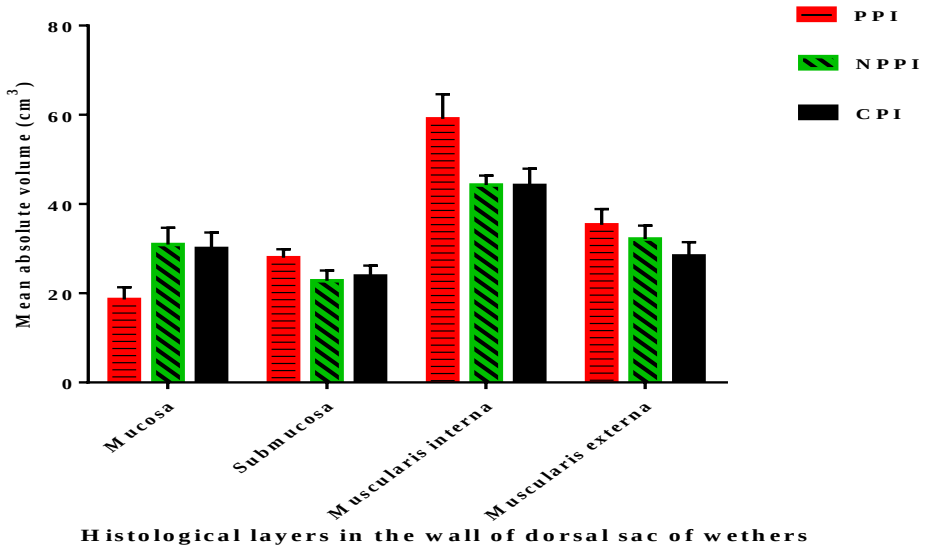


Figure 3.20a: Mean absolute volume of tissues in the cranial ruminal sac of three groups of wethers at 4-week endpoint of Phase I experiment. The results showed reduction in volume of tissues in the cranial sac of the test wethers.
Key: **PPI** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPI** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPI** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.



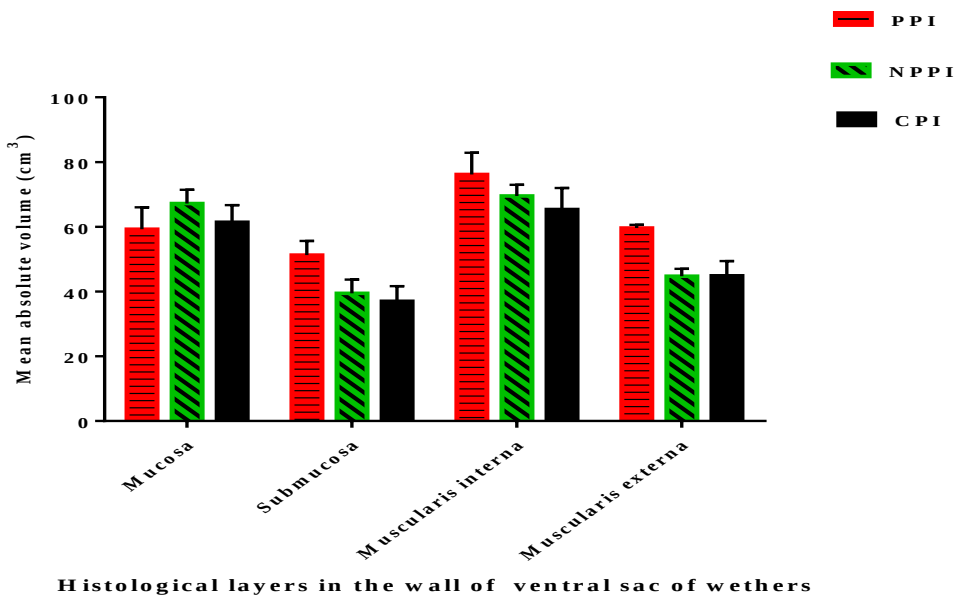
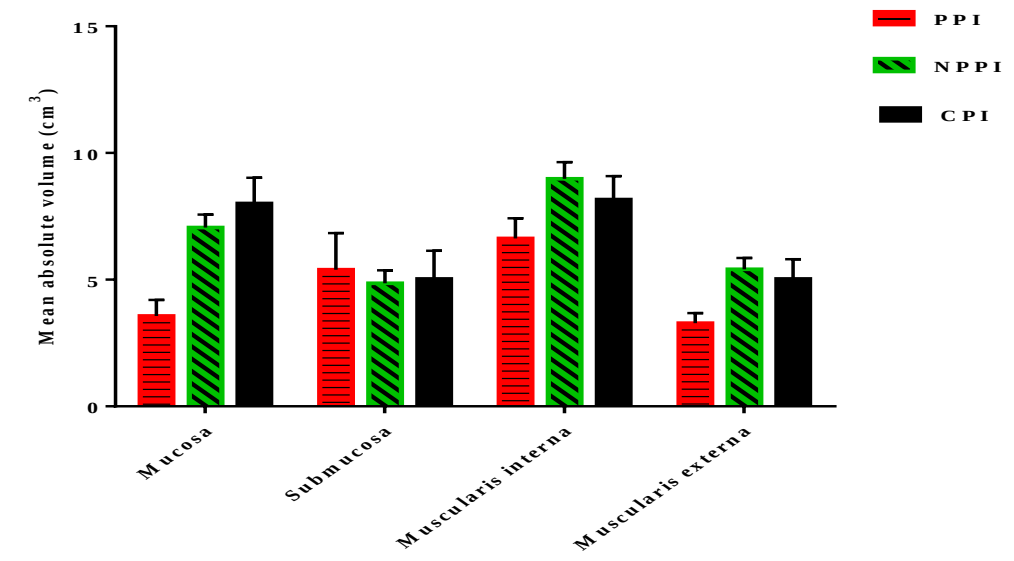


Figure 3.20b: Relative mean absolute volume of tissues in the dorsal and ventral ruminal sacs of three groups of wethers after 4 weeks of Phase I experimentation. The results showed changes in tissue volume of the test wethers.
Key: **PPI** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPI** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPI** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.



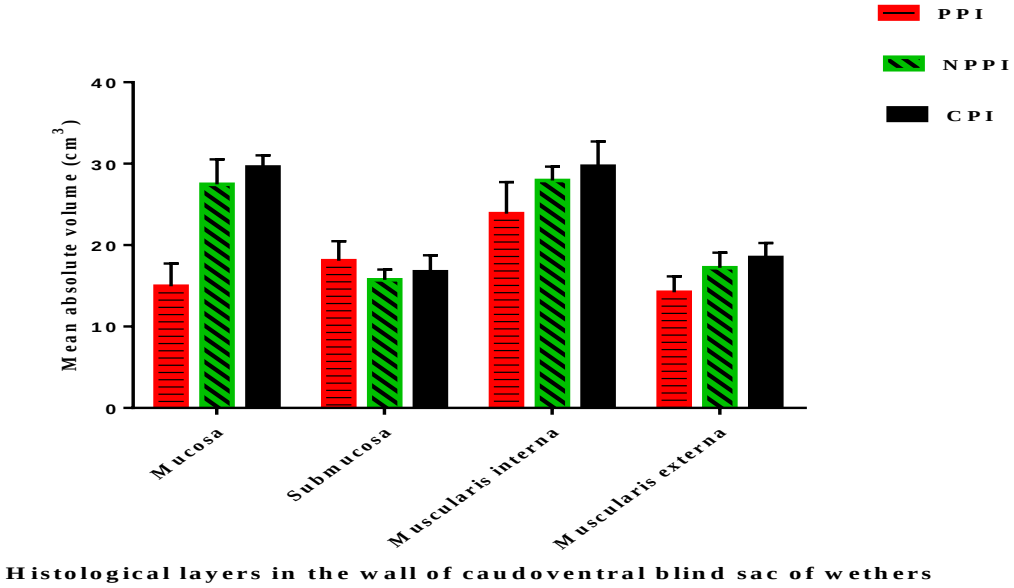


Figure 3.20c: Relative mean absolute volume of tissues in the caudodorsal blind and caudoventral blind sacs of three groups of wethers at 4-week endpoint of Phase I experiment. The results showed significant reduction in mucosal tissues of the test wethers.
Key: **PPI** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPI** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPI** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

3.5.5.3: Quantitative structural changes in the whole rumen of wethers during Phase I experiments

The results of stereological analysis of the total mean surface area and total mean absolute volume of the whole rumen of wethers in the 3 groups evaluated 4 weeks after implanting plastic bags in the rumen during Phase I experiments are presented in Tables 3.7. The calculated body-mass-standardized values for total mean surface area and mean absolute volume per mean body weight of wethers in the 3 groups at 4-week experimental endpoint are presented in Table 3.8.

The total mean surface area of the absorptive mucosal surface of the entire rumen from wethers impacted with plastic bags (PPI) at the end of Phase I experiment was $0.368 \pm 0.016 m^2$. The total mean absolute volume of mucosa in the whole rumen of group PPI was $112.30 cm^3$.

The total mean surface area of the entire rumen of the test group (PPI) decreased by 20% compared to the negative control (CPI) whose total mean surface area was $0.458 \pm 0.014 m^2$ and by 21% compared to the positive control (NPPI) group which had a mean of $0.463 \pm 0.013 m^2$ (Table 3.7). Tukey's multiple comparison test of one-way ANOVA revealed that these differences in the values of total mean surface area were significant comparing group PPI with NPPI ($p = 0.0016$) and with CPI ($p = 0.0024$).

The total mean absolute volume of mucosa in the whole rumen of test group (PPI) decreased by 27% ($p = 0.0017$) compared to the negative control group (CPI) which had a mean of $155.47 cm^3$ and by 31% ($p = 0.0005$) compared to the positive control (NPPI) whose mean was $162.92 cm^3$. The mean absolute volume of the muscularis interna layer in the rumen of the test group (PPI) was 13% and 9% more compared to negative control group (CPI) and positive control group

(NPPI) respectively (Table 3.7). At the end of experimentation the absolute volume of mucosa in the rumen of wethers in group PPI was about 20% of their entire rumen volume which was 579.08cm^3 while that of either of the control group was about 28% of their whole rumen volume.

Table 3.7: Total mean surface area and volume of tissues in the whole rumen of three groups of wethers after 4-week of Phase I experimentation

Group (n = 5)	**Mean body weight (kg)**	**Total mean absolute volume (cm^3) of rumen**					**Total mean surface area of the rumen (m^2)**
		Mucosa	Submucosa	Muscularis interna	Muscularis externa	Serosa	
PPI	23.5 ± 0.6	112.30 ±7.43	118.26	202.13	134.21	12.18	0.368 ± 0.016
NPPI	27.5 ± 1.9	162.92 ±5.89	98.60	184.83	123.75	10.09	0.463 ± 0.013
CPI	27.8± 0.7	155.47 ±6.64	97.40	176.68	121.42	11.21	0.458 ± 0.014

Values are expressed as means. Standard error of means are also indicated

Key: PPI = wethers whose rumen were implanted with plastic bags through rumenotomy, NPPI = wethers that had with rumenotomy performed but not implanted with plastic bags, CPI = wethers with neither rumenotomy nor plastic bags in the rumen, n = number of wethers per group, kg = kilogram, cm^3 = cubic centimeter, m^2 = squared meter

Table 3.8: Body-mass-standardized total surface area and volume of mucosal tissue in the entire rumen of three groups of wethers in Phase I experiment at 4-week endpoint

Body-mass-standardized per parameter	**Experimental Groups**		
	PPI	**NPPI**	**CPI**
Total absorptive surface area (m^2kg^{-1})	0.0157	0.0168	0.0165
Total volume of mucosa in ruminal wall (cm^3kg^{-1})	4.78	5.92	5.59

Key: PPI = wethers whose rumen were implanted with plastic bags through rumenotomy, NPPI = wethers that had with rumenotomy performed but not implanted with plastic bags, CPI = wethers with neither rumenotomy nor plastic bags in the rumen, m^2kg^{-1} = squared meter per kilogram, cm^3kg^{-1} = cubic centimeter per kilogram.

3.5.6 Stereological analysis of structural changes in the rumen of wethers during Phase II experimentation

3.5.6.1 Mean surface density and mean surface area of ruminal sacs at the 8-week experimental endpoint in three groups of wethers

Stereological analysis of the effects of rumen impaction with plastic bags on surface density (Sv) and surface area of the various ruminal sacs: cranial (AR), dorsal (DS), ventral (VS), caudodorsal blind (CDB) and caudoventral blind (CVB) in the test and control groups of wethers are presented in Tables 3.9 and 3.10. The comparison of mean surface density and mean surface area of ruminal sacs between the wethers with plastic bags in the rumen and the control groups are shown in Figures 3.21 and 3.22.

Eight weeks after implanting plastic bags in the rumen, the mean surface density (Sv) estimates of the cranial (AR), dorsal (DS), ventral (VS) caudal dorsal (CDB) and caudal ventral blind (CVB) sacs from rumen in the test group (PPII) were significantly lower ($p < 0.05$) compared to positive (NPPII) and negative (CPII) control groups, which did not have plastic bags (Table 3.9). The mean surface densities of AR, DS and VS from wethers in the test group (PPII) significantly decreased by 38% ($p < 0.0001$), 32% ($p = 0.0007$) and 36% ($p < 0.0001$) respectively when compared with the positive control group (NPPII). Similarly, significant decreases were also observed in mean surface density of AR, DS and VS by 40% ($p < 0.0001$), 32% ($p = 0.0006$) and 37% ($p < 0.0001$) respectively in wethers with plastic bags compared to the negative control group (CPII). The mean surface densities for caudodorsal and caudoventral blind sacs of the test group (PPII) were significantly lower ($p < 0.05$) compared with negative (CPII) and positive control groups (NPPII) (Table 3.9). The mean surface density of the caudodorsal and

caudoventral blind sacs of test group (PPII) significantly decreased by 22% ($p = 0.0254$) and 33% ($p = 0.0003$) respectively compared to positive control group (NPPII). Similarly, comparing the mean surface densities of the caudodorsal and caudoventral blind sacs of the test group (PPII) to those of the negative control group CPII, there was a decrease of 25% ($p = 0.0091$) and 35% ($p < 0.0001$) respectively in group PPII.

Tukey's multiple comparison test of two-way ANOVA revealed significant decrease ($p < 0.05$) in mean surface area of the cranial (AR), dorsal (DS), ventral (VS) and caudoventral blind (CVB) sacs between wethers with rumen plastic bag impaction (PPII) and those without plastic bags in their rumen (Table 3.10). The mean surface area of the cranial sac of the rumen from the test group (PPII) was significantly lower by 51% ($p < 0.0001$) compared to positive control group (NPPII). The mean surface area of the dorsal and ventral sacs from the test group (PPII) was also significantly lower by 30% ($p = 0.0227$) and 41% ($p < 0.0001$) respectively in comparison to the positive control group (NPPII). The mean surface area of AR, DS and VS in group PPII decreased by 52% ($p < 0.0001$), 28% ($p = 0.0376$) and 41% ($p < 0.0001$) respectively in comparison to the negative control group (CPII) (Table 3.10). Mean surface area of caudoventral blind sac of the test group (PPII) was 43% ($p = 0.0031$) lower than the negative control group (CPII), and 38% ($p = 0.0227$) lower than positive control group (NPPII).

Table 3.9: Mean surface densities of ruminal sacs of three groups of wethers at 8-week endpoint in Phase II experimentation

Rumen compartments	**Mean surface density (cm^{-1}) of ruminal sacs (Mean ± SE)**			***P* Value**
	***PPII (n = 5)**	**NPPII (n = 5)**	**CPII (n = 5)**	
Cranial sac (AR)	5.420 ± 0.176	8.728 ± 0.735	8.995 ± 0.659	< 0.0001[a] < 0.0001[b]
Dorsal sac (DS)	5.475 ± 0.221	8.069 ± 0.521	8.096 ± 0.293	0.0006[a] 0.0007[b]
Ventral sac (VS)	5.965 ± 0.290	9.260 ± 0.510	9.387 ± 0.743	< 0.0001[a] < 0.0001[b]
Caudodorsal blind sac (CDB)	6.157 ± 0.231	7.929 ± 0.196	8.181 ± 0.460	0.0091[a] 0.0254[b]
Caudoventral blind sac (CVB)	5.705 ± 0.175	8.449 ± 0.595	8.743 ± 0.532	< 0.0001[a] 0.0003[b]

Values are presented as plus or minus standard error of mean (Mean ± SE) per centimeter (cm^{-1})
Significance at $p < 0.05$
[a]*P*-value = test group PPII compared with negative control group CPII
[b]*P*-value = test group PPII compared with positive control group NPPII
Key:
PPII = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPII = positive control group of wethers that had rumenotomy but no plastic bags in the rumen
CPII = negative control group of wethers that had neither plastic bags in their rumen nor rumenotomy done.
n = number of wethers per group

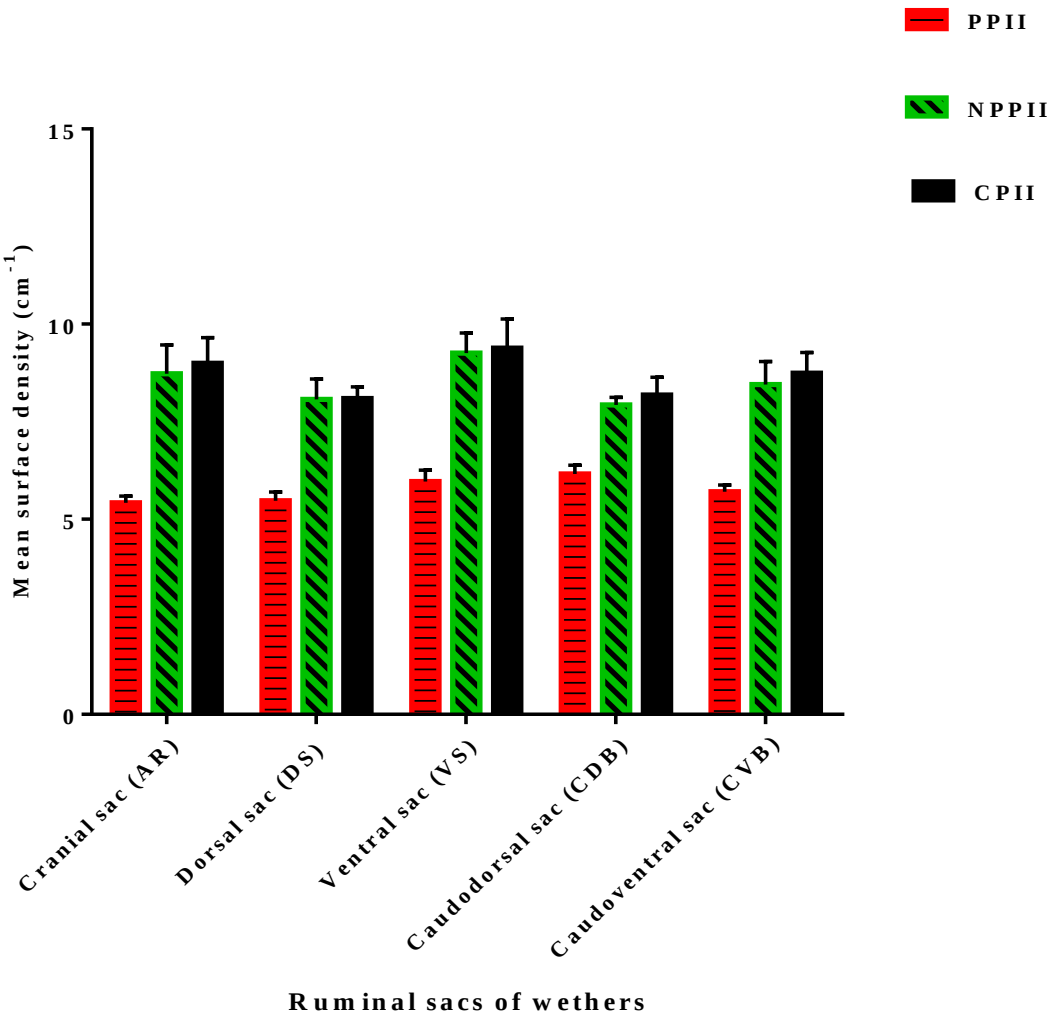


Figure 3.21: Comparison of the mean surface densities of different ruminal sacs in three groups of wethers of Phase II experimentation. The results showed decreased surface densities in ruminal sacs of the test group.

