INFLUENCE OF GRAZING INTENSITY ON CYANOGENIC TOXICITY IN SAVANNA GRASSES IN BARINGO, KENYA

ALFAYO KOSKEI

A THESIS SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN NATURAL RESOURCES MANAGEMENT OF EGERTON UNIVERSITY

EGERTON UNIVERSITY

JUNE, 2016

DECLARATION AND APPROVAL

DECLARATION

This thesis is my original work and has not been submitted or published for any award of a degree or diploma in this university or any other university.

Signature _____ _____

Alfayo Koskei NM 11/3665/13 Date

APPROVAL

This thesis has been presented with our approval as university supervisors.

Dr. Elias K. Maranga (PhD.)

Department of Natural Resources

Faculty of Environment and Resource Development

Egerton University

Signature _____ _____

Date

Dr. Obonyo Meshack (PhD.)

Department of Biochemistry

Faculty of science

Egerton University

Signature _____ _____

Date

ACKNOWLEDGEMENT

I gratefully acknowledge the professional support and mentorship of Dr. E.K.Maranga, Department of Natural Resources and Dr. M. Obonyo, Department of Biochemistry, Egerton University in the development of this thesis, from proposal phase through research phase and writing up of this thesis. Iam sincerely grateful to Dr. E.K.Maranga for field supervision and assistance with field research designs and Dr. M. Obonyo for allowing me to use the laboratory facilities of the Department of Biochemistry for my laboratory tests. I would like to give a special mention to Dr. Kirui, Department of Natural Resources who has been part of this project, reading and providing invaluable comments especially during concept note development and data analysis procedures.

All fieldwork was made possible by the Dryland Grant Research Fund, through the Division of Research and Extension of Egerton University. Prof Kibor coordinated this award, enabling research to run smoothly. I wish to register my appreciation for this noble gesture.

To all the project facilitators, field assistance and all the lecturers in Department of Natural Resources who contributed in one way or another in the preparation of this work; I would like to extend a big thank you. To Barsosio Abraham, thank you for being there at every step during my field work, am deeply humbled by the assistance you offered.

COPYRIGHT

DEDICATION

To my most supportive and loving mum Elizabeth Maina and my siblings Geoffrey, Kenneth, Janet, Emmy and Lily for their compassionate and endless support.

ABSTRACT

The potential role of anti-herbivory mechanisms involving use of qualitative and quantitative compounds in minimizing herbivory is well known. However, synergistic responses to grazing and interactive effects on herbivores are poorly understood. The aim of this study was to quantify the interaction between cyanogenic glycosides in grasses with cattle grazing in Lake Bogoria (00°28'N, 35°59'E), Baringo County. Field experiments were carried out in ten 50×10m exclosures to quantify influence of grazing intensity and age of grasses on cyanogenic concentration. Grazing intensity was varied using simulated grazing method to determine the effect of grazing disturbance on cyanide concentration. Two grazing treatments which embraced heavy grazing (Clipping at 5cm height) and light grazing (clipping at 15cm height). Grasses were also categorized into two age classes; young (leaf blade length <2cm, no florescence and spikelet) and old (leaf blade length >2cm, florescence open and spikelet presence). Grass samples were tested for cyanogenic glycosides using impregnated picrate paper and concentration of the cyanogenic glycosides was determine by hydrolyzing the glycosides and trapping the cyanide evolved in 1M NaOH. The findings showed that five species out of 16 produced cyanogenic glycosides; *Cynodon dactylon, Cynodon plectostachyus, Digitaria scalarum, Sporobolus spicatus and Cyperus laevigatus.* Cyanogenic glycosides decreased with increase in age of plants. Young cuttings yielded more Hydrogen Cyanide than older cuttings of the same grasses though there were no significant difference (p-value>0.05 for all species). For example the concentration levels in young and old materials of *C. dactylon* 1.89 and 1.74 MgCN g^{-1} dw, *C. plectostachyus* 1.42 and 1.32, *S. spicatus* 1.26 and 1.17, *C. laevigatus* 1.24 and 1.47 and *D. scalarum* 1.21 and 1.13 MgCN g^{-1} dw. Grazing intensity had a significant effect on concentration of cyanogenic content in *C. dactylon* (1.14 and 1.50 MgCN g^{-1} dw**,** p-value<0.005) and *C. laevigatus* (1.27 and 1.58 MgCN g^{-1} dw) for control and second clipping respectively while the effect on others were not significant p>0.05 such as *C. plectostachyus* (1.23 and 1.42) *S. spicatus* 1.30 and 1.34) *D. scalarum* 1.22 and 1.28 and *C. laevigatus* (1.32 and 1.58 MgCN g^{-1} dw). On the basis on the findings, it is recommended that grazing regime of managed pastures should consider the age of pastures while allowing utilization of pastures preferably grazed on mature pastures with low levels of cyanogenic glycosides. Consequently the study recommends a response to a less grazing pressure on pastures and apparently avoid overstocking on managed pastures as the forage items concentrate relatively more defense glycosides with increase in disturbance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ACRONYMS