Key:
PPII = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPII = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen
CPII = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

Table 3.10: Mean surface area of ruminal sacs of three groups of wethers at 8-week endpoint in Phase II experiment

Rumen compartment	**Mean surface area (m^2) of ruminal sacs (Mean ± SE)**			***P* Value**
	PPII (n = 5)	**NPPII (n = 5)**	**CPII (n = 5)**	
Cranial sac (AR)	0.048 ± 0.006	0.097 ± 0.008	0.101 ± 0.010	< 0.0001[a] < 0.0001[b]
Dorsal sac (DS)	0.065 ± 0.003	0.092 ± 0.008	0.090 ± 0.007	0.0376[a] 0.0227[b]
Ventral sac (VS)	0.110 ± 0.009	0.186 ± 0.011	0.186 ± 0.010	< 0.0001[a] < 0.0001[b]
Caudodorsal blind sac (CDB)	0.014 ± 0.003	0.019 ± 0.003	0.016 ± 0.001	0.9778[a] 0.8695[b]
Caudoventral blind sac (CVB)	0.045 ± 0.003	0.072 ± 0.006	0.079 ± 0.007	0.0031[a] 0.0227[b]

Values are presented as plus or minus standard error of mean (Mean ± SE) in squared meters (m^2)
Significance at $p < 0.05$
[a]*P*-value = test group PPII compared with negative control group CPII
[b]*P*-value = test group PPII compared with positive control group NPPII
Key:
PPII = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPII = positive control group of wethers that had rumenotomy but no plastic bags in the rumen
CPII = negative control group of wethers that had neither plastic bags in their rumen nor rumenotomy done.
n = number of wethers per group

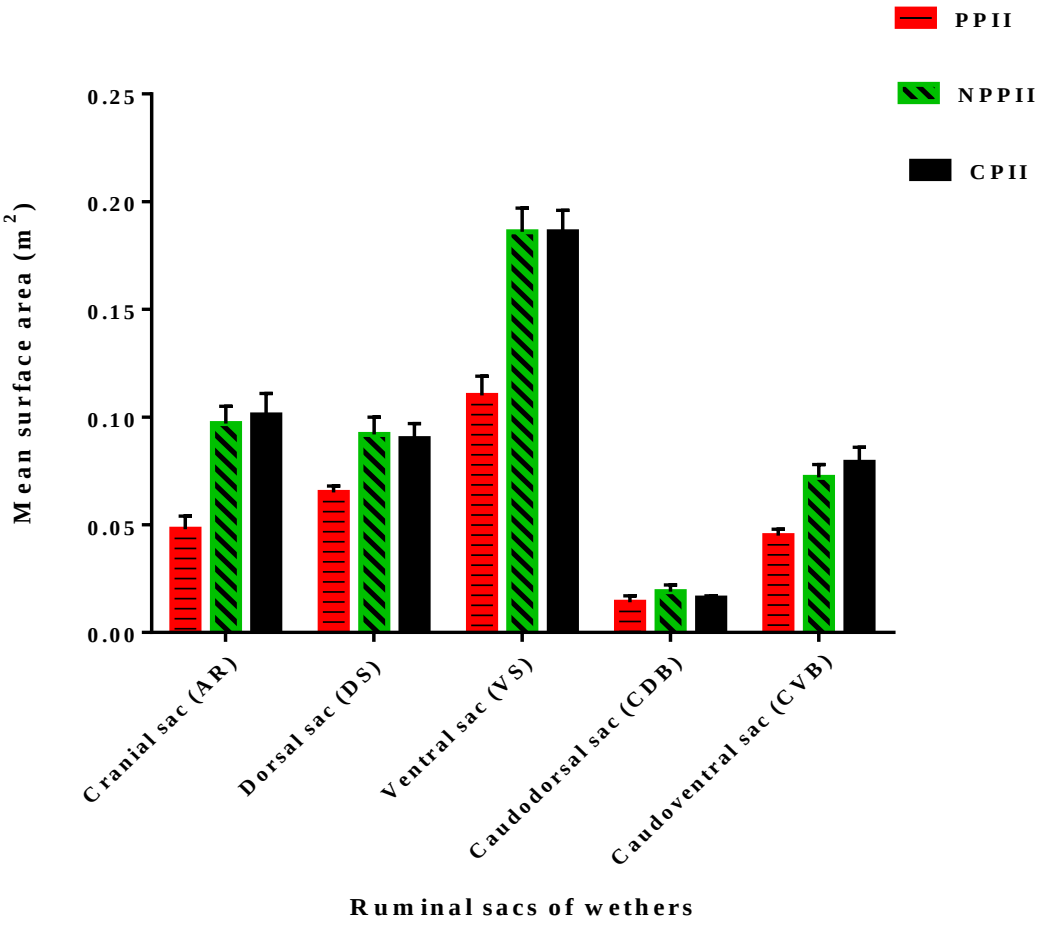


Figure 3.22: A comparison of the mean surface areas of different ruminal sacs from three groups of wethers at the 8-week end point of Phase II experiment. The results showed a significant loss in surface area of the ruminal sacs of the test group

Key:

PPII = test group of wethers whose rumen were implanted with plastic bags through rumenotomy

NPPII = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen

CPII = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

3.5.6.2: Effects of rumen impaction on volume density and absolute volume of tissues in the ruminal sacs in wethers during the Phase II experiments

The results of stereological evaluation of the effect of rumen impacted with plastic bags on the volume density and absolute volume of the mucosa, submucosa, muscularis interna and muscularis externa layers of the ruminal sacs in wethers in one test group and the control groups during Phase II of the experiment are presented in Tables 3.11 and 3.12. Comparative graphical presentation showing how rumen impaction with plastic bags for 8 weeks affects the volume density and the absolute volume of ruminal sacs is given in Figures 3.23 and 3.24 respectively.

The mean volume density of mucosa in the cranial (AR), dorsal (DS), ventral (VS), caudodorsal blind (CDB) and caudoventral blind (CVB) sacs of rumen in wethers whose rumen had plastic bags (PPII) for 8 weeks was significantly ($p < 0.05$) lower compared to the negative control (CPII) and positive control (NPPII) groups of wethers whose rumen did not have plastic bags (Table 3.11). The mean volume density of mucosa in the test group of wethers (PPII) decreased significantly by 11.0% ($p = 0.0004$) in the cranial sac, by 16.0% ($p = 0.0002$) in the dorsal sac, by 9.1% ($p = 0.0019$) in the ventral sac, by 15.6% ($p < 0.0001$) in the caudodorsal blind sac and by 19.0% ($p < 0.0001$) in the caudoventral blind sacs compared with the negative control group (CPII). Likewise Tukey's multiple comparison test revealed significant decrease ($p < 0.05$) in values of mean volume density of mucosa in all the ruminal sacs between the test group (PPII) and the positive control group (NPPII). The decrease in mean volume density of mucosa in AR, DS, VS, CDB and CVB in group PPII were 13% ($p < 0.0001$), 17% ($p < 0.0001$), 7% ($p = 0.0181$), 17% ($p < 0.0001$) and 14% ($p < 0.0001$) respectively in comparison to those from the NPPII group.

The results obtained for mean absolute volume of mucosa in the cranial, dorsal, ventral caudodorsal and caudoventral blind sacs of the rumen all showed differences in values between wethers that had rumen impaction with plastic bags (PPII) and the controls without plastic bags (CPII and NPPII) (Table 3.12). The mean absolute volume of mucosa in all the ruminal sacs of the test group (PPII) decreased compared with negative control group (CPII) and positive control group (NPPII). Tukey's multiple comparison test of two-way ANOVA revealed significant differences between the test group (PPII) and the negative control group (CPII) for mean absolute volume of mucosa in the cranial sac ($p < 0.0001$), dorsal sac ($p = 0.0027$), ventral sac ($p = 0.0070$), caudodorsal blind sac ($p = 0.0065$) and caudoventral blind sac ($p < 0.0001$). This difference included a decrease in the mean absolute volume of mucosa by 50.9% in the cranial sac, by 51.8% in the dorsal sac, by 38.0% in the ventral sac, by 52.7% in the caudodorsal blind sac and by 64.8% in the caudoventral blind sac of the test group (PPII) compared to the positive control group (CPII) after 8 weeks of rumen impaction with plastic bags. Likewise, there was a significant decrease of 52.4% ($p < 0.0001$), 55.1% ($p = 0.0006$), 32.3% ($p = 0.0427$), 58.8% ($p = 0.0004$) and 55.0% ($p = 0.0002$) in mean absolute volume of mucosa in AR, DS, VS, CDB and CVB respectively in the test group PPII in comparison to those of the positive control group NPPII. However there was a 28.6% increase in the mean absolute volume of the muscularis interna layer of the dorsal ruminal sac of the test group (PPII), which was significant ($p = 0.0340$) when compared with the negative control group (CPII) at the 8-week experimental endpoint.

Table 3.11: Mean volume densities of tissues in the ruminal sacs of three groups of wethers at 8-week experimental endpoint

Histological layers in ruminal sacs	**Mean volume density (%) (Mean ± SE)**			***P* Value**
	***PPII (n = 5)**	**NPPII (n = 5)**	**CPII (n = 5)**	
Cranial sac (AR)				
Mucosa	18.6 ± 1.2	31.2 ± 2.6	29.6 ± 2.2	0.0004[a] < 0.0001[b]
Submucosa	22.7 ± 2.5	17.3 ± 1.3	16.6 ± 1.0	0.1187
Muscularis interna	33.5 ± 3.1	29.5 ± 1.9	29.0 ± 0.9	0.3023
Muscularis externa	23.8 ± 0.9	19.5 ± 1.8	21.8 ± 1.7	0.2525
Dorsal sac (DS)				
Mucosa	14.0 ± 0.3	31.2 ± 6.5	30.0 ± 2.0	0.0002[a] < 0.0001[b]
Submucosa	21.6 ± 1.3	15.2 ± 0.4	19.7 ± 0.6	0.2077
Muscularis interna	38.4 ± 2.6	30.8 ± 4.0	29.5 ± 1.7	0.1126
Muscularis externa	24.2 ± 1.4	20.2 ± 2.4	18.2 ± 1.0	0.5334
Ventral sac (VS)				
Mucosa	19.3 ± 0.5	26.4 ± 1.2	28.4 ± 2.2	0.0019[a] 0.0181[b]
Submucosa	23.1 ± 2.3	18.4 ± 1.8	20.4 ± 2.0	0.1568
Muscularis interna	32.4 ± 2.9	31.9 ± 1.7	32.1 ± 2.1	0.9783
Muscularis externa	22.4 ± 1.1	20.6 ± 0.7	17.3 ± 1.1	0.7539
Caudodorsal blind sac (CDB)				
Mucosa	15.6 ± 1.7	32.6 ± 1.2	31.2 ± 2.5	< 0.0001 < 0.0001
Submucosa	25.8 ± 0.8	20.0 ± 0.1	22.0 ± 1.3	0.0210[b]
Muscularis interna	36.9 ± 1.5	28.6 ± 1.4	28.1 ± 1.5	0.0003[a] 0.0007[b]
Muscularis externa	18.6 ± 2.3	16.1 ± 0.3	15.9 ± 1.2	0.4611
Caudoventral blind sac (CVB)				
Mucosa	13.2 ± 1.7	27.3 ± 1.1	32.2 ± 1.9	< 0.0001[a] < 0.0001[b]
Submucosa	33.6 ± 1.5	20.9 ± 1.3	22.6 ± 0.6	< 0.0001[a] < 0.0001[b]
Muscularis interna	29.7 ± 1.8	29.0 ± 1.2	25.5 ± 1.5	0.9366
Muscularis externa	20.8 ± 2.1	20.6 ± 1.1	17.1 ± 0.4	0.9947

Values are presented as plus or minus standard error of mean (Mean ± SE) in percentage (%)
Significance at $p < 0.05$. [a]*P*-value = test group PPII compared with negative control group CPII
[b]*P*-value = test group PPII compared with positive control group NPPII
NB: Serosa values per sac not included because they were negligible.
Key: **PPII** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group that had neither rumenotomy nor plastic bags implanted in their rumen.

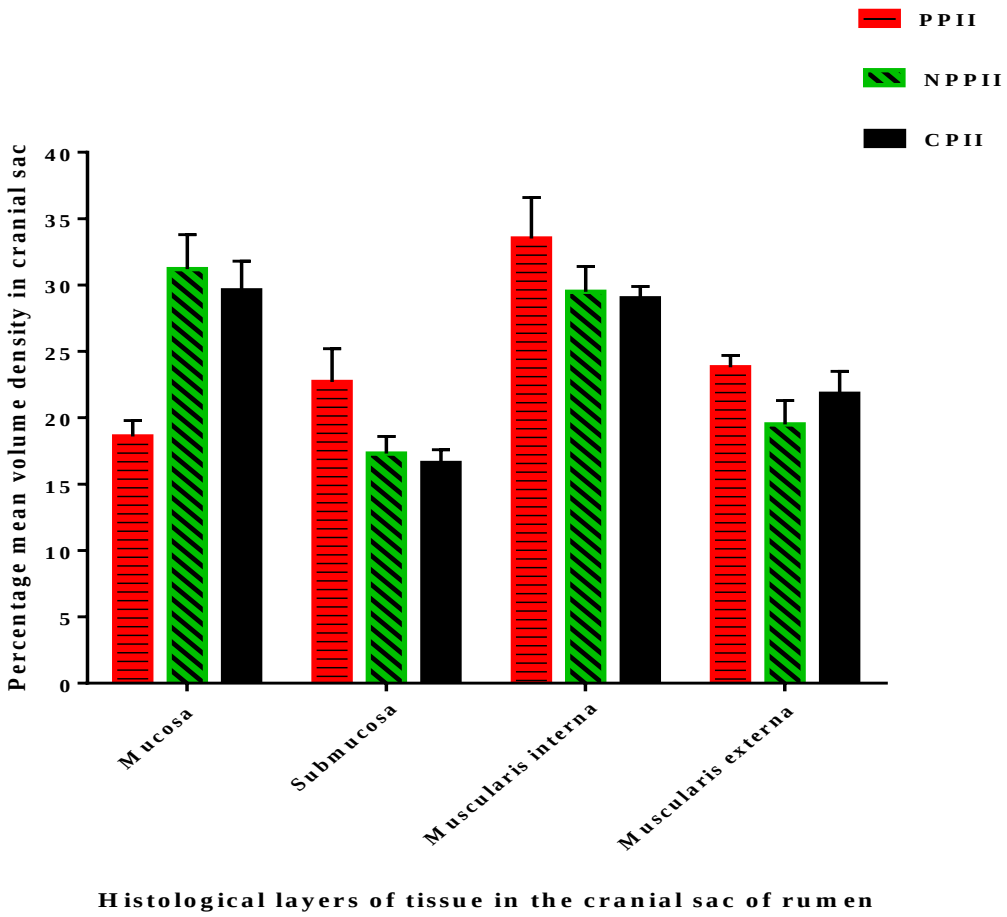


Figure 3.23a: Comparative mean volume densities of tissues in the cranial ruminal sacs in three groups of wethers after 8 weeks of experimentation. The result showed that the volume density of mucosal tissues in the test groups was significantly reduced.

Key:
PPII = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPII = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen
CPII = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

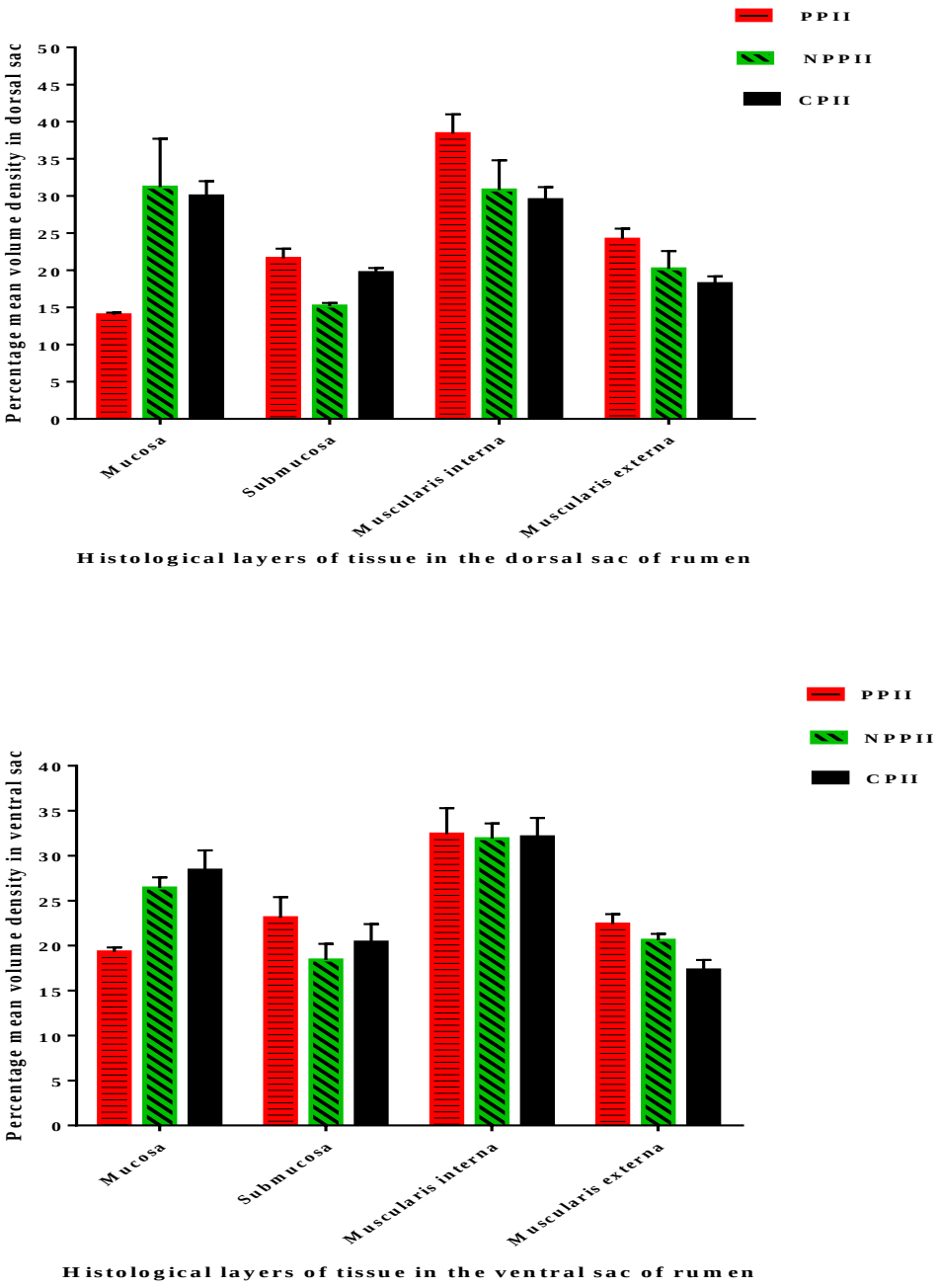
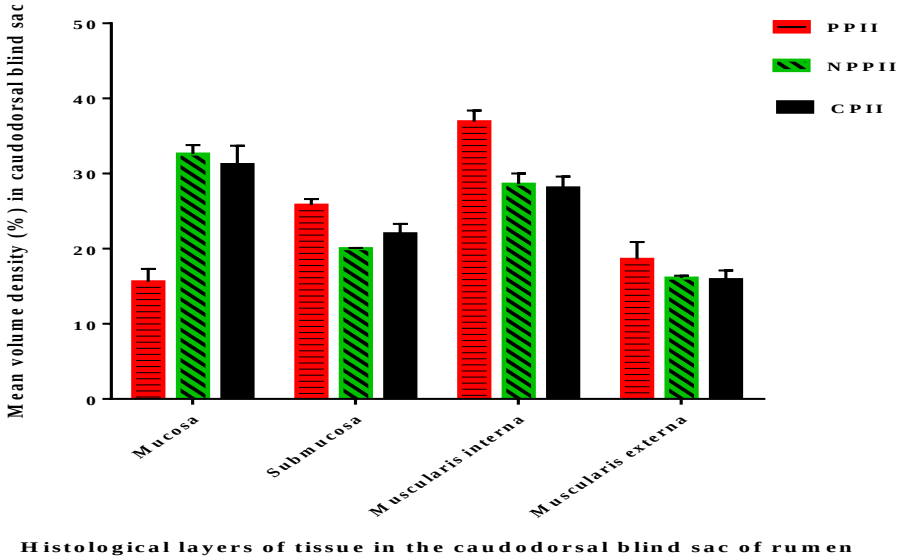


Figure 3.23b: Comparative mean volume densities of tissues in the dorsal and ventral ruminal sacs in wethers of three different groups at the 8-week end point in Phase II experiment. The volume of mucosal tissue was significantly reduced in the test group.
Key: **PPII** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.