ATP	: Adenosine Triphosphate
CNglcs	: Cyanogenic Glycosides
CN	: Cyanide-Nitrogen covalence combination
HCN	: Hydrogen Cyanide
HG	: Heavily Grazed
LG	: Light Grazed
NaCN	: Sodium Cyanide
PH	: Potential of Hydrogen
RCBD	: Randomized Complete Block Design
SPPs	: Secondary Plant Products

CHPATER ONE

INTRODUCTION

1.1 Background Information

Cyanogenic glycosides are a group of plant secondary compounds that contain nitrogen and yields cyanide (cyanogenesis) following their enzymatic breakdown. Natural sources of cyanide, include bacteria, plants and fungi which synthesize and secrete cyanide but the most significant sources of cyanide in the environment are from industrial wastes which enters the soil through solution with rain water and infiltration (Woodrow, Slocum and Gleadow, 2002). Certain plants absorb cyanide in the form of nitrogen and their products such as alkaloids and glycosides contain cyanide.

Cyanide has played a primary role in the evolution of life on earth and remains an important form of nitrogen for plants, microorganisms and fungi (Ubalua, 2010). It is also used in industrial production and natural products. Some of the uses include; measurement of ketone bodies, lowering of blood pressure and applications in vascular research, pest control, fishing industry, jewelry and as a component in the manufacture of food additives. Plants in prunus genera and many other plants known to contain cyanogen glycosides are consumed as food as well as herbal medicines. About ten cyanogenic glycosides including amygdalin, prunasin, dhurrin, linamarin, and taxiphyllin have been reported in edible plants (Francisco and Pinotti, 2000; Vetter, 2000). However when cyanide found naturally in plants is in high concentrations it is toxic and causes poisoning (Ramirez and Barry, 2005).

There is strong evidence that cyanogenesis is one of the mechanisms that can serve the plant as a protective device against predators such as herbivores (Gleadow and Woodrow, 2002) and storage of nitrogen in nitrite form. The co-evolution between plants, herbivores and pathogens may have given some fungi and insects the ability to overcome the defense system based on cyanogenic glycosides, either by their ability to transform the compounds into non-toxic constituents or by sequestration and further use in their own defense (Zagrobelny *et al.*, 2008). The level of cyanogenic glycosides produced is dependent upon the age and variety of the plant, as well as

environmental factors (Engler *et al.,* 2000; Ubalua, 2010). The presence and concentration of cyanide in plants vary greatly (Cho *et al.,* 2013). In their study (Cho *et al.,* 2013) they found that the difference between cyanogen content in the samples of the single species obtained from different locations vary greatly reflecting the variations of cultivation, harvest and or storage. This variation is attributed to soil, climate and species, as different species have different absorptivity rate of nutrients in soil.

Grasses are known to produce an array of secondary metabolites, such as hydroxamic acids (Niemeyer, 1988), condensed tannins (Bernays *et al.,* 1989), cyanogenic glycosides (Jones, 1998) and alkaloids, albeit at levels much lower than dicotyledons (Zagrobelny *et al.,* 2008; Vicari and Bazely, 1993). Secondary metabolites found in grasses have been shown to have adverse effects on the performance of rodents when consumed in artificial diets (Jane *et al.,* 2014). However, only rarely have these compounds been demonstrated to have measurable negative impacts on free-ranging cattle herbivores at the concentrations in which they naturally occur in grasses. Exceptions again involve livestock; cyanogenic glycosides, poisoning cattle (Georgiadis and McNaughton, 1988) and indole alkaloids reducing the palatability of grass for sheep (Simons and Marten, 1971; Marten *et al.,* 1973). Hydrogen cyanide derived from cyanogenic glycoside can cause health concerns including cell death by blocking cytochrome oxidase and the arrest of the ATP production. Certain plant species synthesize cyanogenic glycosides and cyanolipids[1] which when disrupted by grazing are hydrolyzed and in the process liberate Hydrogen Cyanide (HCN) as shown in the equation below

$$2H_2O_{(l)} + 4CN \xrightarrow{\text{(Grazing)}} 4HCN + O_{2(g)}$$

(Dew in grass)

Poisonous Gas

Equation 1: Hydrogen Cyanide liberation process

Production of cyanide is thought to be due to the presence of cyanogenic glycosides that release HCN (hydrogen cyanide) when acted upon by enzymes found within plant cells (Francisco and

[1] Components of the lipids of seeds in the family Sapindaceae mainly, although some are known from the Hippocastaneaceae and Boraginaceae, where they occur with conventional acylglycerols.

Pinotti, 2000). Thus, it appears that HCN does not naturally occur in the free state, but is produced after the plant cell is crushed or disrupted, allowing one or more specific enzymes to make contact with the cyanogenic compound and release HCN. In the intact plant, the enzyme and the cyanogenic glycoside remain separated, but if the plant tissue is damaged both are put in contact and cyanohydric acid is released (Siritunga and Sayre, 2004)

When cattle, goats, sheep and other domestic animals graze early in the morning in some pastures cases of bloating are witnessed. This is attributed to the Hydrogen cyanide gas which is a result of dew which interacts with cyanogenic glycosides, cyanolipids and grazing activity. Grasses are highly tolerant to grazing by means of their rapid regrowth capacity, their underground storage structures, basal meristems and tillering capacity (Dyer *et al.*, 1991; Karban and Baldwin, 2007). Indeed, grasses have been considered to primarily rely on these tolerance traits in lieu of physical or chemical defenses to mitigate damage caused by herbivory. However, a number of mechanisms have been identified in grasses that may deter feeding by grazers, and hence act as defenses (Vicari and Bazely, 1993). The most prominent of these is enhanced silicon (Si) uptake in response to damage (McNaughton and Tarrants, 1983; Massey *et al.*, 2007a).

Timing of grazing affects plants more severely at certain stages of their development than other stages. For example the most critical grazing period is usually from flowering to seed production although the least critical period is dormancy. Research and demonstration work have shown that removing high quantities of forage during dormancy is almost as detrimental to plant productivity as during active growth periods (Briske and Richards, 1994). The actual level of cyanogenic glycosides of a cyanogenic plant is influenced by various factors, both developmental (endogenous) and ecological (exogenous). The development cycle of cyanogenic plants shows characteristic changes in cyanogenic glycoside (and HCN) content (Vetter, 2000). Cyanide can be present in environmental matrices and waste streams in various simple cyanides (e.g. HCN, CN, NaCN), metal cyanide complexes, cyanates and nitriles (Ebbs, 2004). Other environmental variables that influence the presence, levels of cyanogenic glycosides in plants and absorption include soil type, physical and chemical characteristics of soil such as porosity, bulk densities, drainage and soil pH, water holding capacity etc. air temperatures and precipitation; a factor of water stress (Cho *et al.*, 2013; Engler *et al.*, 2000; Ubalua, 2010)