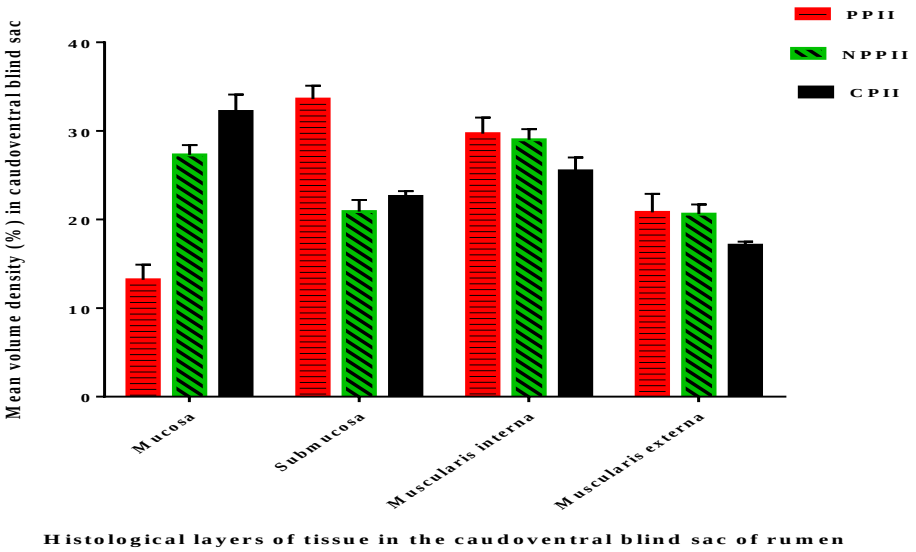


Figure 3.23c: Mean volume densities of tissues in the caudodorsal blind and caudoventral blind sacs in three groups of wethers after 8 weeks of experimentation. The results showed a significant reduction in mucosal tissue volume in the test group.
Key: **PPII** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

Table 3.12: Mean absolute volume of tissues in the different ruminal sacs of three groups of wethers at 8-week experimental endpoint

Histological layers in ruminal sacs	**Mean absolute volume (cm^3) of tissues in ruminal sacs (Mean ± SE)**			***P* Value**
	PPII (n = 5)	**NPPII (n = 5)**	**CPII (n = 5)**	
Cranial sac (AR)				
Mucosa	16.49 ± 2.45	34.66 ± 2.71	33.57 ± 3.86	< 0.0001[a] < 0.0001[b]
Submucosa	19.22 ± 1.81	19.29 ± 1.46	18.66 ± 1.32	0.9998
Muscularis interna	29.43 ± 4.64	32.87 ± 2.10	32.48 ± 1.68	0.6172
Muscularis externa	21.00 ± 2.88	21.80 ± 2.34	24.23 ± 1.49	0.9739
Dorsal sac (DS)				
Mucosa	16.48 ± 0.32	36.72 ± 8.90	34.17 ± 4.66	0.0027[a] 0.0006[b]
Submucosa	25.52 ± 1.52	17.35 ± 1.18	22.10 ± 2.09	0.2442
Muscularis interna	45.39 ± 3.08	34.57 ± 4.07	32.43 ± 1.50	0.0340[a]
Muscularis externa	28.79 ± 2.26	22.66 ± 2.44	20.27 ± 1.67	0.4468
Ventral sac (VS)				
Mucosa	35.96 ± 3.86	53.13 ± 3.04	57.97 ± 8.05	0.0070[a] 0.0427[b]
Submucosa	42.27 ± 4.30	36.83 ± 3.27	40.58 ± 4.06	0.7123
Muscularis interna	61.67 ± 9.77	64.16 ± 3.66	63.92 ± 4.21	0.9309
Muscularis externa	41.16 ± 3.23	41.48 ± 1.85	34.95 ± 3.33	0.9988
Caudodorsal blind sac (CDB)				
Mucosa	3.28 ± 0.50	7.96 ± 1.51	6.93 ± 1.10	0.0065[a] 0.0004[b]
Submucosa	5.45 ± 0.69	4.82 ± 0.79	4.79 ± 0.56	0.8447
Muscularis interna	7.80 ± 1.07	6.71 ± 0.87	6.01 ± 0.39	0.6060
Muscularis externa	3.71 ± 0.22	3.90 ± 0.67	3.39 ± 0.21	0.9847
Caudoventral blind sac (CVB)				
Mucosa	10.50 ± 1.67	23.34 ± 1.19	29.85 ± 4.27	< 0.0001[a] 0.0002[b]
Submucosa	26.75 ± 2.42	17.77 ± 0.84	20.53 ± 1.68	0.0097[b]
Muscularis interna	23.38 ± 1.89	25.08 ± 2.05	22.87 ± 1.65	0.8307
Muscularis externa	16.40 ± 1.95	17.86 ± 1.74	15.62 ± 1.47	0.8720

Values are presented as plus or minus standard error of mean (Mean ± SE) in cubic centimeters (cm^3). Significance at $p < 0.05$. [a]*P*-value = test group PPII compared with negative control group CPII. [b]*P*-value = test group PPII compared with positive control group NPPII

NB: Serosa values per sac not included because they were negligible.

Key: **PPII** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group that had neither rumenotomy nor plastic bags implanted in their rumen, **n** = number of wethers per group.

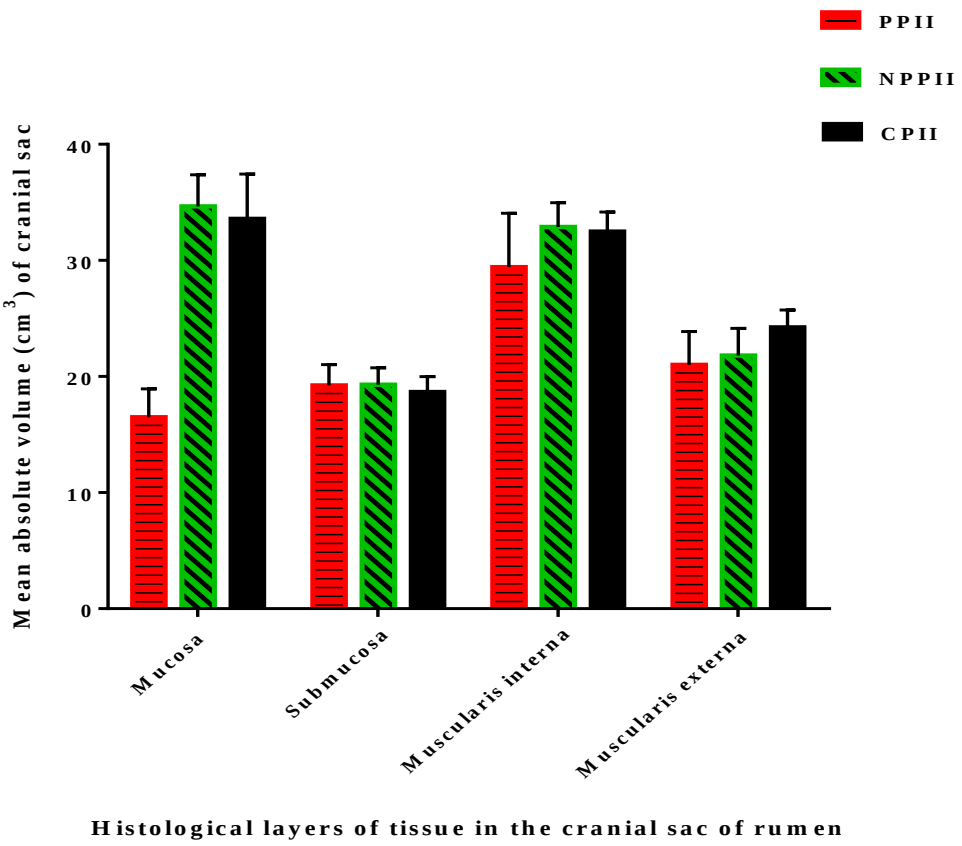


Figure 3.24a: Comparative mean absolute volume of tissues in the cranial ruminal sac of three groups of wethers after 8 weeks experimentation. The result showed a significant reduction in mucosal tissue volume of the test group.
Key: **PPII** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

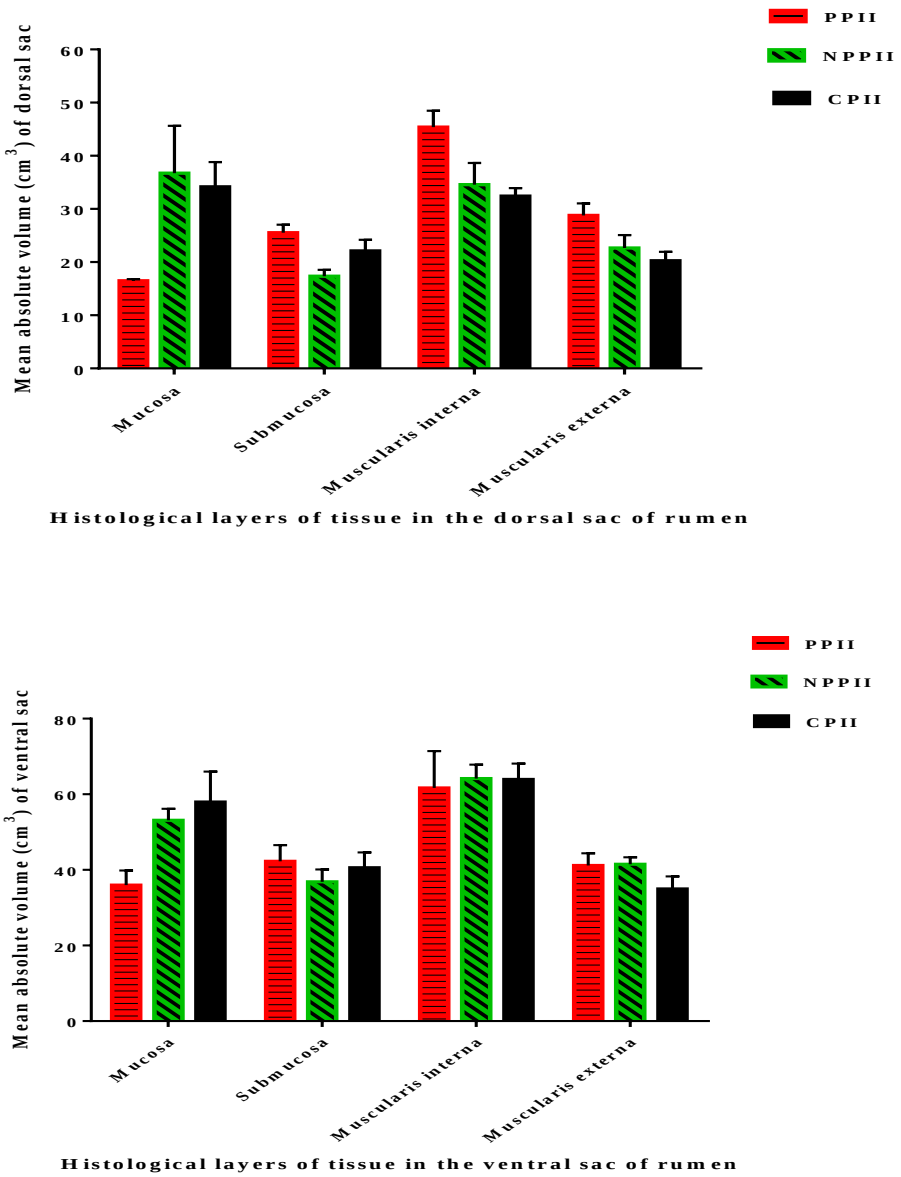
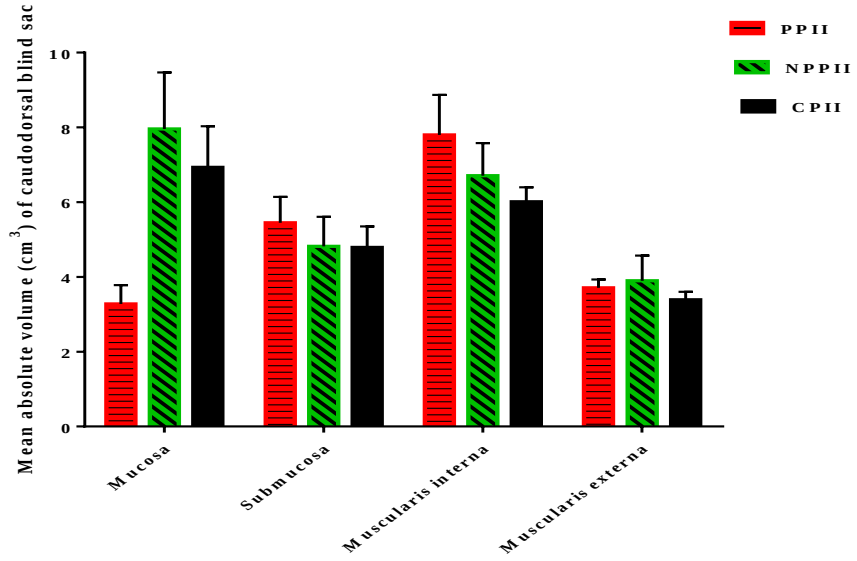
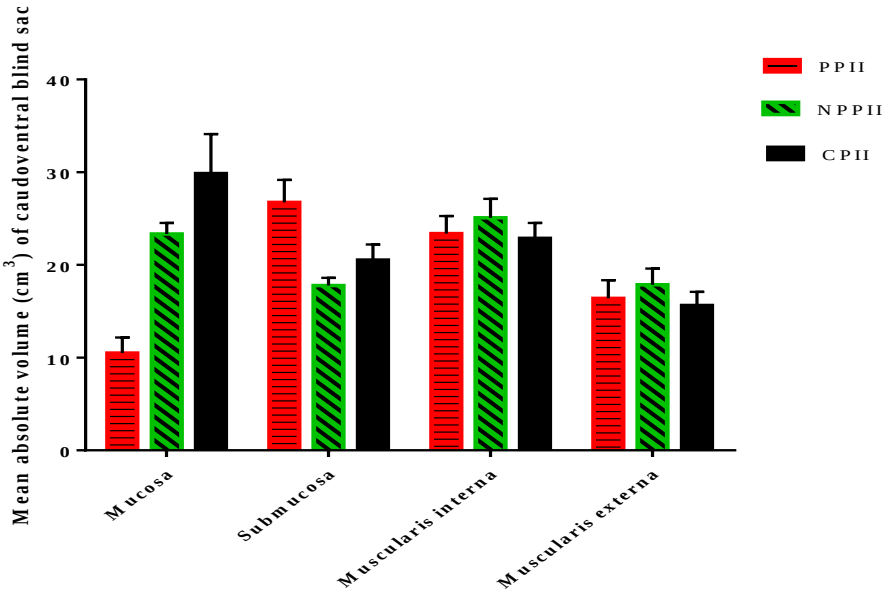


Figure 3.24b: Comparative mean absolute volume of tissues in the dorsal and ventral ruminal sacs of three groups of wethers at the end of 8-week experimentation. The results showed significant decrease in volume of mucosal tissue.

Key: **PPII** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.



Histological layers of tissue in the caudodorsal blind sac of wethers



Histological layers of tissue in the caudoventral blind sac of wethers

Figure 3.24c: Comparative mean absolute volume of tissues in caudodorsal blind and caudoventral blind ruminal sacs in three groups of wethers at an experimental endpoint of 8 weeks. The results showed significant reduction in volume of mucosal tissue in the test group.

Key: **PPII** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

3.5.6.3: Quantitative structural changes in the whole rumen of wethers during Phase II experiments

The results of stereological analysis of the total surface area and absolute volume of the whole rumen 8 weeks after implanting plastic bags during Phase II of the study are presented in table 3.13. The calculated body-mass-standardized values for total mean surface area and mean absolute volume per mean body weight of wethers in the 3 groups at 8-week experimental endpoint are presented in Table 3.14.

The total mean surface area of the absorptive mucosal surface of the entire rumen from wethers impacted with plastic bags (PPII) at the end of Phase II experiment was $0.282 \pm 0.012 m^2$. The total mean absolute volume of mucosa in the whole rumen of group PPI was $82.70 cm^3$ (Table 3.13). After eight weeks of impaction the total mean surface area of the rumen in the test wethers (PPII), reduced significantly by 40.4% ($p < 0.0001$) compared with the negative control group (CPII) whose mean was $0.473 \pm 0.017 m^2$ and by 39.7% ($p < 0.0001$) compared with the positive control group (NPPII) which had a mean of $0.467 \pm 0.017 m^2$. The total mean absolute volume of mucosa in the whole rumen of wethers with plastic bags (PPII) decreased by 49.1% ($p = 0.0002$) compared to the negative control group (CPII) which had a mean of $162.48 cm^3$ and by 46.9% ($p = 0.0004$) compared to the positive control (NPPII) whose mean was $155.82 cm^3$.

At the end of experimentation the absolute volume of mucosa in the rumen of wethers in group PPII formed 17% of their entire rumen volume which was $492.05 cm^3$ while that of either of the control group (NPPII and CPII) formed about 30% of their whole rumen volume (Table 3.13).

Table 3.13: Comparative mean absolute volume and total mean surface area of the entire rumen from three groups of wethers after 8 weeks Phase II experimentation

Experimental group (n = 5)	**Mean body weight (kg)**	**Mean absolute volume of tissue (cm^3) in rumen**					**Total mean surface area of rumen (m^2)**
		Mucosa	Submucosa	Muscularis interna	Muscularis externa	Serosa	
PPII	25.0 ± 1.4	82.70 ± 4.18	119.20	167.67	111.05	11.43	0.282 ± 0.012
NPPII	28.6 ± 0.7	155.82 ± 10.41	96.05	163.39	107.69	13.63	0.467 ± 0.017
CPII	30.9 ± 1.8	162.48 ± 11.91	106.66	157.72	98.45	13.21	0.473 ± 0.017

Values are expressed as means. Standard error of means are also indicated

Key: PPII = wethers whose rumen were implanted with plastic bags through rumenotomy, NPPII = wethers that had with rumenotomy performed but not implanted with plastic bags, CPII = wethers with neither rumenotomy nor plastic bags in the rumen, n = number of wethers per group, kg = kilogram, cm^3 = cubic centimeter, m^2 = squared meter

Table 3.14: Body-mass-standardized total surface area of the rumen and total mean volume of absorptive ruminal mucosa of three groups of wethers at an endpoint of 8 weeks in Phase II experiment

Body-mass-standardized per parameter	**Experimental Group**		
	PPII	**NPPII**	**CPII**
Total absorptive surface area (m^2kg^{-1})	0.0113	0.0163	0.0153
Total volume of mucosa in rumen wall (cm^3kg^{-1})	3.31	5.45	5.26

Key: PPII = wethers whose rumen were implanted with plastic bags through rumenotomy, NPPII = wethers that had with rumenotomy performed but not implanted with plastic bags, CPII = wethers with neither rumenotomy nor plastic bags in the rumen, m^2kg^{-1} = squared meter per kilogram, cm^3kg^{-1} = cubic centimeter per kilogram.

3.6 Discussion

The loss in weekly mean body weight observed in wethers impacted with plastic bags in the rumen during both Phase I (4-week period) and Phase II (8-week period) of the experiment may primarily be attributed to starvation from reduced feed intake. However, it should be noted that in this particular study the animals were weighed weekly instead of daily. Similar findings have been reported in sheep and goats which had indigestible foreign bodies in their rumen (Igbokwe *et al.,* 2003; Ghurashi *et al.,* 2009; Abdelaal and El-Maghawry, 2014; Otsyina *et al.,* 2015). Reduced feed intake may have led to decreased production and absorption of VFAs across the ruminal wall thereby decreasing rate of fattening in animals (Igbokwe *et al.,* 2003). The concentration of VFAs in the rumen are dependent on rumen motility and microbial population (Khan *et al.,* 1999), which were reduced due to the presence of plastic bags occupying rumen space (Otsyina *et al.*, 2015). The more severe weight loss in the wethers with rumen impaction in Phase II experiment than those in Phase I experiments can be explained by the longer duration of the impaction in Phase II, hence prolonged period of starvation from reduced feed intake.

The reduction in mean body weights recorded during the two weeks post-operative in the positive control group that was subjected to rumenotomy without implanting plastic bags may probably be attributed to post-surgical stress from some level of persistent pain despite the pain management protocol that was instituted (Anderson and Muir, 2005). Thus stress from surgery may have resulted in decreased appetite with subsequent loss of body weight during the post-operative period. Secondly, rumen motility may have been affected by surgery hence affecting mixing of ingesta and microbes consequently compromising digestion and weight gain.

The present study revealed that impacting the rumen of wethers with plastic bags for about 4 weeks or more, reduced density of papillae on the mucosal surface and affected the musculature of the ruminal wall. This is supported by previous studies (Beharka *et al.*, 1998; Steele *et al.*, 2011; Liu *et al.*, 2013), which concluded that dietary components, ruminal internal environment and duration of impaction have effects on the size, shape and density of papillae.

The pale colour of the mucosal surface of the rumen observed at the end of 4-and 8-week periods after implanting plastic bags could be attributed to loss of pigmentation, which suggests possible loss of microbial activity as reported previously (Sinclair and Kunkel, 1959) or due to thinning of the ruminal wall. This is in contrast to the control groups whose rumen mucosal surface retained the greenish brown colour as reported previously in sheep (Neiva *et al*., 2006).

The increase in the macroscopic surface area estimate of the entire rumen from wethers with plastic bags implanted in their rumen was evident 8 weeks post-impaction. This is suggestive of thinning of ruminal tissues probably due to excessive ruminal movement with non-squeezable content and the decrease in ruminal space ensuing from the accumulated plastic bags in the rumen. The thinning of ruminal walls in the plastic bag impacted rumen particularly in the cranial sac could have resulted from overstretched smooth muscles in its wall due to excessive pressure exerted by the plastic bags and exacerbated by the initial increase in the force of rumen contractions (Chungath *et al*., 1985; Mahesh, 2008; Poonia *et al*., 2011).

As reported in previous studies (Khan *et al.*, 1999; Abdelaal and El-Maghawry, 2014), obstruction of the rumeno-reticular orifice resulted from churning of plastic bags by continuous movements that mixed them with ingesta forming hard mass that would not pass through, hence blockage of the passage. Consequently it results in general weakness and compromise of the health of the animals as reported previously (Hailat *et al.*, 1996; Hailat *et al.*, 1998; Igbokwe *et*

al., 2003). It also concurs with the findings of Mayer *et al.* (1992) and Kumar and Dhar (2013) who stated that clinical impaction characterized by depression, weakness, inappetance, pale mucous membranes ensues when the rumeno-reticular orifice is blocked with indigestible foreign bodies lodging in the orifice.

Absorption of volatile fatty acids (VFAs) occurs through the ruminal wall as well as in the reticulum after passing through rumeno-reticular orifice (Mahesh *et al*., 2014; Bergman, 1990; Peters *et al*., 1990). Hence obstruction of the rumeno-reticular orifice and impaired ruminal wall may have contributed to decreased absorption of VFAs, water and electrolytes (Singh *et al*., 1983). Additionally, obstruction of the rumeno-reticular orifice may have led to gases being retained in the rumen which could have led to rumen acidosis reported by previous studies on indigestible foreign body impacted rumen (Abdelaal and El-Maghawry, 2014). This may have been exacerbated by the less chewing activity from reduced feed intake which may have resulted in decreased saliva production for the neutralization of acidity in the rumen. Consequently, the optimal function of the rumen and health of the animal may have been compromised.

The varied density of papillae in different regions of the ruminal sacs from the non-impacted rumen is consistent with previous findings by Poonia *et al*. (2011) who reported variations in size, shape and density of papillae in the different regions of the rumen of sheep. Secondly, the finding of the highest density of papillae in the cranial and ventral sacs followed by the caudal blind sacs concurs with earlier findings (Scott and Gardner, 1973; Shen *et al*., 2004). The findings from this study however showed relatively more papillae in the caudoventral blind sacs than that in the caudodorsal blind sacs of the normal rumen of wethers.