1.2 Statement of the Problem

Like other chemical cycles such as Nitrogen, Carbon or Phosphorus, Cyanide enters the food chain through soil and is taken up by plants during physiological processes and eventually passed on to animals. The biological pathways for the transfer of cyanide in soil through plant physiology and metabolism to animals is not yet elucidated in savanna grasses. Production of cyanide is thought to be due to the presence of cyanogenic glycosides that release HCN (hydrogen cyanide) when acted upon by enzymes found within plant cells. There is evidence that HCN does not occur naturally in nature, but as a result of disruption of plant parts to allow certain enzymes to be in contact with cyanogenic glycosides. Cyanogenic glycosides are HCN-producing phytotoxins; which predispose livestock to toxicity. In the study areas cases of acute poisoning and bloating in when animals feed on wet vegetation in mornings or after rains have been reported. Savanna grasses synthesize cyanogenic glycosides. However limited information is available on grass species that synthesize cyanogenic glycosides and effects of grazing regimes that are associated with disruption of plant parts to allow certain enzymes to be in contact with cyanogenic glycosides. Additionally influence of physiological capacity of grass as a result of age variation on cyanide occurrence and concentration in grasses have not yet been elucidated. This study seeks to identify savanna the grasses which synthesize cyanide and attempt to elucidate the biological pathways that link cattle grazing with cyanogen toxicity associated with these grasses in Kenya.

1.3 Objectives of the Study

1.3.1 Broad objective

To study the influence of grazing intensities on cyanogenic concentrations in savanna grasses in drier habitats in L. Bogoria, Baringo County.

1.3.2 Specific objectives

i. To determine the concentration levels of cyanogenic glycosides in savanna grasses
ii. To establish the relationship between concentrations of cyanide and age of grasses
iii. To determine the effect of grazing intensity on the concentration of cyanide.

1.4 Research Hypotheses

i. The levels of cyanogenic glycosides do not vary within species in savanna grasses
ii. The age of grasses do not affect the concentration levels of cyanide in grasses.
iii. Grazing intensity has no effect on the concentration of cyanide in grasses

1.5 Justification of the Study

Since it is highly desirable that the toxicity of cyanogenic plants to humans and livestock be reduced, research is needed to identify the grasses species with low-cyanogens or non-cyanogenic for grazing purposes. An understanding of the mechanisms of interaction between grazing (defoliation) intensity and cyanogenesis is necessary in resolving the problem of toxicity associated with cyanide. The experiment to establish if there is relationship between cyanogenic glycosides and livestock poisoning clearly establish the linkages between cyanide to poisoning of livestock. The aim of this work was to detect cyanogenic glycosides in grass species in the savanna grasslands, Baringo ecosystems and to simulate the effect of age of grasses and grazing intensity on the availability and concentration of cyanide. An understanding of the relationship between age of grasses and concentration of cyanide will provide information for development of grazing regimes to ensure low cyanogenic bio amplification in food chain.

1.6 Assumptions of the Study

The study assumed that other factors (apart form age, species and grazing disturbance) that affects cyanogenesis such as terrain, climate, and rainfall, and soil, waste disposal, farming practices, animal husbandry and eco-zones affect all grasses uniformly and do not form part of the variation. The study also assumed that the grasses under the survey had the same absorptivity of nutrients.

1.7 Scope of Study

The study was carried out in Marigat, part of Baringo ecosystem within Lake Bogoria National Reserve for a duration of six months. The study covered only savanna grasses enclosed within ten plots of each 50m by 10m. The study covered the influence of three variables in the synthesis and levels of cyanogenic glycosides; age, species and grazing intensity. All the species of grasses

enclosed in the quadrat was sampled excluding herbaceous plants not related to grass family in the first experiment

1.8 Limitation of the Study

The method of determination of cyanide presence was insufficient to qualify the types of cyanogenic glycosides. The method only identified the presence of cyanogenic glycosides and the concentration but could no single out the types. The sampling strategy employed in collecting grass species was simple random sampling and for successive experiments was purposive sampling. Simple random sampling may not have been adequate to sample plants in areas of one species dominance or with great diversity since it gives no preference to specific species. Moreover, grazing intensities was classified only into two levels but other levels are also possible.

1.9 Definition of Terms

Acyanogenic plants **:** Plants that do not metabolize cyanogenic glycosides and where the function of cyanogenesis is not revealed through their phenoltypic characteristics

Cyanogenesis **:** Is the ability of some plants to synthesize cyanogenic glycosides, which when enzymically hydrolyzed, release Cyanohydric acid (HCN) known as prussic acid

Cyanogenic glycosides **:** The precursor of cyanide in many plants, arthropods and some bacteria are amino acid-derived

Cyanogenic polymorphism: An adaptive mechanism for plant species where a plant metabolize multiple cyanogenic forms within same species.

Cyanohydrin Is the an unstable hydroxynitrile intermediate, acetone that is yield by hydrolysis of linamarin

Cyanolipids : Are components of the lipids of seeds in the family *Sapindaceae* mainly, although some are known from the *Hippocastaneaceae* and *Boraginaceae*, where they occur with conventional acylglycerols.

Grazing intensity **:** Refers to the amount of grass or browse material that is consumed by grazers

Phagostimulant **:** Any material that stimulates the production of phagocytes

Phytoanticipins **:** Is one of the two Classes of Plant Antibiotics

Toxicity **:** Term for the property of substances indicating the degree to which a compound can damage a cell or organism; it is dose dependent and measured by the effect on the p articular target

CHAPTER TWO

LITERATURE REVIEW

2.1 Cyanogenic Glycosides in Plants

Cyanide played a primary role in the evolution of many life forms on earth and remains a significant form of nitrogen for plants, fungi and microorganisms (Woodrow et al., 2002). Many scholars have hypothesized that plants, herbivores and pathogens may have co-evolved in a constant chemical warfare for about 430 million years, thus plants do not rely on a single defense mechanism, but rather express multiple defenses comprising the constitutive and induced synthesis of many chemical compounds as well as the production of structural traits (Agrawal, 2006; Kursar, 2003). Presumably, such an amalgamation of different traits may have led to the evolution of multiple defense syndromes, since the association with specific ecological interactions results in co-variation of defensive traits

The co-evolution between plants, herbivores and pathogens may have afforded some insects and fungi the ability to overcome the defense system based on Cyanogenic glycosides (CNglcs), either by their ability to transform the compounds into non-toxic constituents or by sequestration and further use in their own defense (Bak *et al.,* 2006). Consequently, this evolutionary path is supported by the fact that ancestral angiosperms like Magnoliales contain tyrosine-derived cyanogenic glycosides (Buhrmester *et al.,* 2000). Hence within monocotyledons, Liliales are known to contain aromatic cyanogenic glycosides, and within Poales both aromatic and aliphatic cyanogenic glycosides occur.

Bak *et al.,* (2006) further proposed that the widespread occurrence of cyanogenic glycosides in nature implies that they are ancient biomolecules in terrestrial plants and that the specific presence of aromatic cyanogenic glycosides in ferns and gymnosperms indicate that the cyanogenic glycosides initially in nature were aromatic and that these served as progenitors for aliphatic cyanogenic glycosides. Interestingly, in eudicota wide distribution of aromatic as well as aliphatic cyanogenic glycosides is observed, but the amino acid precursor used within a given family is generally cones.

As part of co-evolutionary origins, cyanogenesis has been extensively studied in some bacteria. Amongst them are the fluorescent pseudomonads, especially *Pseudomonas flourescens* and *Pseudomonas aeruginosa* (Gallagher and Manoil, 2001). Cyanogenesis has also been reported in *Chromobacterium violaceum* and has often been reported to occur in the case of cyanobacteria such as *Anacystis nidulans*, *Nostoc muscorum* and *Plectonema boryanum* (Olsen *et al.,* 2013. Some strains of *Rhizobium leguminosarium* have also been reported to produce cyanide as free-living bacteria (Goodger *et al*. 2006).