The stunted, short, highly branched papillae and thin epithelia observed in the different sacs of impacted rumen is similar to previous findings, which revealed that gross histological changes occurred due to indigestible foreign bodies in the rumen of Sambar stag (*Rusa unicolor*) (Kumar and Dhar, 2013). This study has for the first time shown histological changes that occurred in the five different ruminal sacs of wethers as a result of rumen impaction with plastic bags. The severe histological changes in the ruminal epithelia may be attributed to pressure exerted on the mucosal surface by the impacting plastic bags. It is speculated that branching of papillae observed in this study is likely to be a compensatory mechanism to increase the absorptive capacity of the mucosa following the destruction of papillary surface. This luminal extension of papillae on the mucosal surface of the rumen has been shown to increase the absorptive surface area (Steven and Marshall, 1970), which subsequently enhances ruminal function.

The different surface density and volume density results of the absorptive mucosa of the different sacs of the wethers in the control groups without rumen impaction corroborates earlier studies that, the absorptive surface varies in the different regions of the rumen (Dobson *et al*., 1956; Scott and Gardner, 1973; Liebich, 1999; Poonia *et al*., 2011). Significantly low values in surface area of the absorptive mucosal surface and absolute volume of tissues in the ruminal sacs of wethers whose rumen were impacted with plastic bags could be attributed to the long period of rumen impaction.

The reduction in surface density of papillae in the ruminal sacs of the impacted wethers could be attributed to erosion caused by the plastic bags. The continuous physical contact and firm pressure exerted by the plastic bags on the absorptive mucosal surface exacerbated by strong ruminal contractions may have led to the gradual denudation of papillae. Generally, the dry

nature of ingesta found in the rumen of the impacted wethers could contribute to friction of ingesta on the mucosal surface.

The decrease in mean surface area of the absorptive mucosal surface in the ruminal sacs of impacted wethers which was more pronounced after 8 weeks post-impaction could be attributed to the prolonged effect of the plastic bags on the ruminal surface whose effect correlates with prolonged duration of impaction. The continual existence of the plastic bags in the rumen and their possible toxic chemicals may have obstructed and damaged the functional surfaces of the ruminal sacs leading to the decrease in surface area. Although it is difficult to compare data due to lack of previous quantitative studies in the different regions of impacted rumen which this study addressed the reduction is supported by Soveri and Nieminen (1995). The authors reported that the lower the volume of ingesta received in the rumen the decreased amount of volatile fatty acids and thus decreased surface density of papillae. This is because the production and absorption of volatile fatty acids in the ruminal wall is important for the development and maintenance of overall health of ruminal papillae which is a function of surface density and surface area estimates. Thus starvation from reduced feed intake in those impacted wethers as a result of the plastic bags in their rumen may have led to decreased ingesta hence less volatile fatty acids produced consequently changing surface character of the rumen.

The loss in mean surface area of cranial sac, ventral sac and caudoventral blind sac suggests that the impact of plastic bags on papillae density and absorptive surface occurred more drastically in these regions of the rumen. The cranial, ventral and caudal blind sacs of sheep have been shown to have dense papillae (Hofmann *et al*., 1976; Berg and Edvi, 1976) as revealed in the non-

impacted rumen in the current study. These areas with dense papillae are the main sites for absorption of volatile fatty acids (Hofmann and Schnorr, 1982; Josefsen *et al*., 1996), which are also affected by dietary factors, concentration of volatile fatty acids (VFAs) and internal ruminal factors (Sakata *et al*., 1980). In particular butyrate, one of the VFAs acts locally by increasing blood supply to the papillae (Thorlacius, 1972) for optimal function. Therefore a decrease in VFAs production and absorption from reduced feed intake due to impaction of the rumen with plastic bags may have impaired blood flow to these papillae for their nourishment. Eventually, this could have led to necrosis of papillae in that region of the rumen consequently contributing to a decrease in surface area of ruminal sacs from wethers with impaction reported in this study.

The small papillae volume and surface area found in the ruminal atrium or cranial sac in the current study is comparable to those of reindeer calves that grazed freely under natural but poor conditions, though no mention of indigestible foreign bodies in them (Soveri and Nieminen (1995). Furthermore, the continuous ruminal movement and churning of ingesta with plastic bags may have resulted in interwoven compact masses that pressed on the surfaces of these sacs, subsequently compromising their space (Khan *et al.,* 1999). Moreover, the movement of the compact mass of plastic bags and the limited space in these sacs could have led to severe abrasions of the absorptive surfaces leading to the loss in mean surface area. It is speculated that the toxic compounds in plastic bags may have leached out onto the absorptive surface predisposing to atrophy of the papillae.

The absorptive surface area is directly related to food intake (Susan and Rosa, 1973), ingesta weight in rumen (Clauss *et al*., 2009), rate of fermentation (Amaral *et al*., 2005) and volatile fatty acid absorption capacity (Daniel and Resende Junior, 2012) which in turn influences rumen

efficiency (Gäbel *et al*., 1987; Bannink *et al*., 2008) and animal health. Butyrate has been shown to have potent effects on papillae size (Sander *et al*., 1959; Tamate *et al*., 1962) and possess anti-apoptotic factors (Kauffold *et al*., 1977; Sakata and Tamate, 1978; Mentschel *et al*., 2001). Thus the loss in mean surface area could also be attributed to decreased feed intake and reduced production of volatile fatty acid hence the subsequent loss of the beneficial protective function of butyrate which led to damage of the absorptive surface of the rumen. Pronounced papillae degeneration has been linked to poorly digestible carbohydrates which results in low VFA concentration (Hofmann and Schnorr, 1982).

The decrease in mean volume density of mucosa in the walls of all the ruminal sacs of the wethers whose rumen were implanted with plastic bags suggests the effects of these plastic bags on the epithelial lining of the rumen, which together with ruminal movements exerted pressure that eroded the epithelial surface as previously reported in qualitative studies (Hailat *et al.*, 1996; Hailat *et al.*, 1998). These qualitative studies indicated that impaction of the rumen with indigestible foreign materials especially plastic bags leads to sloughing and thinning of mucosal wall which could affect the digestive and absorptive function of the rumen (Ghurashi *et al*., 2009). The mucosa of the rumen comprises keratinized stratified squamous epithelium which is responsible for absorption, transport and metabolism of VFAs (Barnett and Reid, 1961; McGilliard *et al*., 1965), as well as protecting underlying tissues from abrasive effects from any mass in the rumen (Lavker *et al*., 1969). This fact compares closely with a recent study which reported a reduction in the volume of stratum granulosum cells of the epithelial layer in goats fed on high grain diet relative to those fed on high forage diet, thus reducing barrier function of the epithelial layer, hence promoting entry of pathogens (Liu *et al*., 2013). In addition to this,

pressure from compact masses of plastic bags probably exerted enough pressure on the ruminal epithelium to weaken the tight cell junctions (Gonzalez-Mariscal *et al*., 2008), hence the probability of allowing toxic chemicals leached from these plastic bags to permeate through and consequently damage the epithelial cells. Therefore, impaction of the rumen with plastic bags may alter normal microbial digestion due to starvation, reduced feed intake and availability. This likely alters the production and absorption of VFAs within the rumen and these could affect ruminal epithelial integrity.

The substantial loss in mean absolute volume of mucosa in the ruminal sacs 8 weeks after implanting plastic bags in the rumen suggests a progressive damage of ruminal mucosa due to prolonged exerted pressure from the impaction and continual friction with gradual sloughing off of the epithelial cells, since morphological changes in the ruminal epithelia occur slowly (Gabel and Aschenbach, 2002). The physical presence of plastic bags in the rumen may have reduced the appetite (Reece, 2005) in those animals, leading to insufficient fermentable carbohydrate for VFA production needed for cell proliferation (Dirksen *et al*., 1985) or even obstruction of microbial activity (Khan *et al.*, 1999). Low concentration of VFA particularly butyrate in the rumen could have resulted in degradation of ruminal mucosa (Mentschel *et al*., 2001), hence loss of mucosal volume. Structural differences in ruminal epithelia due to dietary fermentability have been reported in cows (Steele *et al*., 2011), calves (Schurmann, 2013) and goats (Liu *et al*., 2013) fed a high grain diet.

Increase in mean volume density and mean absolute volume of inner circular muscle layer of tunica muscularis in the walls of the cranial and dorsal sacs is a contrast to a previous report

stating that physical structure of diet does not influence rumen muscle thickness (Beharka *et al*., 1998). The diet referred to in this previous report could possibly be fermentable carbohydrates as opposed to indigestible materials as was the case in the present study. However, the findings of the current study partially compare with those of Scheidemann and Huthmann (2011) who reported muscular thickening in gastric impaction in horses. The increase in mean absolute volume of muscularis interna layer could be attributed to hyperplasia or hypertrophy from excessive pressure of plastic bags on the ruminal wall. These authors reported that lesions found in the muscles such as vacuolation of smooth muscle cells, focal fibrosis and focal myositis could be suggestive of the increase in tissue volume (Scheidemann and Huthmann, 2011). The increased volume of muscularis interna muscle layer could probably be due to oedema ensued from injury of the abrasive effects of the compacted masses of plastic bags. The intensity of the injury may have been less pronounced on the outer smooth muscle, the muscularis externa perhaps being further away from the direct impact of the plastic bags possibly explaining why there was no significant increase in its volume.

Twice the loss in total mean surface area of absorptive mucosal surface and in total mean absolute volume of mucosa in the entire rumen of the impacted wethers at 8 weeks compared to loss at 4 weeks indicates that duration of impaction has exacerbating effects on the rumen histoarchitecture. This also suggests that duration of plastic bags in the rumen has severe consequences on ruminal morphology and function. The loss in total surface area of the entire rumen could imply decrease in VFA absorption and transport and hence energy requirement of the animal may not be met. This could have compromised ruminal functional efficiency and

health. Possibly this may have led to the death of 2 wethers with impacted rumen at before the 8 weeks endpoint.

The loss of 50% total mean absolute volume of mucosa in the entire ruminal wall of impacted wethers concurs with Liebich *et al*. (1987) that diet and ruminal content has influence on ruminal epithelia. The fact that in the present study total mean absolute volume of mucosa in the impacted rumen formed about 20% of the entire ruminal volume, it could be implied that the impaction reduced ruminal efficiency, which may have had negative effects on health of the animals. This corroborates earlier studies that rumen impaction with plastic bags affects health of animals (Igbokwe *et al*., 2003; Otsyina *et al.*, 2015). The total surface area and the total volume of the rumen have a significant influence on nutrient transport (James *et al*., 1983), hence when these are diminished there will be significant reduction in function that subsequently has an overall negative effect on health and productivity of the animals. All these reduction in structural quantities of the rumen are likely to affect the rate of fattening of the animals (Igbokwe *et al*., 2003), which could account for decreased body-mass-standardized total mean surface area and total mean absolute volume that was found 8 weeks after implanting plastic bags in the rumen of wethers.

3.7 Conclusions

This study has for the first time shown the effect of rumen impaction with plastic bags on wethers and quantitative structural changes in their rumen. The structural quantities of tissues in the normal rumen of wethers are also reported. Thus it can be concluded that:

a) Rumen impaction with plastic bags led to severe loss in body weights of the wethers
b) There were severe histological changes in ruminal mucosae of wethers with rumen impaction
c) There was a decrease in surface density of the absorptive mucosae in all the sacs of the rumen at the end of 4 weeks and 8 weeks post-impaction.
d) There was a significant reduction in mean surface area of the absorptive mucosa in the cranial, ventral and caudoventral blind sacs of the rumen by the end of 8 weeks post-implantation.
e) Total surface area of the entire absorptive ruminal mucosa significantly decreased 8 weeks post-implantation.
f) There was loss in volume density of mucosa in the walls of all the ruminal sacs at 4 week and 8 week endpoints of the experiments.
g) There was 30% loss in total absolute volume of mucosa in the entire rumen at 4 weeks and 50% loss of the same at 8 weeks post-implantation.
h) The total volume of mucosal tissue reduced forming 20% of the entire ruminal tissue volume in wethers with impaction with plastic bags.
i) The total surface area and absolute volume of tissues in the entire rumen of wethers with impaction at 8-week endpoint reduced to $0.282 \pm 0.012 m^2$ and $480 cm^3$ respectively.
j) The total surface area and absolute volume of tissues in the entire rumen of normal wethers without impaction was $0.473 \pm 0.017 m^2$ and $540 cm^3$ respectively.
k) The body-mass-standardized total surface area and volume of mucosal tissue in the rumen decreased progressively the longer the rumen was impacted.

CHAPTER FOUR

4.0 PLASMA CORTISOL LEVELS, AN ESTIMATOR OF STRESS IN WETHERS IMPACTED WITH PLASTIC BAGS IN THE RUMEN

4.1 Introduction

Rumen impaction with indigestible foreign bodies is increasingly gaining much attention especially in Africa because of the negative effect it has on animal health and productivity. Previous studies have shown the incidence of impaction of the rumen with non-biodegradable foreign bodies in sheep and goats (Igbokwe *et al*., 2003; Ghurashi *et al*., 2009; Abebe and Nuru, 2011; Mersha and Desiye, 2012; Khurshaid *et al.*, 2013; Abdelaal and El-Maghawry, 2014; Otsyina *et al.,* 2015).

Sheep and goats raised by smallholder households in urban and peri-urban areas roam and scavenge on dump sites, polluted pastures and roadsides in search for food and water (Verbeek *et al.,* 2007). They inevitably ingest waste plastic bags (Ghurashi *et al.,* 2009), which gradually accumulate in the rumen, subsequently leading to impaction and consequently resulting in digestive problems. The prolonged presence of these indigestible materials such as plastic bags in the rumen interferes not only with digestion but also feed intake and energy balance in the body. These may affect other physiological functions of the body (Abebe and Nuru, 2011) that could culminate into stress for the animal. Stress becomes critical when it results in significant physiological changes that eventually affect the well-being of the animal (Moberg, 2000).

Stress has been defined as the disease of adaptation (Selye, 1946), where the mechanisms to cope with stressors become overextended and eventually break down. The stressor may be an internal

stimuli (within the animal body) or external stimuli (within the environment of the animal) (Hristov *et al*., 2012). The Hypothalamic-pituitary-adrenal (HPA) axis together with autonomic nervous system referred to as the neuroendocrine system mediates stress response (Manteuffel, 2002; Mormede *et al.*, 2007). The primary active stress hormone produced by the HPA axis for most mammals such as sheep, cattle, horse and pig is cortisol, whiles corticosterone is produced in birds and laboratory rodents (Palme *et al.,* 2005; Mormede *et al.,* 2007). Both of these stress hormones are glucocorticoids which are cholesterol-derived steroids synthesized in the zona fasciculata of the adrenal cortex.

Any environmental or biological factor that continually stimulates the HPA axis for several days will lead to chronic increase in cortisol secretion. Increased cortisol level over a prolonged period due to chronic stress (prolonged stressor) is detrimental to animal health, inhibiting inflammatory processes and suppressing immune response which greatly increases susceptibility of the animal to pathogens (Spraker *et al*., 1984; Lynch *et al*., 1992). Ingestion and accumulation of indigestible plastic bags in sheep may create physiological disturbances that could result in significant stress. Hence the reason for cortisol assays to assess presence or absence of stress in the present study using wethers whose rumen were experimentally implanted with plastic bags. Measurement of plasma cortisol levels to elucidate stress in rumen impaction in sheep has not been done previously. Currently, enzyme-linked immunosorbent assay (ELISA) is one of the methods for the assay of plasma cortisol levels in biological samples.

4.2 Materials and methods

4.2.1 Acquisition of animals and experimental set-up

The acquisition of animals and the experimental design is as described in section 3.2.1. The experiment included 30 healthy wethers, of which 15 were used in Phase I and the other 15 in Phase II of the experiment. For each phase the wethers were further divided into three experimental groups each with 5 animals. Phase I comprised of the test group coded PPI (n = 5) whose rumen were implanted with plastic bags through rumenotomy, positive control group coded NPPI (n = 5) in which rumenotomy was performed but no plastic bags were implanted in the rumen and negative control group coded CPI (n = 5) in which neither rumenotomy was performed nor plastic bags were implanted in the rumen. The groups in Phase II were coded PPII (n = 5) for the wethers whose rumen were implanted with plastic bags through rumenotomy, NPPII (n = 5) for the positive control group in which rumenotomy was performed but no plastic bags implanted and CPII (n = 5) for the negative control group in which wethers had neither rumenotomy nor implanted with plastic bags.

4.2.2 Rumenotomy procedure and implantation of plastic bags in the rumen

The procedure for rumenotomy and implanting of plastic bags in the rumen is described in details in section 3.2.3.

4.2.3 Sampling procedure

The jugular vein was the site of choice for ease of collection of blood samples from all experimental wethers. The sampling site was first swabbed and sterilized with ethyl alcohol to prevent infection. Four milliliters of blood sample was drawn by venipuncture with 18 gauge

needle within one minute. The blood was immediately transferred into vacutainer tubes containing anticoagulant EDTA and labelled with each animal's number and group identification code. The vacutainer tubes with blood samples were placed in a cool box containing ice until centrifuged in the laboratory. The blood samples in EDTA vacutainers were centrifuged at 2500g for 15 minutes within 1-2 hours after collection. After centrifugation plasma samples were harvested into individual clean Eppendorf tubes. The Eppendorf tubes were labelled with the group code, each animal's number and sampling date. Plasma samples were stored frozen at -20°C until analysis at a later date.

4.2.3.1 Baseline blood sampling

Blood samples were collected from all groups of wethers before rumenotomy. The results from these samples were considered as baseline values.

4.2.3.2 Post-implantation blood sampling

4.2.3.2.1 Short-term blood sampling

Once rumenotomy was completed, 4ml of blood samples were collected from all groups of wethers in Phase I and Phase II of the experiments over a period of 72 hours post-implantation. These blood samples were collected at 6hours, 24hours, 48hours and 72hours post-implantation and the results considered as short-term values.

4.2.3.2.2 Long-term blood sampling

Weekly blood samples were collected only from the groups of wethers in Phase II. They were collected weekly beginning the first week post-implantation and continued to week 8 post-implantation for the three groups.

4.2.4 Plasma cortisol assay

Frozen plasma samples (described earlier in section 4.2.3) were thawed and analyzed for the concentration of total cortisol. Total plasma cortisol concentration was measured by a cortisol competitive enzyme linked Immunosorbent assay (ELISA) commercial kits (Creative Diagnostics, NY, USA; Cat. No: DEIA0708H). The procedure for analysis was carefully followed as detailed in the manufacturer's protocol. Intra- and inter-assay coefficients of variation ranged between 5.6%-14.7% and 6.3%-10.9% respectively. The limit of detection was determined as 45.4pg/mL. Sensitivity of the assay was determined as 17.3pg/mL. The absorbance of generated colour was read out using an automatic microtitre plate reader connected to a computer at a wavelength of 450 nm. The concentrations of the standards were log-transformed and a standard curve was plotted with their corresponding absorbance using GraphPad software version 6.0 (GraphPad, San Diego, USA). The total cortisol concentration for each sample was interpolated from the standard curve as log transformed and then converted back to normal values by antilog function in Microsoft excel.

4.3 Data management and statistical analysis

The raw data collected for all parameters were verified, validated and entered into Microsoft Office 2010 excel spread sheet. The data sets were coded with letters and numbers representing each sample in an experimental group. Data were managed in Excel 2010 and values obtained for each data set were expressed as "Mean ± S.E.M". The data obtained were imported into GraphPad Prism software version 6.0 (GraphPad Prism Statistical Software, Inc. California, USA) for graphical presentation and statistical analysis. Mean plasma cortisol concentrations were plotted against hourly and weekly time points. One-way and Two-way Analysis of variance

(ANOVA) with Tukey's Multiple Comparison (Post hoc) Test were performed using GraphPad Prism software (version 6.0) to compute associations and comparisons between the different parameters and experimental groups. Comparisons of the means and associations were considered significant at the level of $p < 0.05$.

4.4 Results

4.4.1 Short-term assessment of stress in wethers in Phase I and Phase II

4.4.1.1 Short-term measurement of plasma cortisol levels in Phase I

The mean plasma cortisol concentrations measured in the 3 groups of wethers in Phase I experiments over 72 hour period after implantation are presented in Table 4.1. The comparative effects of impacted rumen on the plasma cortisol concentration, between the test group and the control groups are represented graphically in Figure 4.1. The overall analysis of variance and differences in overall means of plasma cortisol concentration between the experimental groups are presented in Table 4.1a and Table 4.1b respectively.

Generally, the concentration of plasma cortisol significantly ($p < 0.05$) increased in the test wethers (PPI) in samples collected at the designated intervals between 6 hours to 72 hours post-implantation. The increased plasma cortisol levels ranged from baseline value of 72.0 ± 6.8nmol/L to 314.1 ± 31.9nmol/L at 6 hours post-implantation which was a 3-fold increase. This increase was significant ($p < 0.0001$) when compared with the negative control group CPI. After 48 hour post-implantation the concentration of plasma cortisol had significantly ($p < 0.0001$) increased further to 341.9 ± 107.4nmol/L about 4-fold from baseline but thereafter declined

considerably to 126.6 ± 26.7nmol/L by the end of 72 hours. The values of mean plasma cortisol concentration at the 72 hour endpoint were still higher in PPI wethers but these values were not significant when compared with the control groups. The positive control group (NPPI), in which rumenotomy was performed without implanting plastic bags in their rumen had two times increase in the levels of plasma cortisol 6 hour post-implantation, but the levels declined to almost baseline values at 72 hours. The increase in mean plasma cortisol concentration in the positive control group (NPPI) was however not significant when compared with the negative control group that had neither rumenotomy nor plastic bags in their rumen (CPI). Overall the effect of interaction between the experimental groups and time was significant ($p = 0.0420$) (Table 4.1a).