Cyanogenic glycosides are widely distributed among 100 families of flowering plants (Francisco and Pinotti, 2000). They are also found in some species of ferns, fungi and bacteria (Harborne, 1972, 1993). There are many economical important plants highly cyanogenic, including white clover, linum, almond, sorghum, the rubber tree and cassava. According to Bak *et al.,* (2006) cyanogenesis, the ability of organisms to produce hydrocyanic acid (HCN), is widespread in the plant kingdom. This ability has been reported in approximately 1000 plant species, but the identity of the cyanogenic compound(s) has been established for less than 100 of these species

Bak *et al.,* (2006) documented evidence of CNglcs in more than 2,650 higher plant species distributed among 130 families in pteridophytes (ferns), gymnosperms and angiosperms (Conn, 1998; Siegler and Brinker, 1993) implying that in plants the ability to synthesize cyanogenic glycosides is at least 300 million years old. Since its first description in plants in 1803, the phenomenon of cyanogenesis has been recognized in over 3000 species of higher plants distributed throughout 110 different families of ferns, gymnosperms, and both monocotyledonous and dicotyledonous angiosperms (Ramirez and Barry, 2005). *Trifolium repens*, *Lotus tenuis*, and *L. corniculatus* are among several plant species which are polymorphic for the cyanogenic character.

It is estimated that between 3,000 and 12,000 plant species produce and sequester cyanogenic glycosides, including many important crop species (Guilloton *et al.,* 2002; Vetter, 2000) such as sorghum, almonds, lima beans (non-domesticated), and white clover. Cyanogenesis is also known in animals, but is restricted to the arthropods, notably to certain centipedes, millipedes, and insects. In fungi and bacteria, HCN may originate via oxidative decarboxylation of glycine (Gleadow and Woodrow, 2002). The grass in genus *Cynodon* is a successful widespread grass in African grasslands. Grasses in this genus are known to produce cyanide (Gleadow and Woodrow, 2002).

Many economically important food plants are highly cyanogenic and have caused numerous cases of acute cyanide poisoning of animals including man. Additionally, in areas of the world where cyanogenic plants such as cassava and lima beans comprise the major item of the diet, chronic cyanide poisoning and associated pathological conditions still exist (Gleadow *et al.*, 2009; Gleadow *et al.,* 2008; Jonathan, 2009).

2.2 Age of Vegetation and Cyanide Concentration

Cyanogenic glycosides (CNglcs), the precursor of cyanide in many plants, arthropods and some bacteria are amino acid-derived β-glycosides of α-hydroxynitriles. Howe and Noble, (2005) carried out an experiment that showed cyanide concentrations in *Tanacetum vulgare* were variable, but the plant appeared to concentrate cyanide where soil concentration increased. The ability to concentrate cyanide may be related to plant age; i.e., younger cuttings tended to yield more HCN than older plants taken from the same cyanogenic soil (Howe and Noble, 2005).

Generally, the level of cyanogenic glycosides produced is dependent upon the age and the variety of the plant, as well as environmental factors (Francisco Pinotti, 2000; Ubalua, 2010). More than 60 different CNglcs are known to be present in more than 2,500 plant species including ferns, gymnosperms, and angiosperms (Bak *et al.*, 2006; Buhrmester *et al.*, 2000; Seigler *et al.*, 2002) and it is not common to find cyanogenic and acyanogenic plants within the same species, where the function of cyanogenesis is revealed through their phenoltypic characteristics (Francisco and Pinotti, 2000)

Cyanogenic glycoside concentration also varies with leaf age and environmental conditions (e.g., Massey et al. 2007b; Bernays *et al.*, 1977; Gleadow *et al.*, 1998; Gleadow and Woodrow, 2000). A particular plant may be innocuous under one set of conditions, but lethal under another (Bernays *et al.*, 1977). Studies that show little or no influence of cyanogenic glycosides on feeding preferences could be testing plants that are essentially nontoxic to the herbivore. This has been particularly well documented in sorghum, which is highly toxic to grazing stock when young, but becomes suitable for pasture as plants mature (Burns *et al.* 2002). Gleadow and Woodrow (2000a), for example, detected a significant inverse correlation between the concentration of cyanogenic glycosides in the young leaves of *Eucalyptus cladocalyx* and the amount of damage by herbivores. Similarly, Schappert and Shore (1999) found that the concentration of cyanogenic glycosides was

important not only in the selection of *Turnera ulmifolia* as a source of food, but also as an oviposition site for *Euptoieta hegesia*.

CNglcs may accumulate in all parts of a plant [e.g., as in cassava (McMahon *et al.*, 1995)], only in the aboveground parts [e.g., as in Eucalyptus (Gleadow and Woodrow, 2000) and white clover (Hughes, 1991; Stochmal and Oleszek., 1997)], or only in vegetative tissues [e.g., as in sorghum (Gleadow and Woodrow, 2000)]. In plants that synthesize more than a single CNglc, the ratio between the different types of CNglcs may differ in above- and belowground parts. This pattern may vary with reproductive stage as well. Some *T. ulmifolia* populations, for example, lose their cyanogenic capacity around flowering, whereas others do not (Schappert and Shore, 2000). Vegetative and reproductive tissues of plants may also contain different types of CNglcs, and the content in roots may differ from that in shoots. Most Prunus species contain prunasin in the leaves but store the related diglucoside, amygdalin, in the seeds (Sanchez-Perez, 2012). *H. brasiliensis* also contains a monoglucoside (linamarin) and a diglucoside (linustatin) (Sanchez-Perez, 2012), which allows CNglcs to be transported within the plant as linustatin without the risk of being degraded by the β -glucosidase specific for the monoglucoside (Sanchez-Perez, 2012).

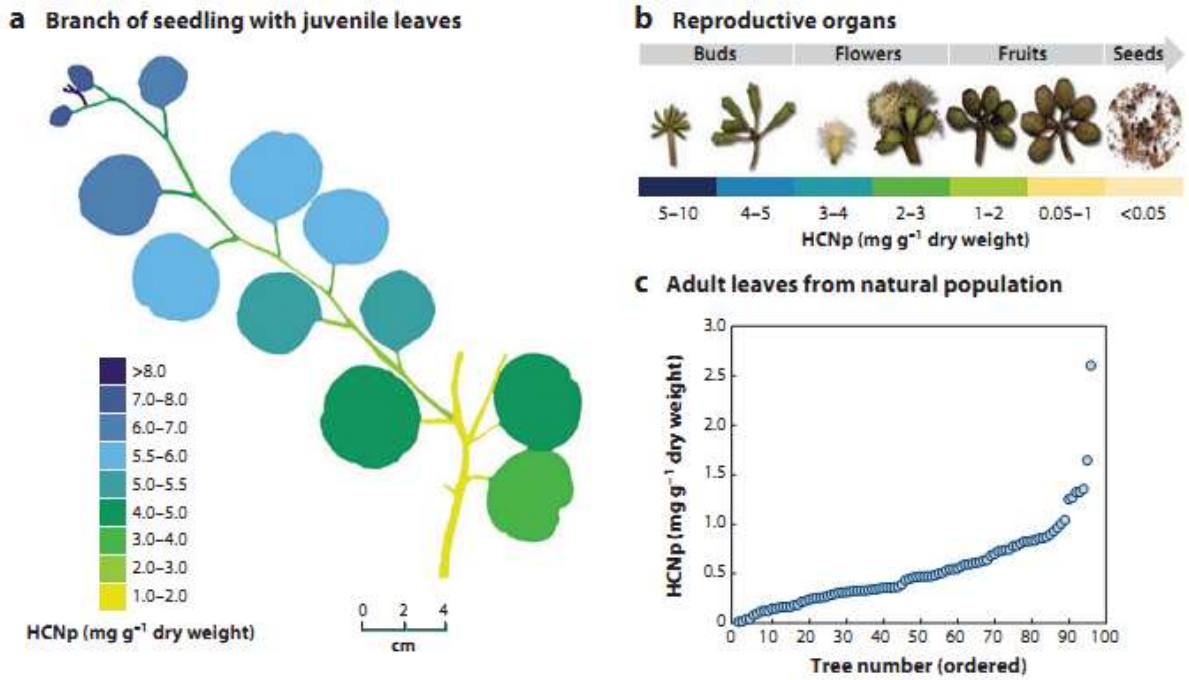

Figure 1: Variation in cyanide potential (HCNp) in *Eucalyptus cladocalyx*.