Table 4.1: Mean plasma cortisol concentration in three groups of wethers during the first 72 hour period in Phase I experimentation

Time (hours)	**Mean (± SE) plasma cortisol concentration (nmol/L)**			***P* value**
	PPI (n = 5)	**NPPI (n = 5)**	**CPI (n = 5)**	
0	72.0 ± 6.8	72.8 ± 8.0	51.4 ± 7.7	0.9093
6	314.1 ± 31.9	195.7 ± 20.7	85.4 ± 18.6	0.0001[a]
24	179.5 ± 38.6	131.5 ± 38.8	68.8 ± 10.9	0.0884
48	341.9 ± 107.4	151.0 ± 31.1	90.0 ± 20.4	< 0.0001[a] 0.0013[b]
72	126.6 ± 26.7	88.7 ± 29.9	62.0 ± 16.7	0.4262

Data are presented as means with plus or minus the standard error (± SE)
Significance at $p < 0.05$
[a]*P*-value = test group PPI compared with negative control group CPI
[b]*P*-value = test group PPI compared with positive control group NPPI
Note: Plasma cortisol concentration of 42 – 82nmol/L is the normal range of values in sheep (Jackson and Cockcroft, 2002)
Key:
PPI = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPI = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen
CPI = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.
nmol/L = nanomoles per litre, n = number of wethers per group

Table 4.1a: Analysis of variance for mean values of plasma cortisol concentration measured over 72 hours in three groups of wethers in Phase I experimentation

Source of variation	**d.f**	**s.s**	**m.s**	**v.r**	***P* value**
Experimental groups	2	230913	115456	17.42	<.0010
Time	4	213913	53478	8.07	<.0010
Experimental group.Time	8	115381	14423	2.18	0.0420
Residual	60	397779	6630		
Total	74	957986			

d.f is degrees of freedom, s.s is sum of squares, m.s is mean square and v.r is variance ratio

Table 4.1b: Differences in overall means of plasma cortisol concentration in three groups of wethers during the first 72 hour period in Phase I experimentation

Experimental groups	**Overall mean**	**Difference in overall mean**
PPI	206.8	135.3
NPPI	127.9	78.9
CPI	71.5	
LSD	46.07	

Key:
PPI = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPI = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen
CPI = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.
LSD = Least significant difference

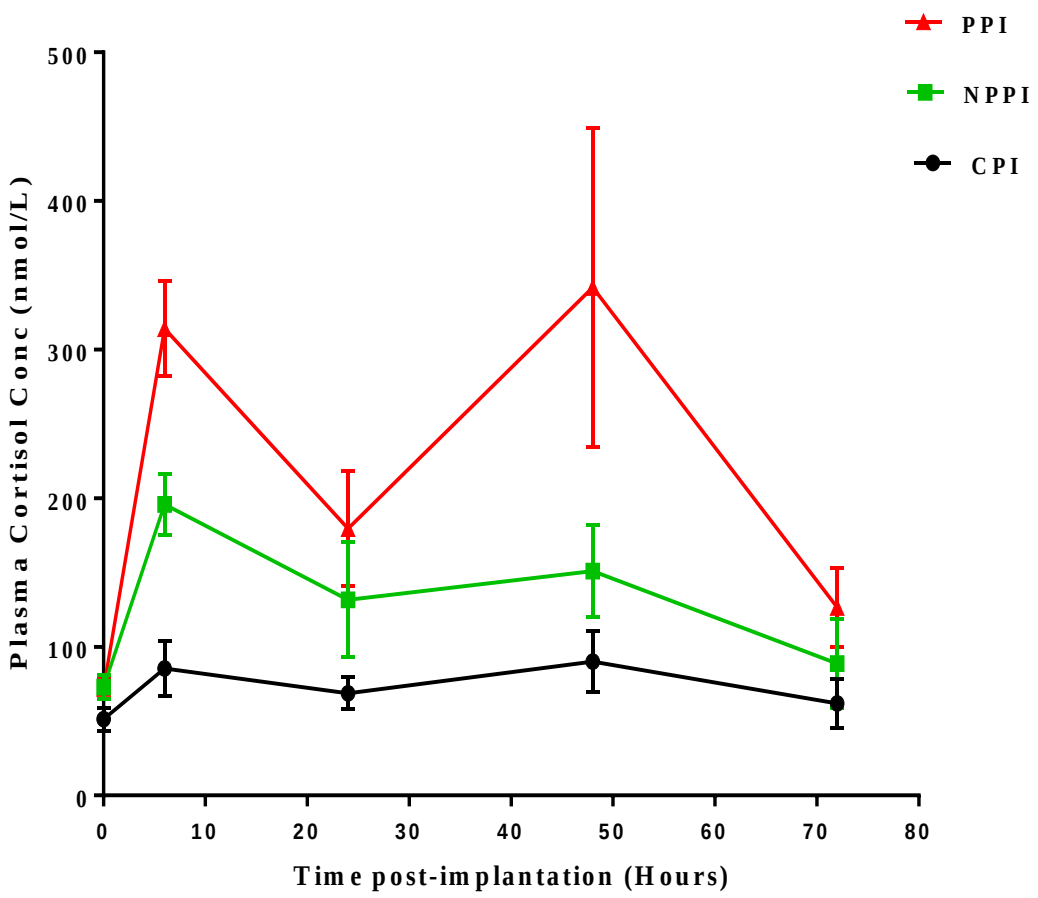


Figure 4.1: Comparative mean values of plasma cortisol levels between three groups of wethers during the first 72 hour period in Phase I experimentation. The results showed significantly elevated cortisol levels in plasma of the test group.

Key: **PPI** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPI** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPI** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

4.4.1.2 Short-term measurement of plasma cortisol concentration in Phase II

The plasma cortisol concentration measured during the first 72 hours after implanting plastic bags in the rumen of wethers in Phase II of the experiments is summarized in Table 4.2 and the graphical comparisons between the test group and control groups is also presented Figure 4.2. The overall analysis of variance and differences in overall means of plasma cortisol concentration between the experimental groups are presented in Table 4.2a and Table 4.2b respectively.

The wethers from group PPII had mean values of plasma cortisol rising significantly ($p < 0.0001$) from baseline value of 62.3 ± 8.5nmol/L to 316.0 ± 4.0nmol/L in the plasma sample collected 6 hours after implanting plastic bags in the rumen in the Phase II experiment. This rise in mean values of plasma cortisol was a 4-fold increase. The increased cortisol levels persisted for the 72 hour period of plasma sampling, but progressively declined to 172.0 ± 20.5nmol/L at 48 hours, which was still about 2-fold increase from baseline value and still significant ($p < 0.0001$) when compared with the negative control group (CPII). At the end of 72 hour period of rumen impaction the mean plasma cortisol levels in wethers with impacted rumen, had decreased to 162.6 ± 17.9nmol/L and yet significant ($p = 0.0355$) in comparison to the negative control group (CPII).

The positive control group of wethers (NPPII) which were subjected to rumenotomy but had no plastic bags implanted in their rumen in Phase II experiment showed significantly ($p = 0.0125$) elevated levels in mean values of plasma cortisol at 6 hours after rumenotomy, when compared with the negative control group (CPII) which had neither rumenotomy nor plastic bags. The increase was about 1.5 times the mean baseline values. However, the concentration of plasma

cortisol in the positive control wethers gradually declined over the remaining period and at 72 hour endpoint plasma cortisol concentration was comparable with normal reference values. Overall the effect of interaction between the experimental groups and time was significant ($p < 0.0010$) (Table 4.2a).

Table 4.2: Mean plasma cortisol concentration in three groups of wethers during the first 72 hour period in Phase II experimentation

Time (hours)	**Mean (± SE) plasma cortisol concentration (nmol/L)**			***P* value**
	PPII (n = 5)	**NPPII (n = 5)**	**CPII (n = 5)**	
0	62.3 ± 8.5	71.6 ± 10.2	63.6 ± 6.2	0.9624
6	316.0 ± 4.0	174.4 ± 48.6	85.0 ± 3.1	< 0.0001[a] < 0.0001[b] 0.0125[c]
24	274.3± 49.9	148.5 ± 17.3	100.9 ± 9.6	< 0.0001[a] 0.0003[b]
48	172.0 ± 20.5	122.9 ± 14.8	99.1 ± 6.4	0.0499[a]
72	162.6 ± 17.9	113.8 ± 17.3	85.4 ± 10.7	0.0355[a]

Data are presented as means with plus or minus the standard error (± SE)
Significance at $p < 0.05$
[a]*P*-value = test group PPII compared with negative control group CPII
[b]*P*-value = test group PPII compared with positive control group NPPII
[c]*P*-value = positive control group NPPII compared with negative control group CPII
Note: Plasma cortisol concentration of 42 – 82nmol/L is the normal range of values in sheep (Jackson and Cockcroft, 2002)
Key:
PPII = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPII = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen
CPII = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.
nmol/L = nanomoles per litre, n = number of wethers per group

Table 4.2a: Analysis of variance for mean values of plasma cortisol concentation measured over 72 hours three groups of wethers in Phase II experimentation

Source of variation	**d.f**	**s.s**	**m.s**	**v.r**	***P* value**
Experimental group	2	157152	78576	30.06	<.0010
Time	4	146624	36656	14.02	<.0010
Experimental group.Time	8	88034	11004	4.21	<.0010
Residual	60	156851	2614		
Total	74	548661			

d.f is degrees of freedom, s.s is sum of squares, m.s is mean square and v.r is variance ratio

Table 4.2b: Differences in overall means of plasma cortisol concentration in three groups of wethers during the first 72 hour period in Phase II experimentation

Experimental groups	**Overall mean**	**Difference in overall mean**
PPII	197.4	110.6
NPPII	126.2	39.4
CPII	86.8	
LSD	28.93	

Key:
PPII = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPII = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen
CPII = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.
LSD = Least significant difference

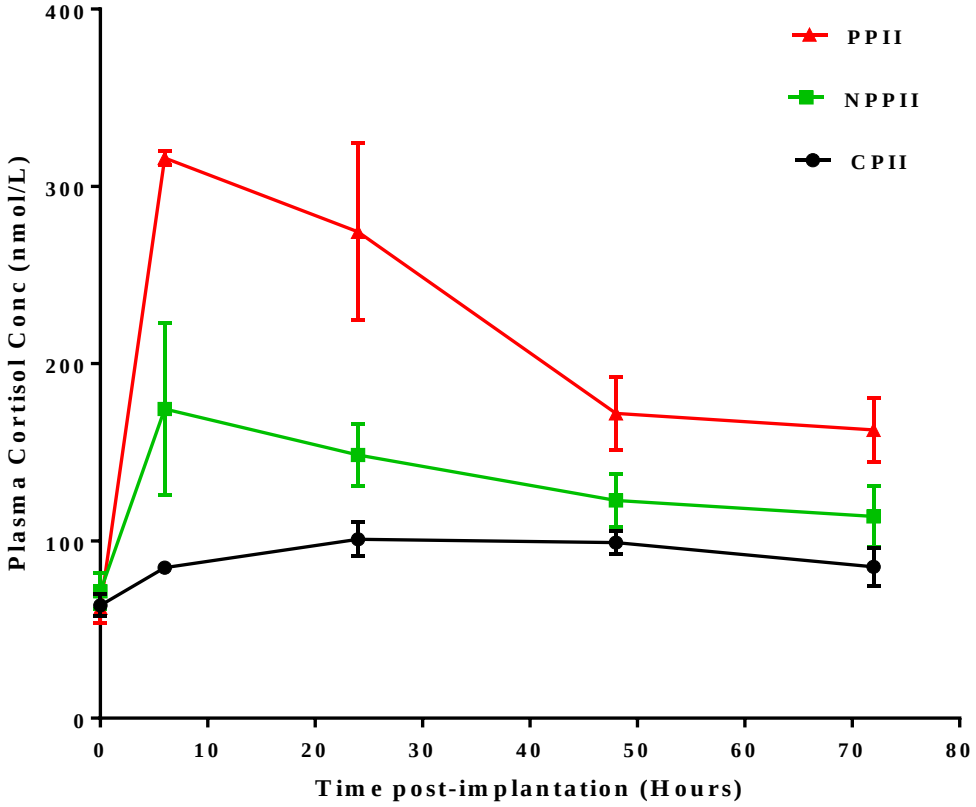


Figure 4.2: Comparative mean plasma cortisol levels in three groups of wethers during the first 72 hour period in Phase II experimentation. The results showed significantly elevated cortisol levels in plasma of the test group.

Key: **PPII** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

4.4.1.3 Comparisons of mean values of plasma cortisol in assessment of acute stress between wethers inPhase I and Phase II experiments

The mean values of plasma cortisol obtained during the first 72 hours of the experiments in the groups of wethers whose rumen were implanted with plastic bag in both Phase I (PPI) and Phase II (PPII) experiments, revealed about 4-fold significant ($p < 0.0001$) increase from baseline values within 48 hours post-implantation compared to the control wethers CPI. At 72 hours of rumen impaction the mean values of plasma cortisol remained elevated above baseline values at 126.6 ± 26.7nmol/L and 162.6 ± 17.9nmol/L in both the test groups PPI and PPII respectively.

The wethers in the positive groups NPPI and NPPII in Phase I and Phase II respectively, which were subjected to rumenotomy without implanting plastic bags had significant ($p = 0.0125$) 1.5-fold increase in mean values of plasma cortisol 6 hour post-implantation, but declined to near normal values by the end of 72 hours.

4.4.2. Plasma cortisol levels for assessment of chronic stress in wethers with rumen impaction in Phase II experiment

The mean plasma cortisol concentration over the 8 week period in Phase II experiments representing long-term measurements are presented in Table 4.3 and Figure 4.3. The overall analysis of variance and differences in overall means of plasma cortisol concentration between the experimental groups are presented in Table 4.3a and Table 4.3b respectively.

Overall, the mean values of plasma cortisol concentration significantly ($p < 0.05$) increased over the first 3 weeks after the rumen were implanted with plastic bags (PPII). Thereafter it progressively declined to values within normal range (Table 4.3). Specifically, after one week of rumen impaction, the mean plasma cortisol concentration in the test group PPII rose to 189.0 ±

33.0 nmol/L, of which the increase was 2-times the baseline value of 62.3 ± 8.5nmol/L. Tukey's multiple comparison test of two-way ANOVA indicated this was significantly different ($p = 0.0004$) from the mean values of plasma cortisol concentration of the negative control group CPII. Similar differences were found between the test group PPII and the positive control group NPPII ($p = 0.0074$). The significantly ($p < 0.05$) high levels of plasma cortisol persisted for 3 weeks post-implantation after which they dropped to normal values of 66.1 ± 6.4 nmol/L. This decline in mean plasma cortisol levels in wethers whose rumen were impacted, persisted to the end of the experiment. One week after rumenotomy, the positive control group (NPPII) had about a 100% significant rise ($p = 0.0074$) in mean plasma cortisol concentration compared with their baseline value, which declined by the second week to normal values that persisted throughout the experimental period. The negative control group (CPII), which had neither rumenotomy done nor implanted with plastic bags in their rumen also showed 63% increase in mean values of plasma cortisol from baseline values during the first week of commencement of the experiment, but decreased to normal values in the weeks that followed. Overall the effect of interaction between the experimental groups and time was significant ($p = 0.007$) (Table 4.3a).

Table 4.3: Mean values of plasma cortisol concentration in three different groups of wethers over the 8-week period of Phase II experimentation

Time (Weeks)	**Weekly mean plasma cortisol concentration (nmol/L)**			***P* value**
	***PPII (n = 5)**	**NPPII (n = 5)**	**CPII (n = 5)**	
0	62.3 ± 8.5	71.6 ± 10.2	63.6 ± 6.2	0.9267
1	189.0 ± 33.0	169.8± 23.5	103.5 ± 3.6	0.0004[a] 0.0074[c]
2	181.7± 28.6	108.1 ± 28.0	92.8 ± 6.5	0.0002[a] 0.0026[b]
3	153.6 ± 35.1	65.7 ± 13.7	44.9± 7.7	< 0.0001[a] 0.0003[b]
4	66.1 ±6.4	48.8 ± 4.3	55.4 ± 11.7	0.8728
5	43.6 ± 5.1 (n = 4)	40.8 ± 6.1	42.7 ± 4.7	0.9991
6	69.1 ± 25.0 (n = 4)	42.3 ± 8.0	29.0 ± 4.8	0.1895
7	75.9 ± 5.4 (n = 3)	33.7 ± 3.5	50.4 ± 6.0	0.5621
8	82.3 ± 12.7 (n = 3)	54.0 ± 6.8	48.6 ± 5.3	0.3678

Data are presented as means with plus or minus the standard error (± SE)
Significance at $p < 0.05$
[a]*P*-value = test group PPII compared with negative control group CPII
[b]*P*-value = test group PPII compared with positive control group NPPII
[c]*P*-value = positive control group NPPII compared with negative control group CPII
Note: i) Plasma cortisol concentration of 42 – 82nmol/L is the normal range of values in sheep (Jackson and Cockcroft, 2002). ii) *PPII: 2 wethers died before the 8th week endpoint
Key:
PPII = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen, nmol/L = nanomoles per litre, n = number of wethers in the group

Table 4.3a: Analysis of variance for mean values of plasma cortisol concentration measured over 8 weeks in three groups of wethers in Phase II experimentation

Source of variation	**d.f**	**s.s**	**m.s**	**v.r**	***P* value**
Experimental groups	2	52486	26243	22.68	< 0.0010
Time	8	182327	22791	19.69	< 0.001
Experimental groups.Time	16	42345	2647	2.29	0.007
Residual	108	118044	1157		
Total	128	386750	3021		

d.f is degrees of freedom, s.s is sum of squares, m.s is mean square and v.r is variance ratio

Table 4.3b: Differences in overall means of plasma cortisol concentration over 8 weeks in three groups of wethers in Phase II experimentation

Experimental groups	**Overall mean**	**Difference in overall mean**
PPII	104.07	44.42
NPPII	71.80	12.15
CPII	59.65	
LSD	14.65	

Key:
PPII = test group of wethers whose rumen were implanted with plastic bags through rumenotomy
NPPII = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen
CPII = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.
LSD = Least significant difference

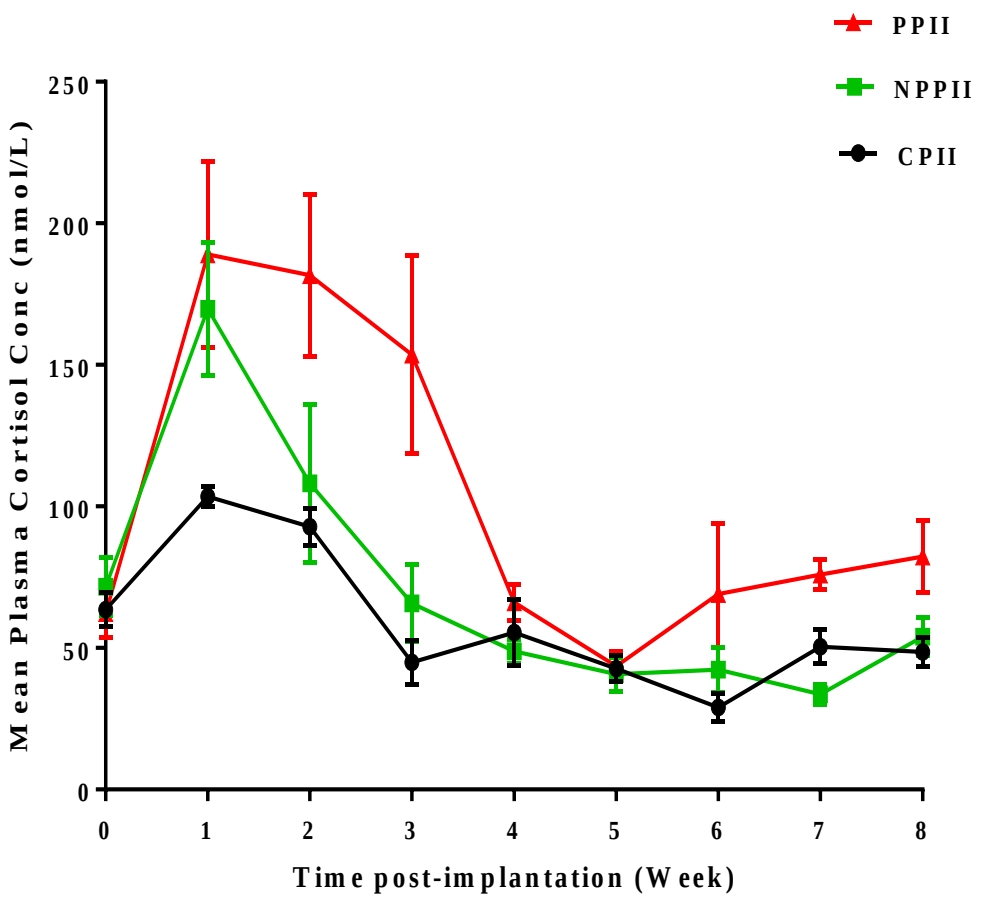


Figure 4.3: Plasma cortisol concentration measured over 8 weeks in three groups of wethers with or without rumen impaction with plastic bags in Phase II experimentation. The results showed significantly elevated levels of cortisol in the plasma of the test group within the first 3 weeks.

Key: **PPII** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

4.5 Discussion

The present study has shown for the first time that rumen impaction in wethers simulated by implanted plastic bags caused acute stress in the first 72 hours, which was demonstrated by significant 4-fold increase ($p = 0.0001$) in levels of plasma cortisol concentration in both Phase I and Phase II of the experiments. It is worth noting that stress from some level of pain from surgery of rumenotomy could have also contributed to the significantly increased cortisol levels during the acute phase. The acute elevated plasma cortisol levels in wethers with impacted rumen in this study remained high beyond the third day owing to the excessive stress from rumen impaction while that in the positive control declined. The differences in observations could be attributed to the additional stress exacted on the animal as a result of impaction of the rumen with indigestible plastic bags. Variation in the type and intensity of the stress stimulus leads to varying levels of cortisol secretion in animals (Ferguson *et al*., 2008). The pressure employed by the plastic bags served as an internal stressor in the rumen, which caused stimulation of the HPA axis to initiate a stress response, consequently causing the adrenal gland to secrete more cortisol hormones.

The ability of an animal to produce enough cortisol on stimulation of the HPA axis by a stressor is key for the animal to be able to quickly adapt to the stressor (Mormede *et al*., 2007 and 2011). Thus increased secretion of cortisol in response to an acute stressor may not necessarily be bad at all, except when the intensity of the stressor results in detrimental changes to the animal's biological function (Moberg, 1985). It is therefore implied that the increased levels of plasma cortisol concentration in the wethers was a reflection of their HPA axis' responsiveness to those foreign materials in the rumen. The results support numerous earlier reports that plasma cortisol

may be a good estimator of acute stress (Kilgour and de Langen, 1970; Harlow *et al*., 1987; Boissy and Neindre, 1997; Sylvester *et al*., 1998; Ruizu-de-la-Torre *et al.,* 2001; Doubek *et al*., 2003; Moolchandani *et al*., 2008; Sheriff *et al*., 2010; Cingi *et al.,* 2012).

The increased mean values of plasma cortisol concentration found in the positive control group can be attributed to stress from the surgical procedure of rumenotomy even without presence of plastic bags in the rumen. Surgical stress is likely to be immediate but later as the animal adjusts, the stress diminishes. This is probably the reason plasma cortisol concentration increased spontaneously 6 hours after surgery but declined thereafter to normal values by the end of 72 hour period. This observation concurs with documented previous findings of increased plasma cortisol concentration in Holstein-Friesian dairy cows that were subjected to surgery on the abdomen (Mudron *et al*., 2005). Since the highest concentrations of plasma cortisol were immediately after surgery, it is likely that pain which is normally severest just after surgery may have served as the potent stressor (Lay, 2000) that elicited the HPA axis to stimulate increased production of cortisol by the adrenal cortex.

The marginal increased levels of mean values of plasma cortisol concentration in the negative control group during the first one week period of the experiments could be a result of circadian fluctuating patterns of cortisol secretion as time progresses (Mostl and Palme, 2002). Other contributing factors to the marginal rise in plasma cortisol levels could have resulted from confinement and handling. The marginal increase in the levels of plasma cortisol in the negative group of wethers in this study is comparable to those reported previously in sheep stressed by confinement (Degabriele and Fell, 2001). Sheep that were also subjected to restraint and

isolation reportedly had increased plasma cortisol concentration on the first day, the concentrations declined to normal values by the third day (Moolchandani *et al.,* 2008). Additionally, the marginal elevated plasma cortisol levels could probably be due to time of the day when blood samples were collected, duration taken to sample each animal or probably stress of repeated blood sampling despite the fact that all wethers were acclimatized before commencement of the study.

Induction of long-term stress by rumen impaction with plastic bags manifested through increased values of plasma cortisol concentration during the first 3 weeks but steadily declining towards normal values in the next five weeks may imply that the animal got adapted to the extent of the adrenal gland not responding any further to the stressor. This can be compared to a similar report by McNatty and Young (1973), which indicated low levels of plasma cortisol concentration in sheep that had fully adapted to their new environment as compared to high cortisol levels in non-adapted sheep. These authors also reported that sheep may require a period of 7 - 28 days to completely adapt to a new stressor. This study observed a period of 28 to 35 days for wethers to adapt to stress of rumen impaction with plastic bags. Similar findings in baboons revealed that decreased production of cortisol is present in chronic stress (Sapolsky, 1983). Conversely, continuous elevation of plasma cortisol concentration over a period of 35 days was reported in sheep exposed to stress of loud noise (Harlow *et al*., 1987).

It has been suggested that animals do adapt to prolonged stress, which results in the reduction of plasma cortisol levels (Mason *et al*., 1957). This is because increased concentration of plasma cortisol inhibits further release of ACTH through negative feedback mechanism (Norman and

Litwack, 1987; Nussey and Whitehead, 2001; Smith and Dobson, 2002), subsequently inhibiting further secretion of cortisol by the adrenal glands in order to maintain homeostasis. Stricklin and Mench (1990) also suggested that low plasma cortisol in prolonged stress, does not mean absence or reduced stress, but could imply exhausted adrenal glands. Others suggest that the sensitivity of adrenal glands as seen in acute stress declines over time with the continuous presence of the stressor (McNatty and Thurley, 1973; Ader, 1975). The findings of the current study agree with earlier reports, which were of the opinion that plasma cortisol concentration may not be a good estimator for assessment of long-term or prolonged stress (Baldwin and Stephens, 1973; Fell and Shutt, 1989; Cockram *et al*., 1994; Dalin *et al*., 1993). Hence there is need to estimate other biomarkers of chronic stress.

4.6 Conclusions

To our knowledge, the present study is the first to determine the effect of rumen impaction with plastic bags on plasma cortisol levels in ruminants. The study concluded that:

a) Rumen impaction with plastic bags induced acute stress effects in sheep up to three weeks which was manifested through increased plasma cortisol levels.
b) Wethers with persistent rumen impaction are likely to adapt well to prolonged stress shown by return to normal values of mean plasma cortisol concentration.
c) Plasma cortisol concentration is a good estimator for assessment of acute stress rather than chronic stress. It is a quick diagnostic tool for stress-related studies.

CHAPTER FIVE

5.0 FAECAL CORTISOL METABOLITE LEVELS AS AN INDICATOR OF STRESS IN WETHERS IMPACTED WITH PLASTIC BAGS IN THE RUMEN

5.1 Introduction

Faecal cortisol metabolite (FCM) as a non-invasive method, for measuring stress in farm animals, has been established and is increasingly being used in stress-related studies. Stress has been indicated by numerous studies as a marker of an animal's well-being thus forming a core component for animal welfare evaluations (Möstl and Palme, 2002; Sheriff *et al.*, 2010). This is because the animal's response to a stressor leads to homeostatic imbalance, which is indicated by increased secretion of stress hormones such as cortisol or corticosterone (glucocorticoids) in the adrenal cortex (Möstl and Palme, 2002). Cortisol is the key hormone that is widely measured to assess stress in animal populations (Schatz and Palme, 2001; Möstl and Palme, 2002; Kleinsasser *et al.*, 2010; Sheriff *et al.*, 2010). Measuring cortisol levels in the faeces of farm animals has been shown to be the most effective way of assessing stress (Möstl and Palme, 2002). The reason for this is that faecal samples can easily be obtained without causing more stress to the animal, the procedure is non-invasive and faecal samples can easily be analyzed (Möstl and Palme, 2002).

Although traditionally, plasma is the specimen of choice for assay of glucocorticoids in animal welfare studies, it has a few shortcomings (Mormede *et al.*, 2007). The shortcomings include the fact that collection of blood is invasive, the capture, handling, and bleeding procedures can also

cause an increase in blood cortisol concentration within 3 minutes (Romero and Romero, 2002). Furthermore, blood sample represents the concentration of cortisol at a single time point. Since glucocorticoids exhibit regular patterns of secretion as well as episodic changes over time (Sapolsky *et al*., 2000; Romero, 2004) single point measurements may not give a true reflection of hormone levels (Touma and Palme, 2005). Thus a non-invasive approach for measuring stress hormones would be preferred. A number of non-invasive sampling procedures that measure cortisol metabolites levels in urine, saliva or milk have been developed to assess physiological responses to stress in many species (Möstl and Palme, 2002; Palme, 2005; Touma and Palme, 2005). However, among these non-invasive procedures, faecal samples have advantages over the rest due to the fact that faeces can be collected easily and over a long period of time, individual animal serves as its own baseline control and these procedures are nearly stress-free (Möstl and Palme, 2002; Palme, 2005). Therefore, collection of faecal sample does not add any more stress to the animal. Hence assessments of faecal cortisol metabolite (FCM) levels, allows more representative conclusions to be drawn about the stress level of the animal (Rauch *et al.*, 2014).

Cortisol is metabolized by the liver and excreted as conjugates through the kidney into urine or through the bile into the gut and cortisol metabolite is eventually excreted in the faeces (Palme *et al.*, 1996; Möstl and Palme, 2002). In ruminants such as cattle and sheep where FCM has been used as a parameter to measure stress (Palme *et al.*, 1999 and 2000; Merl *et al.*, 2000; Möstl *et al.*, 2002; Touma and Palme, 2005), the time delay for the metabolites to be excreted in faeces of sheep is about 12 hours (Palme *et al.*, 1999; Möstl and Palme, 2002; Touma and Palme, 2005). There is no published data on the use of cortisol metabolite analysis to assess if rumen impaction with indigestible foreign materials particularly plastic bags causes stress in the animal. The

current study was carried out to determine cortisol levels in wether sheep experimentally implanted with plastic bags in their rumen. Faecal cortisol metabolite (FCM) levels were determined.

Two group specific 11-oxoaetiocholanolone enzyme immuno-assays; EIA I developed by Palme and Mostl (1997) and EIA II by Mostl *et al.* (2002) at the Institute of Biochemistry, University of Vienna, Austria have successfully been used in numerous animal welfare studies to measure the concentration of cortisol metabolites in faeces. Enzyme immuno-assay I measures 11,17-dioxoandrostanes (11,17-DOA) metabolites, whereas EIA II measures metabolites with a 5ß-3α-hydroxy-11-oxo structure. Both assays have been analytically validated and used in a number of studies. The EIA I has been proven to be suited for assessing stress in animal populations such as sheep (Palme and Mostl, 1997), goats (Kleinsasser *et al.*, 2010) and cattle (Palme *et al.*, 2000; Morrow *et al.*, 2002). In the present study EIA I was chosen to measure the FCM in faeces of wethers.

5.2 Materials and methods

5.2.1 Acquisition of animals and experimental design

The acquisition of animals and design of the experiment are described in sections 3.2.1 and 3.2.2.2 respectively. For this experiment, only the 15 healthy wethers in Phase II experiment which had an endpoint of 8 weeks were used. The 15 wethers were divided into 3 experimental groups and each group had 5 wethers. The groups in Phase II included: PPII (n = 5) wethers whose rumen were implanted with plastic bags through rumenotomy, NPPII (n = 5) as positive

control group in which wethers had rumenotomy done but no plastic bags implanted and CPII (n = 5) as negative control group in which wethers had neither rumenotomy done nor implanted with plastic bags.

5.2.2 Rumenotomy procedure and implantation of plastic bags in the rumen

The procedure for rumenotomy and implanting of plastic bags in the rumen is described in details in section 3.2.3.

5.2.3 Faecal sampling

Ten grams of fresh faecal samples were collected directly from the rectum of each wether using the finger in a gloved hand into zip lock bags. The samples were labelled with the experimental group code, individual number of the wether, time and date of sample collection. The faecal samples in labelled zip lock bags were immediately stored in a cool box with ice to prevent enzymatic degradation of cortisol metabolites (Palme, 2005). The faecal samples in zip lock bags were then stored at -20°C until processed. Storage of samples at -20 °C was done within one hour after collection.

5.2.3.1 Pre-implantation baseline faecal sampling

Ten grams of faecal samples were collected using a Mettler balance (PM4600 DeltaRange, Switzerland) from each wether prior to the commencement of rumenotomy and these were for baseline measurements.

5.2.3.2 Post-implantation faecal sampling

Once rumenotomy was completed which marked the beginning of the experiment, 10g of faecal samples were again collected at two-time points: hourly and weekly sampling and considered as post-implantation samples. The hourly faecal sampling was done at 12, 24, 48, and 72 hours post-implantation both in the test wethers (PPII) and in positive control group wethers coded (NPPII) as well as in negative control group wethers coded (CPII). Weekly faecal samples were collected exactly one week after implanting plastic bags into the rumen and repeated each subsequent week until the 8^{th} week for each group.

5.2.4 Measurements of faecal cortisol metabolite levels

The concentration of faecal cortisol metabolite (FCM) for all the groups of wethers were analyzed using Enzyme Immuno-assay I (EIA I) as described by Palme and Mostl (1997). The FCM measurement was done in two stages, which were extraction and analysis. The extraction of faecal cortisol metabolite was done at the immunology laboratory of Department of Public Health, Pharmacology and Toxicology, University of Nairobi. Analysis of cortisol metabolite in faeces using EIA I was done at Professor Rupert Palme's laboratory in the Department of Biomedical/Biochemistry, University of Veterinary Medicine, Vienna, Austria.

5.2.4.1 Extraction of faecal cortisol metabolites

Frozen faecal samples (as described earlier in section 5.2.3) were thawed and weighed. In order to extract faecal cortisol metabolites from faecal samples, 0.5 g of the sample was weighed into a centrifuge tube (10 ml, pyrex). A total of 5 ml of 80% methanol (Sigma, Aldrich) was added to the tube and the mixture was vortex-mixed for 30 minutes. Tubes were then centrifuged at 2500g

for 15 minutes. A total of 0.5 ml of the supernatant was drawn from each tube with pipette and put into Eppendorf tubes. The Eppendorf tubes were labelled with the experimental group identity, the individual number of the animal and the sampling time point. These labelled tubes with supernatant were then dried on a sand heat block. All the tubes well labelled were fixed onto the sand heat block, which was set at approximately 60°C.

5.2.4.2 Analysis of faecal cortisol metabolites using enzyme immunoassays

Analysis of FCM with EIA I followed the standard laboratory protocol of the Department of Biomedical Sciences/Biochemistry, University of Veterinary Medicine-Vienna, detailed in the July, 2014 version for measuring faecal steroid metabolites with enzyme immunoassay (EIA) on microtitre plates using biotinylated steroids as labels. The absorbance of generated colour was read using an automatic microtitre plate reader connected to a computer with a reference filter of wavelength 620 nm and a measuring filter of 450 nm. The concentrations of cortisol metabolite in the faecal samples were then calculated by a special software programme connected to the plate reader.

5.3 Data analysis

The raw data collected was verified, validated and entered into Microsoft Office 2010 Excel Spread Sheet. The data sets were coded with letters and numbers representing each sample in an experimental group. Data were managed in Excel 2010 and values obtained for each data set were expressed as “Mean ± S.E.M”. The data obtained were imported into GraphPad Prism software version 6.0 (GraphPad Prism Statistical Software, Inc. California, USA) graphical presentation and statistical analysis. Mean plasma cortisol concentrations were plotted against

hourly and weekly time points. One-way and Two-way Analysis of variance (ANOVA) with Tukey's Multiple Comparison (Post hoc) Test were performed using GraphPad Prism software (version 6.0) to compute associations and comparisons between the different parameters and experimental groups and parameters. Comparisons of the means were considered significant at the level of $p < 0.05$.

5.4 Results

5.4.1 Effects of rumen impaction with plastic bags on faecal cortisol metabolite levels of wethers in Phase II experiment

The results of faecal cortisol metabolite (FCM) concentration obtained for test group, positive control group and negative control group of wethers in Phase II experiments are presented in sections 5.4.1.1 and 5.4.1.2.

5.4.1.1 Hourly mean faecal cortisol metabolite levels in wethers in Phase II

The differences in hourly mean values of FCM concentration in the test group of wethers whose rumen were impacted with plastic bags (PPII) and the positive control (NPPII) and negative control (CPII) groups of wethers without plastic bags in the rumen over the first 72 hour period of the experiment are summarized in Table 5.1. The comparative graphical presentation of the effects of rumen impaction on FCM levels in wethers are also shown in Figure 5.1.

Wethers whose rumen were impacted with plastic bags (PPII) had significantly ($p < 0.05$) increased concentrations of faecal cortisol metabolite over the first 72 hour period post-implantation compared with their own baseline measurements and compared with both the

negative and positive control groups (Table 5.1). After 24 hours of implanting plastic bags in the rumen of the test group, the mean value of FCM concentration increased to 124.4 ± 72.3ng/g, which was an increase of more than 5 times from baseline value of 20.0 ± 6.0ng/g. This mean value dropped marginally after 48 hours, but at the end of 72 hour period the mean value of 90.8 ± 24.8ng/g was still over 3 times higher than baseline measurements.

Tukey's multiple comparison test of two-way ANOVA revealed significant differences in the mean values of FCM concentration between the test group PPII and negative control group CPII at 24 hours ($p = 0.0023$) and 72 hours ($p = 0.0294$) post-implantation. Significant differences in mean values of FCM concentration existed between the impacted wethers PPII and positive control group NPPII at 24 hours ($p = 0.0101$) and 48 hours ($p = 0.0073$) post-implantation.

Table 5.1: Hourly mean values of faecal cortisol metabolite concentrations in three groups of wethers with or without rumen impaction during the first 72 hours of Phase II experimentation

Time (Hours)	**Hourly mean (± SE) values of FCM concentration (ng/g)**			***P*- value**
	PPII (n = 5)	**NPPII (n = 5)**	**CPII (n = 5)**	
0	20.0 ± 6.0	10.3 ± 2.5	10.6 ± 2.5	0.9546
12	37.3 ± 6.6	23.8 ± 2.6	20.7 ± 7.3	0.8653
24	124.4 ± 72.3	26.5 ± 7.2	10.1 ± 3.8	0.0023[a] 0.0101[b]
48	121.0 ± 40.0	19.4 ± 9.2	49.4 ± 9.2	0.0073[b]
72	90.8 ± 24.8	13.1 ± 3.4	6.0 ± 1.5	0.0294[a]

Data are presented as means with plus or minus the standard error (± SE)
Significance at $p < 0.05$
[a]*P*-value = test group PPII compared with negative control group CPII
[b]*P*-value = test group PPII compared with positive control group NPPII

Key:
PPII = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen, ng/g = nanogram per gram, n = number of wethers in the group

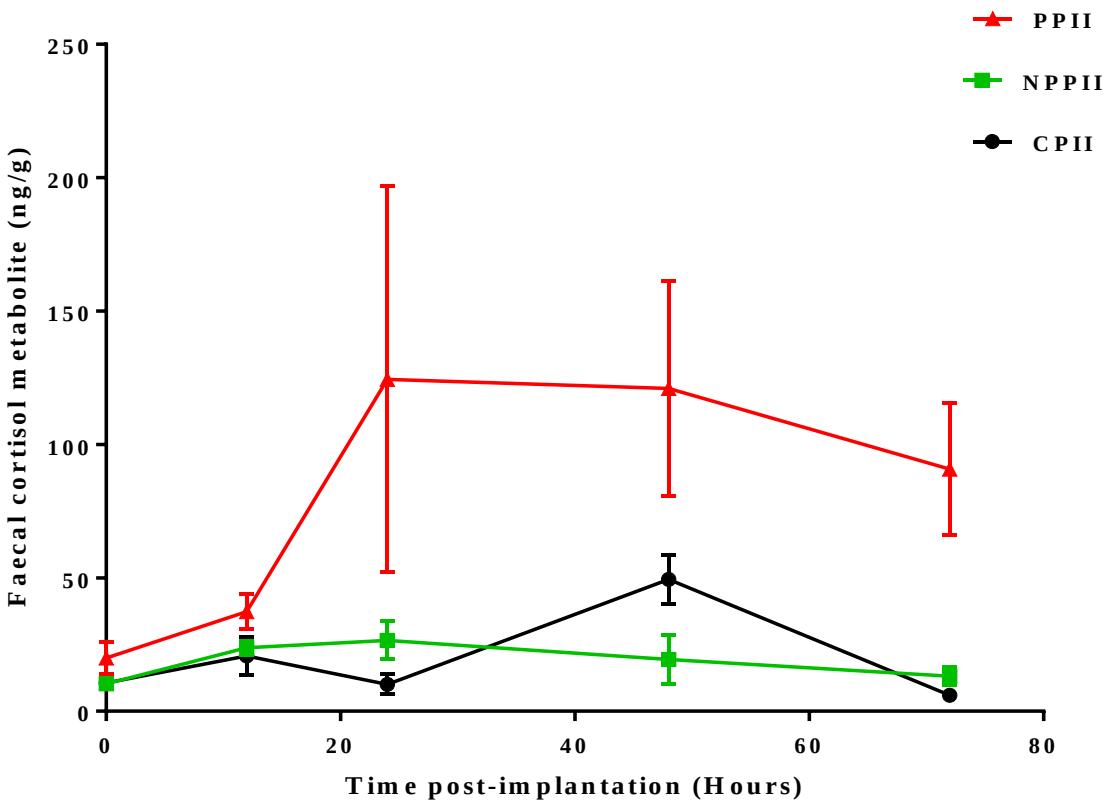


Figure 5.1: Faecal cortisol metabolite concentration in three groups of wethers with or without rumen impaction during the first 72 hour experimentation in Phase II. The results showed significant rise in levels of cortisol metabolites in faeces of the test group.

Key: **PPII** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

5.4.1.2 Weekly mean faecal cortisol metabolite concentration in the wethers in Phase II experiments

Weekly mean faecal cortisol metabolite concentration measured over 8-week post-implantation period and differences in values of mean FCM between test group and control groups are given in Table 5.2. Comparative graphical presentation of these effects on FCM levels between the three groups are presented in Figure 5.2.

Wethers whose rumen were implanted with plastic bags showed increased levels of faecal cortisol metabolite compared with their baseline measurements and also when compared with the control groups, but these levels declined to almost normal by the end of 8 weeks of the experiments. At 2 weeks post-implantation, the wethers in the test group PPII had their mean values of FCM increased 5-fold from their baseline measurements. This increase was significantly high when compared with the values in the negative control group (CPII) ($p < 0.0001$) and also when compared with the positive control group (NPPII) ($p < 0.0001$). The mean FCM concentration then decreased from 120.3 ± 51.4ng/g at week 2 post-implantation to 75.7 ± 52.2ng/g at week 6, which was still 3 times higher and significant ($p = 0.0189$) when compared with their baseline values and when compared with the control groups (Table 5.2). The wethers whose rumen were impacted with plastic bags had mean values of FCM decrease to those comparable with control groups, but still recorded 40% mortality at the end of the 8 week experimental period.

Table 5.2: Weekly mean values of faecal cortisol metabolite concentration in three groups of wethers over an experimental period of 8 weeks

Time (Weeks)	**Weekly mean (± SE) FCM concentration (ng/g)**			***P* value**
	PPII (n = 5)	**NPPII (n = 5)**	**CPII (n = 5)**	
0	20.0 ± 6.0	10.3 ± 2.5	10.6 ± 2.5	0.9234
1	61.5 ± 34.8	15.6 ± 3.6	7.2 ± 0.9	0.0765
2	120.3 ± 51.4	13.5 ± 2.2	14.5 ± 3.3	0.0001[a] 0.0001[b]
3	76.6 ± 37.9	19.5 ± 5.8	29.9 ± 5.8	0.1470
4	53.9 ± 21.0	12.2 ± 5.4	15.5 ± 5.1	0.2706
5	29.5 ± 13.0 (n = 4)	9.7 ± 4.1	24.0 ± 6.6	0.9760
6	75.7 ± 52.2 (n = 4)	3.5 ± 1.1	14.1 ± 4.2	0.0189[b]
7	6.9 ± 4.9 (n = 3)	8.0 ± 1.7	18.4 ± 5.0	0.9145
8	5.8 ± 2.5 (n = 3)	3.1 ± 1.7	9.5 ± 2.1	0.9908

Data are presented as means with plus or minus the standard error (± SE)
Significance at $p < 0.05$
[a]*P*-value = test group PPII compared with negative control group CPII
[b]*P*-value = test group PPII compared with positive control group NPPII

Key:
PPII = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen, ng/g = nanogram per gram, n = number of wethers in the group

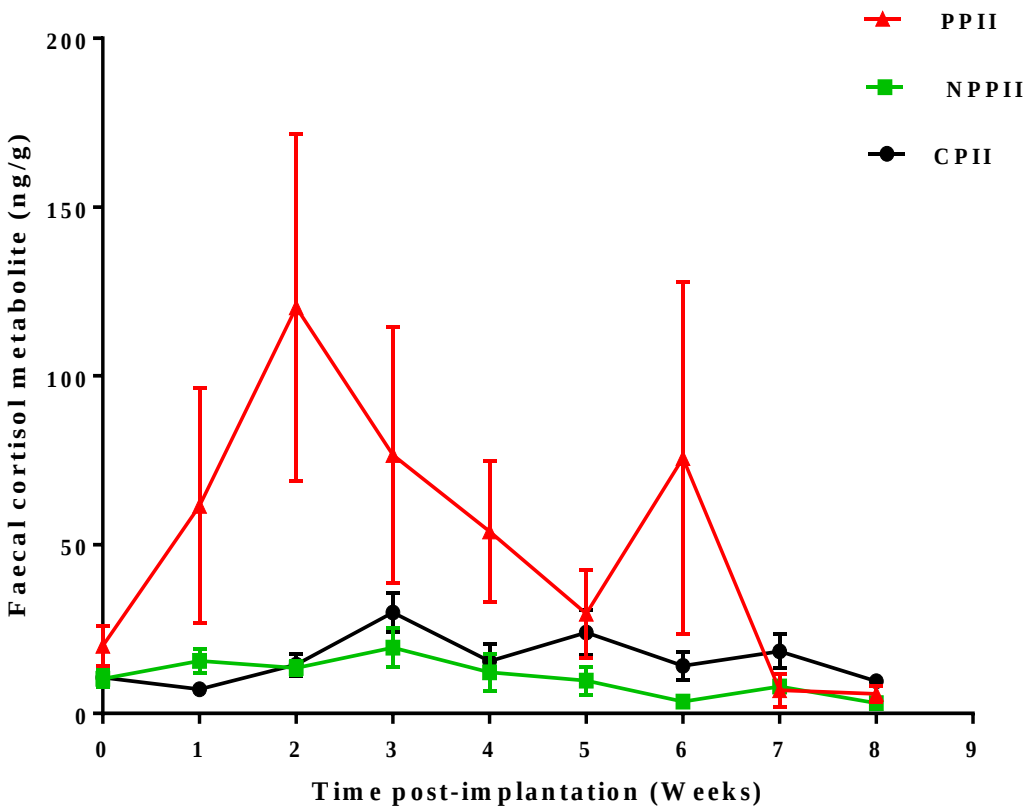


Figure 5.2: Faecal cortisol metabolite concentration measured over 8 weeks in three groups of wethers with or without rumen impaction. The results showed significantly elevated levels in faecal cortisol metabolites in the test group.

Key: **PPII** = test group of wethers whose rumen were implanted with plastic bags through rumenotomy, **NPPII** = positive control group of wethers that had rumenotomy but no plastic bags implanted in their rumen, **CPII** = negative control group of wethers that had neither rumenotomy nor plastic bags implanted in their rumen.

5.4.2 Correlation between weekly mean faecal cortisol metabolite concentration and weekly mean body weight of wethers in Phase II experiments

The correlation between weekly mean body weight (BW) and weekly mean FCM concentration of the three groups PPII, NPPII and CPII are presented in Figures 5.3a, 5.3b and 5.3c. Results of weekly mean body weight of wethers in phase II experiment are shown in the previous Figure 3.7 in section 3.5.1.2.

There was a negative correlation between weekly mean BW and weekly mean FCM concentration for all the groups. The increase in values of weekly mean body weight of control groups of wethers not implanted with plastic bags (NPPII and CPII) negatively correlated with their respective weekly mean FCM concentration which remained stable throughout the experiment. The mean body weight of the group NPPII decreased during the 2 weeks post-implantation but increased over the 6 weeks period that followed while that of group CPII increased over the whole 8-week experimental period. The decrease in values of weekly mean BW of the impacted wethers throughout the experimental period also showed a negative correlation with weekly mean FCM concentration which although increased over 6 weeks post-implantation had declined to normal values at the 8-week endpoint.

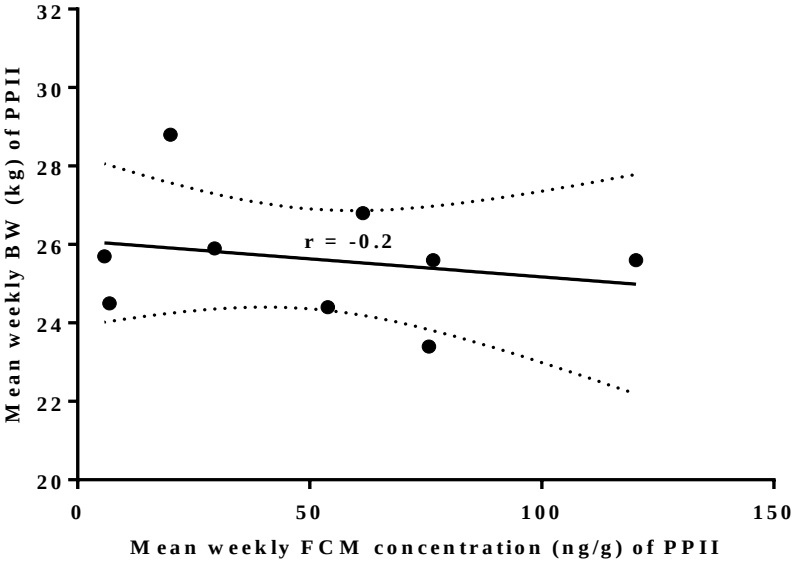


Figure 5.3a: Correlation between weekly mean body weight and mean faecal cortisol metabolite concentration over the 8-week experimental period in wethers whose rumen were implanted with plastic bags (PPII).

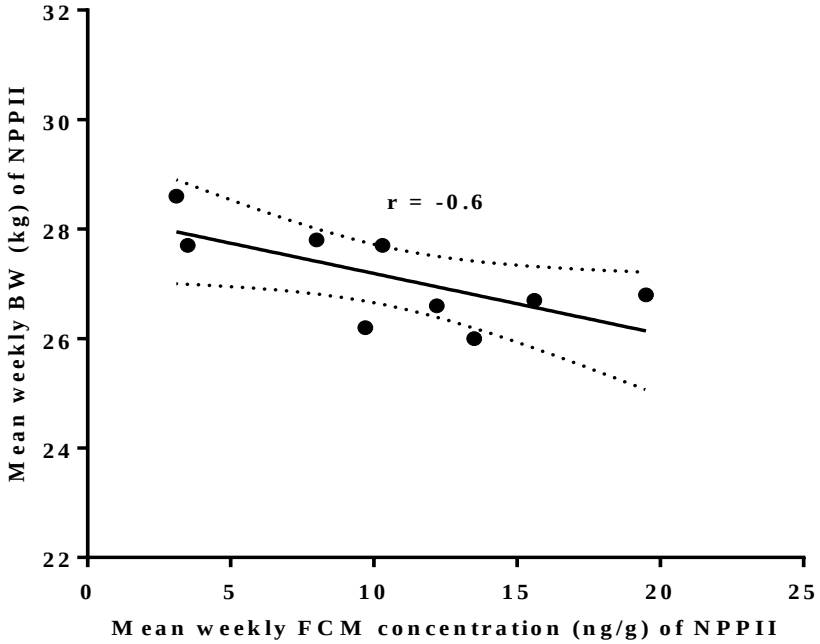


Figure 5.3b: Correlation between weekly mean body weight and faecal cortisol concentration during the 8-week experimental period in positive control group of wethers which had rumenotomy done but no plastic bags in rumen (NPPII)

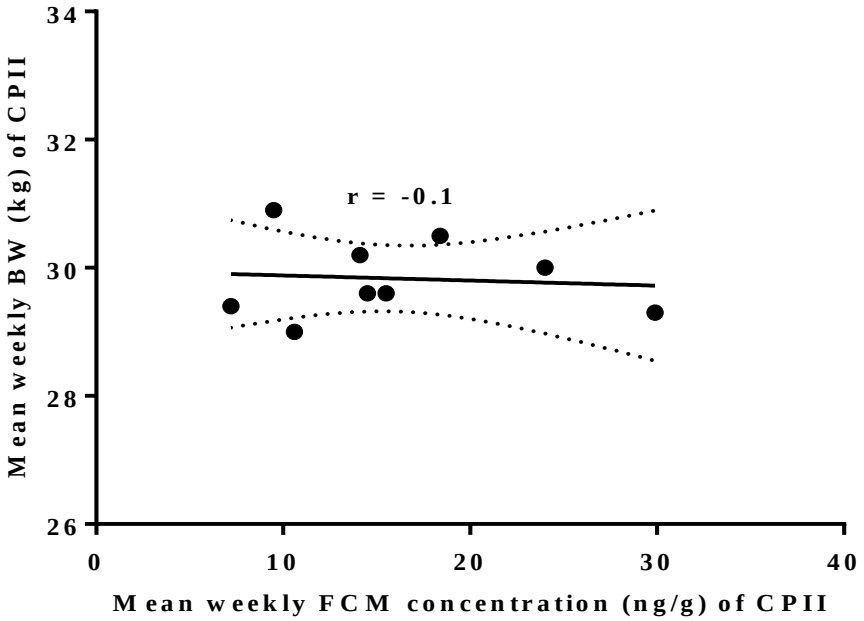


Figure 5.3c: Correlation between weekly mean body weight and mean FCM concentration during the 8-week experimental period in negative control group of wethers which had neither rumenotomy done nor plastic bags in the rumen (CPII).

5.5 Discussion

The significantly ($p < 0.05$) increased concentration of mean faecal cortisol metabolite (FCM) which persisted over the entire 72 hour period suggests that impaction of the rumen with plastic bags induced acute stress in the animals. It increased FCM by over 5-folds. Similar findings of elevated levels of cortisol metabolites in the faeces of animals that are subjected to different stress conditions are reported (Palme *et al.*, 2000; Merl *et al.*, 2000; Mostl *et al..*, 2002; Sheriff *et al.*, 2010; Davies *et al.*, 2013), although there are no previous reports of these metabolites in ruminal impaction. Kleinsasser *et al*. (2010) reported about 9-fold increase in FCM concentration in goats which were subjected to an acute stressor by administering adrenocorticotropin hormone (ACTH). However, most of these studies assessed stress by administering ACTH in animals to elicit a stress response (Mostl and Palme, 2002; Sheriff *et al.*, 2010).

It is suggested that persistent presence of impacting quantities of plastic bags in the rumen in the current study served as an internal stimulus for stress, which disrupted homeostasis and triggered the stress response system to secrete excess cortisol. It is well known from published data that any adverse stimuli be it internal or external that disrupts homeostasis stimulates the hypothalamic-pituitary-adrenal (HPA) axis both in animals and humans to initiate adaptive response (Selye, 1936; Mormede *et al.*, 2007; Tsigos and Chrousos, 2002; Hough *et al.*, 2013). The main active hormone of the HPA axis in sheep is the glucocorticoid cortisol excreted in faeces as a group of cortisol metabolites known as 11,17-dioxoandrostanes (11,17-DOA) (Mostl and Palme, 2002; Mormede *et al.*, 2007). Therefore the continuous stimulation of the HPA axis to secrete cortisol as a result of stress from rumen impaction led to the increased levels of faecal cortisol metabolites secreted in the faeces.

Drastically reduced feed intake and poor absorption of available nutrients in wethers whose rumen were impacted with plastic bags resulting in starvation may also explain the rise in faecal cortisol metabolite concentration. Starvation has been reported to have significant effect on the levels of cortisol (Lane, 2006). Starvation has been implicated as a stressor which triggers increased secretion of cortisol to breakdown muscle protein after depletion of body fats and carbohydrates. However, the effect of this on the concentration of cortisol metabolites is not well known (Millspaugh and Washburn, 2004; Palme, 2005; Palme *et al*., 2005). Starvation is therefore an important factor for sheep and goats that scavenge on waste dumping sites and roadsides that may be contaminated with waste plastic bags (Igbokwe *et al*., 2003; Ghurashi *et al*., 2009; Otsyina *et al.*, 2015). These are likely to ingest the plastic bags, subsequently leading to rumen impaction and consequently reducing feed intake as well as hindering processes of digestion (Igbokwe *et al*., 2003; Khan *et al*., 1999). Since plastic bags take long to accumulate in the rumen from scavenging, the faecal cortisol metabolites are best suited for assessing the ensuing long-term stress. Further studies should be undertaken to evaluate this for natural long-term impaction in the urban and peri-urban scavenging sheep and goats.

The significant short-term 5-fold elevation in the levels of faecal cortisol metabolites of wethers with rumen impaction followed by gradual decline to normal levels over the 8-week experimental period is comparable to other stress-related studies. Similar observations were made in snowshoe hares (*Lepu americanus*), which were naturally stressed by being exposed to a dog for a period of time (Sheriff *et al.*, 2008). Presence of the dog stressor significantly increased FCM concentration of the hares almost 2-fold, 10 hours after the initial exposure then returned to low levels 24 hours later. Similar trends were also described in cows transported into a new stall in which their FCM concentration were markedly increased for about 1 week and thereafter

returned to normal levels (Möstl *et al.*, 2002). In another report, goats that were individually introduced into already established small groups of goats had their FCM concentration increased between the day 1 and day 4. The FCM levels decreased to baseline concentration by day 11 after the goat was returned to its original group (Patt *et al.*, 2012).

The decline in concentration of faecal cortisol metabolites of wethers whose rumen were impacted with plastic bags could probably be attributed to the effect of negative feedback mechanism on the HPA axis that might have inhibited further release of ACTH. Subsequently, this may have inhibited the adrenal cortex from secreting cortisol, hence the decreased levels of faecal cortisol metabolites (Norman and Litwack, 1987; Nussey and Whitehead, 2001; Smith and Dobson, 2002). It has been shown that elevated cortisol levels exert negative feedback on the HPA axis, which ensures the return of its activity to basal levels after stimulation (Matthews, 1998; Meyer *et al.*, 1998) and prevents the effects of chronic elevation of cortisol, such as suppression of inflammatory processes and immune responses (Manteuffel, 2002). Although there are increasing reports on prevalence of indigestible foreign bodies particularly plastic bags in the rumen of ruminants (Igbokwe *et al.*, 2003; Singh, 2005; Abebe and Nuru, 2011; Khurshaid *et al.*, 2013; Otsyina *et al.*, 2015), none of the reports included faecal cortisol metabolite measurements. However, the reports have consistently implied that rumen impaction has adverse effects on the health of the animal. Singh (2005) reported that almost all stray cattle in India suffered various illnesses from indigestible foreign bodies, which occasionally led to death. In addition, Igbokwe *et al.* (2003) reported changes in blood biochemistry in sheep with impacted rumen. Hence rumen impaction with indigestible materials particularly among the small ruminants should not be underrated, but should be keenly addressed.

The fact that FCM concentration returned from high levels to baseline values in wethers that had rumen impaction, should not be taken to mean that stress was resolved. Rather, the beneficial effect of elevated cortisol restored the HPA axis back to baseline value in order for the wethers to cope with the existence of the plastic bags form of stressor in the rumen. Perhaps measuring other biomarkers of chronic stress in impacted rumen after a prolonged period of time may be more informative. This observation is in line with Selye's (1950) General Adaptation Syndrome (GAS) theory which describes the second phase as the resistance phase or allostasis. This phase is characterized by the body's attempt to maintain homeostasis while the stressor is still present. If elevated glucocorticoid persists, then the animal enters the exhaustion phase, which is the final stage that the stress response system is overextended. This leads to various pathologies such as increased susceptibility of the animal to diseases, impaired growth and decreased reproductive abilities (Selye, 1950; Hough *et al.*, 2013). It can therefore be assumed from this study, that the wethers with impacted rumen that died may have suffered the detrimental effects of persistently elevated cortisol, while those of the same group that survived may have benefited from the negative feedback of elevated cortisol, which enabled them to adapt to the stressor.

Failure to adapt to a stressor may be as a result of decreased sensitivity of cortisol negative feedback, accomplished by the interaction of cortisol receptors with transcription factors induced by corticotropic releasing hormone (CRH) and vasopressin (Aguilera, 1994). Anderson *et al.* (1996) also reported that the HPA axis activation in response to an adverse stimulus also depends on the control the animal can have on the stressor. It can therefore be assumed that even within the same experimental group of wethers under the same stressor of rumen impaction with plastic bags, some of the wethers probably coped with the stressor better than others.

Mortalities could also have resulted from starvation due to reduced feed intake caused by diminished rumen space from accumulated plastic bags. Hunger and satiety are regulated by a variety of psychological, gastrointestinal, metabolic and nutritional factors as well as by neuronal and endocrine mechanisms (Plata-Salaman, 1991). It is thus obvious that reduced feed intake as a result of anorexia may have resulted in decreased production and absorption of volatile fatty acids. This implied that energy requirements of the animals were not met, hence decreased body weight, which may also have contributed to death of the wethers with rumen impaction. Chronic activation of the HPA axis has been shown to lead to weight loss (Gertz *et al.*, 1987; Mormede *et al.*, 1990). A study using animal models revealed that activation of CRH receptors alters gastrointestinal functions (Tache *et al.*, 1993). Chronic elevation of cortisol has been reported to cause decreased body weight by reducing the appetite resulting also in diminished food utilization efficiency (Klasing, 1985). In ruminants, stress may have inhibitory effects on rumination, consequently reducing feed digestibility and thereby affecting weight gain (Asres and Amha, 2014).

The absence of correlation between body weight and faecal cortisol metabolites in wethers with impacted rumen and control groups without rumen impaction, suggests that the loss or gain in body weight may not be strongly inversely proportional to the concentration of cortisol metabolites excreted in the faeces. This may be due to the body maintaining a normal balance of cortisol. Furthermore, during stressful conditions the stress response system fights to bring elevated levels to baseline values although the negative effect of stress could affect the body weight. However, studies in roadside mice (*Apodemus sylvaticus*) showed a positive correlation between FCM and individual BW (Navarro-Castilla *et al.*, 2014), which contrasted the findings

of the present study. The differences may be due to differences in the type of animal model used and the snapshot measurements that do not represent long-term assessment. Further work on this is therefore recommended.

5.6 Conclusions

From the current study, it is concluded that:

a) Elevation of faecal cortisol metabolite levels in wethers whose rumen were impacted by implanted plastic bags, was more acute than chronic, hence the need to investigate other biomarkers of chronic stress.

b) Faecal cortisol metabolite concentration is a useful indicator of presence of stress in the sheep measurable in the early stages of stress.

c) Faecal cortisol metabolite measurements should be evaluated in natural occurring rumen impaction to ascertain its usefulness in determining presence of stress.

CHAPTER SIX

6.0 GENERAL DISCUSSION

The results of the present study showed decreased mean body weight in wethers whose rumen were implanted with plastic bags, which corresponded to the prolonged presence of these bags in the rumen. Similar previous reports of loss in body weight in small ruminants with rumen indigestible foreign bodies have been documented (Igbokwe *et al.,* 2003; Ghurashi *et al.,* 2009; Abdelaal and El-Maghawry, 2014; Otsyina *et al.*, 2015). Presence of large quantities of plastic bags in the rumen leads to reduced feed intake, production and absorption of volatile fatty acids. All these may lead to decreased rate of fattening (Igbokwe *et al.,* 2003). Concentration of volatile fatty acids in the rumen depends on rumen motility and microbial population (Khan *et al.,* 1999), which may reduce due to plastic bags occupying rumen space. Hunger and satiety have also been shown to be regulated by psychological, gastrointestinal, metabolic and nutritional factors as well as by neuronal and endocrine mechanisms (Plata-Salaman, 1991).

Furthermore, chronic activation of the HPA axis and CRH receptors reduces appetite, alters gastrointestinal functions and this leads to decreased food utilization efficiency, consequently resulting in weight loss (Klasing, 1985; Gertz *et al.*, 1987; Mormede *et al.*, 1990; Tache *et al.*, 1993). Another contributory factor is the inhibitory effects of plastic bags in the rumen on rumination, which may have led to reduced feed digestibility and thereby interfering with weight gain (Asres and Amha, 2014).

The decrease in mean body weight of positive control group of wethers subjected to rumenotomy with no plastic bags could be attributed to pain and stress from surgery despite administration of

pain management drugs (Anderson and Muir, 2005). The pain stress may have activated neuronal pathway and endocrinal mechanisms that possibly affected appetite (Plata-Salaman, 1991).

Loss of papillae on the mucosal surface of rumen is most likely attributed to the abrasions by the compacted mass of plastic bags. This is supported by previous documented findings that dietary components, ruminal internal environment and duration of impact have effects on the size, shape and density of ruminal papillae (Beharka *et al.*, 1998; Steele *et al.*, 2011; Liu *et al.*, 2013).

The pale coloured mucosa of the impacted rumen which was severe after prolonged period of impaction may be suggestive of reduced pigmented product on the rumen mucosa due to reduced microbial activity (Sinclair and Kunkel, 1959). Moreover the colour of rumen mucosa in the positive and negative control groups remained unchanged, but was greenish-brown (Neiva *et al*., 2006).

Obstruction of the rumeno-reticular orifice resulted from rumen mass formed by mixture of plastic bags and ingesta through the rumen movements. Similar reports have been published previously in ruminants with indigestible foreign bodies in their rumen (Khan *et al.*, 1999; Kumar and Dhar, 2013; Abdelaal and El-Maghawry, 2014). This blockage resulted in decreased flow of ingesta, hence diminished digestive contents with subsequently low inadequate energy leading to general weakness of the wethers (Mayer *et al*., 1992; Hailat *et al.*, 1996; Hailat *et al.*, 1998; Igbokwe *et al*., 2003; Kumar and Dhar, 2013).

The stunted, bent over and slender papillae as well as the thin epithelia on the mucosal surface of rumen that were observed in impacted rumen were similar to those previously described in ruminant that had rumen indigestible foreign bodies (Kumar and Dhar, 2013). The cause of the morphological changes of the papillae is likely to be the pressure exerted on the mucosal surface by the compacted plastic bags. Branching of the papillae as observed in the current study may be compensatory mechanisms probably to increase the absorptive mucosal surface area. The finding of varied sizes, shapes and density of papillae studded on the rumen mucosal surface of different rumen sacs in the control groups of wethers that had no plastic bags, is consistent with previous reports on the normal ruminal morphology (Scott and Gardner, 1973; Yamamoto *et al.,* 1998; Shen *et al*., 2004; Poonia *et al*., 2011).

The loss of surface density, volume density and absolute volume of the absorptive mucosa in the different ruminal sacs as revealed by stereological estimations, suggested worsening damage as duration of rumen impaction with plastic bags increased. The prolonged pressure and friction exerted by compacted plastic bag masses in the rumen gradually led to sloughing and loss of the papillae and epithelial cells, which is consistent with the fact that morphological changes in the ruminal epithelia is a slow protracted but progressive (Gabel and Aschenbach, 2002). Although there is no previous quantitative data on rumen morphology, the results of the current study are partly supported by qualitative reports that impaction of the rumen with indigestible foreign materials leads to sloughing and thinning of mucosal wall (Hailat *et al.*, 1996; Hailat *et al.*, 1998), which could affect the digestive and absorptive functions of the rumen (Ghurashi *et al*., 2009). These findings agree with a recent study which reported a reduction in the volume of stratum granulosum cells of the rumen epithelial layer in goats fed on high grain diet relative to

those fed on high forage diet (Liu *et al*., 2013). Furthermore, it is speculated that toxic compounds released from plastic bags (Bashir, 2013) may have been leached out onto the rumen mucosal surface, which may have weakened the tight epithelial cell junctions (Gonzalez-Mariscal *et al*., 2008), subsequently damaging it's structural morphology.

The increased mean volume density and mean absolute volume of muscularis interna in the cranial and dorsal sacs of the impacted rumen could be attributed to hyperplasia or hypertrophy predisposed by overstretching of the ruminal walls as a result of excessive pressure from the prolonged impaction. This is comparable to muscular thickening and dilatation occurring with mucosal ulceration in gastric impaction in horses (Scheidemann and Huthmann, 2011). Additionally, muscular lesions such as vacuolation of smooth muscle cells, focal fibrosis and myositis (Scheidemann and Huthmann, 2011) may further have contributed to the observed increase volume density and mean absolute volume found in the current study.

Double loss in the magnitude of total mean surface area of absorptive mucosal surface and higher loss in total mean absolute volume of mucosa in the rumen impacted for 8 weeks compared to the loss in these parameters at 4 weeks implies that duration of impaction has severe consequences on ruminal function. The rumen is important for the breakdown of diet to produce VFA which provides as much as about 80% of the animal's energy requirement (Bergman, 1990). The papillary surface increases the surface area for the absorption and transport of these end-products of microbial fermentation. The high loss in total surface area could be a reflection of decreased VFAs absorption and transport leading to negative energy balance in the animal and consequently interfering with the overall health of the animal that may have resulted in the

deaths that occurred in the present study. Previous studies indicated that rumen impaction with indigestible foreign materials affects health and productivity in ruminants (Ghurashi *et al.,* 2009; Abebe and Nuru, 2011; Mersha and Desiye, 2012; Khurshaid *et al.,* 2013; Otsyina *et al.,* 2015), which may result in mortalities (Singh 2005; Kumar and Dhar, 2013). The 50% loss in total mean absolute volume of mucosa in the entire ruminal tissue of impacted wethers concurs with what is previously reported that diet and ruminal content has influence on ruminal epithelia (Liebich *et al*., 1987). The total ruminal surface area and mucosal volume have a significant influence on absorption and nutrient transport (James *et al*., 1983), thus the likelihood of reduced ruminal functional efficiency in wethers whose rumen were impacted.

The acute elevated levels in plasma cortisol concentration in wethers impacted with plastic bags in their rumen over the 72 hour period post-implantation is partly comparable to previous reports, which indicated increased plasma cortisol concentration in sheep that were stressed by confinement or restraint in isolation (Degabriele and Fell, 2001; Moolchandani *et al.,* 2008). However, the type and intensity of the stress stimulus could account for differences in plasma cortisol level responses (Ferguson *et al*., 2008). Therefore, acute elevation of plasma cortisol levels observed in this study could be attributed to the body responding to plastic bags in the rumen as an internal stressor, stimulating HPA axis to initiate a stress response that subsequently caused the adrenal gland to secrete more cortisol. It is known that the ability of an animal to produce enough cortisol on stimulation of the HPA axis by a stressor is key for the animal to be able to quickly adapt to the stressor (Mormede *et al*., 2007 and 2011). Thus increased secretion of cortisol in response to an acute stressor may not necessarily be a negative thing, except when the intensity of the stressor results in detrimental changes to the animal's biological function

(Moberg, 1985). It is therefore implied that the acute increased levels of plasma cortisol concentration in the wethers was a reflection of their HPA axis' responsiveness to those foreign plastic bags in the rumen. The results thus lend support to earlier reports that plasma cortisol may be a good estimator of acute stress (Kilgour and de Langen, 1970; Harlow *et al*., 1987; Boissy and Neindre, 1997; Sylvester *et al*., 1998; Ruizu-de-la-Torre *et al.,* 2001; Doubek *et al*., 2003; Moolchandani *et al*., 2008; Sheriff *et al*., 2010; Cingi *et al.*, 2012).

The sudden increased mean plasma cortisol concentration in the positive control group within a few hours after rumenotomy and decreasing to normal levels within 72 hours is similar to that reported in cows, which showed a rise in the concentration of plasma cortisol shortly after an abdominal surgery (Mudron *et al*., 2005). The reason for the sudden rise in cortisol is likely to be due to pain from surgery which elicited the HPA axis to stimulate the increased production of cortisol by the adrenal cortex. Similar accounts have been observed by Lay (2000).

The decline in the mean values of plasma cortisol concentration to normal levels by the fourth week and remaining within normal throughout the 8-week period of the experiment may be attributed to adaptation to the stress of rumen impaction. There is a possibility that the adrenal glands responded to the stressor of plastic bags in the rumen and secreted more cortisol to initiate the negative feedback mechanism of the HPA axis which inhibited further secretion. A similar observation of low levels of plasma cortisol was reported in sheep that had fully adapted to their new environment as compared to high cortisol levels in non-adapted sheep (McNatty and Young, 1973). Conversely, Harlow *et al*. (1987) reported continuous elevated plasma cortisol concentration in sheep exposed to stress of loud noise over 35 days. It is known that the continuous secretion of cortisol under chronic stress is detrimental to the health of the animal

(Wingfield, 2001), which may reduce immune competence and increase animal's susceptibility to diseases. It could possibly be implied that wethers that survived after rumen impaction, had a better control on the stress of impaction considering that activation of the HPA axis in response to adverse stimulus also depends on the control the animal has on the stressor (Anderson *et al*., 1996). However in prolonged stress, low concentration of cortisol may not necessarily be a result of absence of any more stress but also the effect of exhausted adrenal glands (Stricklin and Mench, 1990). Others have suggested that sensitivity of the adrenal gland declines over time in persistence of the stressor (McNatty and Thurley, 1973; Ader, 1975). Therefore, there is need to determine other biomarkers that may be more sensitive to chronic stress.

The increase in faecal cortisol metabolites (FCM) concentration that was found in wethers whose rumen were impacted with plastic bags agrees with previous studies in which animals subjected to different stress conditions, also had elevated levels of cortisol metabolites in their faeces (Merl *et al.*, 2000; Palme *et al.*, 2000; Mostl *et al.*, 2002; Sheriff *et al.*, 2010; Davies *et al.*, 2013). Most of these studies however assessed stress by administering adrenocorticotropin hormone (ACTH) to the animals in order to elicit a stress response (Mostl and Palme, 2002; Sheriff *et al.*, 2010). For example, Kleinsasser *et al*. (2010) reported about 9-fold increase in FCM concentration in goats which were subjected to an acute stressor by administering ACTH.

In the current study, continuous presence of large quantities of plastic bags in the rumen of wethers together with their indigestible characteristic and impaction was perceived as an internal stimulus for stress, disrupting homeostasis and probably triggering the stress response system to

secrete more cortisol. It is established that any adverse stimuli be it internal or external that disrupts homeostasis stimulates the hypothalamic-pituitary-adrenal (HPA) axis both in animals and humans to initiate adaptive responses (Selye, 1936; Mormede *et al.*, 2007; Tsigos and Chrousos, 2002; Hough *et al.*, 2013). Cortisol is the main active hormone of the HPA axis in sheep which is excreted in faeces as a group of cortisol metabolites known as 11,17-dioxoandrostanes (11,17-DOA) (Mostl and Palme, 2002; Mormede *et al.*, 2007). Therefore the continuous stimulation of the HPA axis to secrete cortisol as a result of stress from rumen impaction led to the increased amounts of cortisol metabolites excreted in their faeces as compared to low levels recorded in wethers which had no rumen impaction.

Additionally, the elevated faecal cortisol metabolites concentrations in wethers whose rumen were impacted, could be due to starvation as a result of reduced feed intake due to reduced appetite as well as occupation of most rumen space by plastic bags. Previous reports on rumen impaction with indigestible materials indicated reduced feed and interference with digestion processes (Khan *et al*., 1999; Igbokwe *et al*., 2003). Starvation has been reported to have significant effect on the levels of cortisol (Lane, 2006), owing to the body perceiving starvation as a stressor that triggers increased secretion of cortisol to breakdown muscle protein for energy once that of body fat and carbohydrate have been depleted (Millspaugh and Washburn, 2004; Palme, 2005; Palme *et al*., 2005). Since FCM are end-products of metabolized cortisol, it therefore follows that an increase in cortisol would correspondingly lead to an increase in their metabolites. However, the initial elevation of FCM concentration that later declined to normal level by the end of the 8 week experimental period, is a finding comparable to the trends

previously found in cows that were transported into a new stall, after which their FCM concentration markedly increased for about 1 week and thereafter returned to normal levels.

The decline in faecal cortisol metabolites concentration could also be attributed to negative feedback mechanism of HPA axis that inhibited further release of ACTH, subsequently preventing adrenal cortex from secreting excess cortisol (Norman and Litwack, 1987; Nussey and Whitehead, 2001; Smith and Dobson, 2002). Elevated levels of cortisol exert negative feedback on the HPA axis that ensures the return of its activity to basal levels after stimulation (Matthews, 1998; Meyer *et al.*, 1998). This negative feedback is critical for the animal in preventing the damaging effects of chronically elevated cortisol such as suppressing inflammatory and immune responses (Manteuffel, 2002). Although previous reports on indigestible foreign bodies particularly plastic bags in the rumen of ruminants (Igbokwe *et al.*, 2003; Singh, 2005; Abebe and Nuru, 2011; Khurshaid *et al.*, 2013; Otsyina *et al.*, 2015) did not measure metabolites of stress hormone, they however reported that rumen impaction has adverse effects on the health of the animal. Present study reported mortalities in some of the wethers whose rumen were impacted with plastic bags, which concurs with that of Singh (2005) who stated that various conditions such as indigestible foreign bodies in the rumen are associated with deaths of the animals. Therefore, the surge of rumen impaction with indigestible materials especially among small ruminants should not be underrated.

It is important to note that the return of the high levels of FCM concentration to baseline values may not necessarily mean that stress has been resolved. Rather stress may still be present but the initial benefit of elevated cortisol restored the HPA axis back to baseline levels, or probably due

to an overworked less efficient adrenal gland (Selye, 1950) which could have resulted in the lower values. Probably measuring other biomarkers of chronic stress as well as the effect on immune function may be more informative.

CHAPTER SEVEN

7.0 OVERALL CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

The following conclusions were made from the present study:

a) The body weights of wethers decreased progressively with increasing duration of rumen impaction and the animals could subsequently die if the impaction persists.

b) Severe macroscopic and histological changes observed in rumen impacted with plastic bags included discolouration of the ruminal mucosa, obstruction of rumeno-reticular orifice, degeneration of ruminal papillae, stunted and bent over ruminal papillae, thin ruminal epithelial layer and thin ruminal walls.

c) Stereological evaluation of ruminal sacs of wethers whose rumen were impacted with plastic bags showed a decrease in surface density of absorptive mucosal surface and loss in volume density of mucosa, which were more pronounced with increased duration of rumen impaction. Other stereological parameters that decreased in a similar pattern were: mean surface area of the absorptive mucosa in the cranial sac, ventral sac and caudo-ventral blind sac, mean absolute volume of mucosa in the ruminal walls of cranial sac, dorsal sac, ventral sac, caudo-dorsal blind sac and caudo-ventral-blind sac.

d) The total mean volume of the mucosa in the entire impacted rumen volume was 17%, while that of the control groups was 30% each of their whole rumen volume.

e) The total mean surface area of the entire absorptive ruminal mucosa of the impacted rumen decreased by 40% over the whole period of rumen impaction with plastic bags.

f) The total mean absorptive surface area and total mean absolute volume of ruminal mucosa in the rumen of the controls were much higher than that of the impacted and this could reduce the ruminal functional efficiency in animals with rumen impaction.

g) The total surface area and absolute volume of tissues in the entire rumen of normal wethers without impaction was $0.473 \pm 0.017 m^2$ and $540 cm^3$ respectively.

h) Body-mass-standardized total surface area and total absolute volume of mucosa diminished further as duration of rumen impaction increased.

i) Rumen impaction induced acute stress in wethers, which was effectively indicated by increased levels of plasma cortisol concentration.

j) Increased faecal cortisol metabolite concentration is a positive indicator of stress reactions in rumen impaction.

k) Through measurements of plasma cortisol levels and faecal cortisol metabolites concentration, the current study is the first to positively demonstrate that impaction of the rumen induces stress in sheep, a fact that can probably be extrapolated to other ruminants.

All these mentioned altered parameters of the rumen would correspondingly interfere with rumen function and efficiency and subsequently affect the overall health of the animal. Depending on the persistence of rumen impaction and the extent of ruminal tissue damage, this leads to remarkable reduction in feed intake and digestibility that could ultimately result in the death of the animal.

7.2 Recommendations

The following recommendations are made from the present study:

a) Waste plastic bags can be devastating to small ruminant production and may subsequently affect the livelihoods of those who depend on these animals. Therefore, it is recommended that education and public awareness on the devastating effect of plastic bags and proper disposal of it should be carried out through seminars, media and other information dissemination methods.

b) Small ruminants and indeed animals in general should neither be let to scavenge on the waste dumping sites nor be grazed on plastic bag polluted road sides or pastures.

7.3 Further research

The possible areas of research that could help fill the gaps of knowledge are:

a) Stereological assessments of the effects of rumen impaction on papillae numbers and ruminal epithelial cells. This should not only be impaction with indigestible materials but also excessive digestible feeds that over-engorge the rumen for a prolonged period.

b) Ultrastructural investigation of the effects of rumen impaction on ruminal epithelial cells.

c) Estimation of other biomarkers of chronic stress and biomarkers of collagen biosynthesis and degradation that were not evaluated in the current study and its overall effects on other body systems and functions due to rumen impaction.

d) Investigation of the effect of rumen impaction with indigestible pastic bags on microbial activity and volatile fatty acid production and absorption.

e) Estimation of toxic chemicals contained in plastics that leached out into the blood stream.

CHAPTER EIGHT

8.0 REFERENCES

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APPENDICES

Appendix 1

Work flow of STEPanizer stereological software used to upload digitally captured images of histological sections of ruminal tissues for stereological estimations.

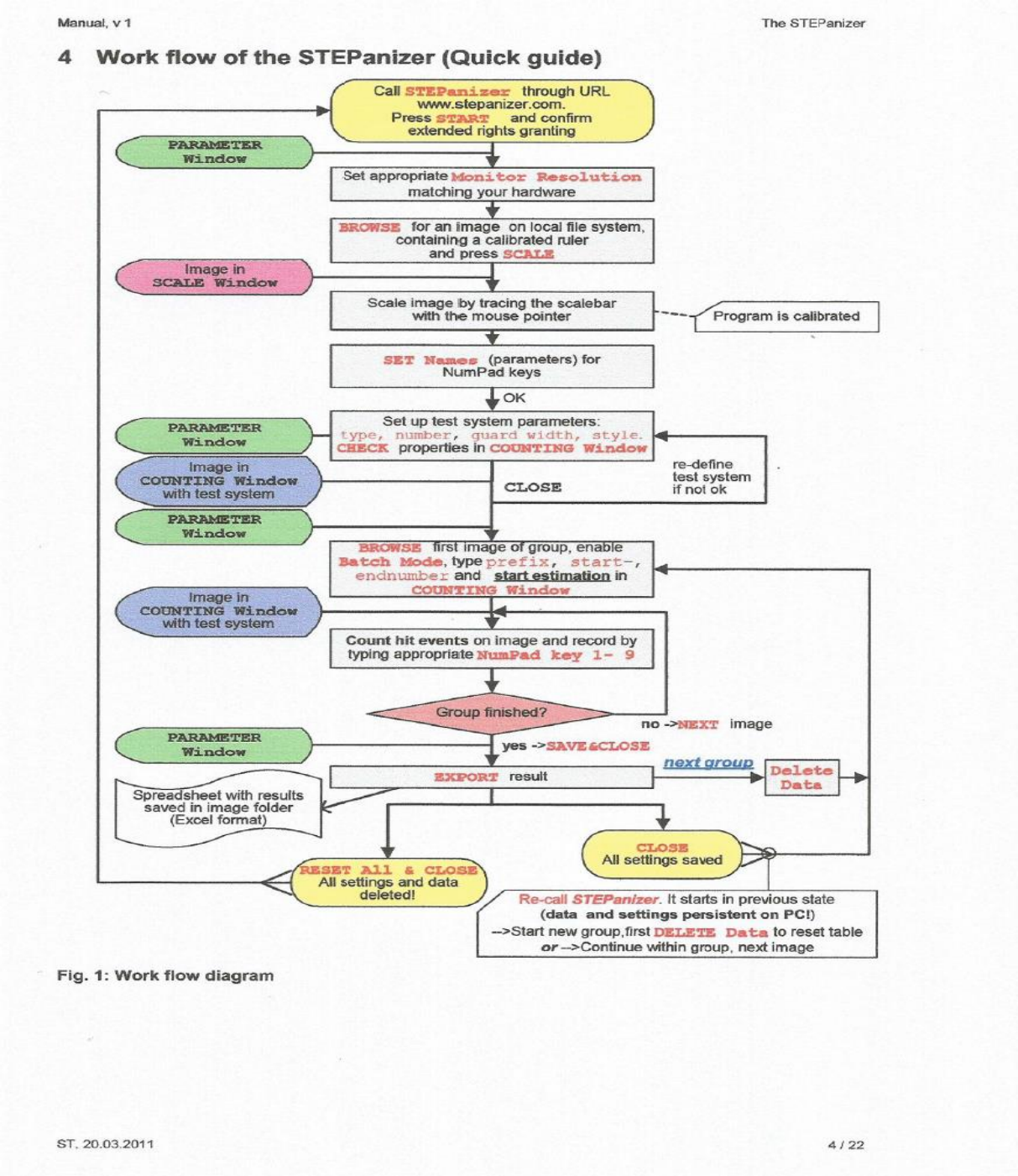

Fig. 1: Work flow diagram

Appendix 2

ETHICAL APPROVAL

Ethical approval for this research work was granted by the Biosafety, Animal use and Ethics Committee of the Faculty of Veterinary Medicine, University of Nairobi, Kenya.

UNIVERSITY OF NAIROBI
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Ms Ann Nancy Etsey
c/o Dept of Vet. Anatomy and Physiology

03/04/2014

Dear Ms Etsey,

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

Structural and physiological changes in selected organs in rams and bucks implanted with plastic bags in the rumen

By Ann Nancy Etsey

We refer to the above proposal that you submitted to our committee on 18th March 2014. We have now reviewed your proposal and are satisfied that the animals will be handled humanely as per the proposed methods. Furthermore we are satisfied that the numbers proposed are necessary for the study.

We hereby approve your study as per your proposal.

Yours sincerely

Rodi O. Ojoo BVM, M.Sc, Ph.D
Chairman, Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine.

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