(a) Branch of a seedling, showing the decrease in HCNp with leaf age in a natural tree population from Kangaroo Island, South Australia. Panels a and b adapted from Reference 69; panel c adapted from Reference 68.

2.3 Cyanide Toxicity

Cyanogenesis, the ability of plants and other living organisms to release hydrogen cyanide, has been known for several centuries. Most cyanogenic glycosides are derived from the ten hydrophobic protein amino acids tyrosine, phenylalanine, valine, leucine, and isoleucine. Upon tissue disruption, cyanogenic glycoside degradation is initiated by cleavage of the carbohydrate moiety by one or more, B-glycosidases, yielding the corresponding cyanohydrin (Massey *et al.,* 2007b). This intermediate may decompose either spontaneously or enzymically in the presence of a-hydroxynitrile lyase to release HCN and an aldehyde or ketone (Selmar *et al.,* 2004). This view is supported by mixed enzyme incubations in which various ratios of *hydroxynitrile lyase* to (3-glucosidase were analyzed for rapidity of HCN evolution (Li *et al.,* 2008). As with other secondary products, cyanogenic were originally viewed as excretory substances (18), but their turnover (seasonal and even diurnal) argues strongly against this hypothesis.

Symptoms of consumption of cyanogen containing foodstuff including vomiting, nausea, dizziness, weakness and occasional death. Chronic intake has been linked to goiter especially in iodine deficiency cases (Cardoso *et al.,* 2005). Cyanide is highly toxic for most living organisms because it forms very stable complexes with transition metals that are essential for protein function, i.e., iron in cytochrome oxidase (Quinn *et al.,* 2014). Consequently, organisms growing in the presence of cyanide must have a cyanide-insensitive metabolism, such as the alternative oxidase described for plants (Berthold *et al.,* 2000) or the cytochrome (or cyanide insensitive oxidase) in bacteria (Massey *et al.,* 2007b)

Cyanohydric acid is extremely toxic to a wide spectrum of organisms, due to its ability of linking with metals (Fe^{++}, Mn^{++} and Cu^{++}) that are functional groups of many enzymes, inhibiting processes like the reduction of oxygen in the cytochrome respiratory chain, electron transport in the photosynthesis, and the activity of enzymes like catalase, oxidase (Cho *et al.,* 2013; Ubalua, 2010). Hydrogen cyanide (HCN) inactivates the enzyme cytochrome oxidase in the mitochondria of cells by binding to the Fe3+/Fe2+ contained in the enzyme (Francisco and Pinotti, 2000). This

causes a decrease in the utilization of oxygen in the tissues. Cyanide causes an increase in blood glucose and lactic acid levels and a decrease in the ATP/ADP ratio indicating a shift from aerobic to anaerobic metabolism. Cyanide also activates glycogenolysis and shunts glucose to the pentose phosphate pathway decreasing the rate of glycolysis and inhibiting the tricarboxylic acid cycle. Hydrogen cyanide will reduce the energy availability in all cells, but its effect will be most immediate on the respiratory system and heart (Massey *et al.*, 2007b)

The potential toxicity of a food produced from a cyanogenic plant depends on the likelihood that its consumption will produce a concentration of hydrogen cyanide (HCN) that is toxic to exposed humans (Cho *et al.*, 2013). Factors important in this toxicity are: (i) the plant may not be sufficiently detoxified during processing or preparation and, therefore, HCN may remain in the food; (ii) if the plant is consumed raw or insufficiently processed, HCN may be released in the body, until the low pH of the stomach deactivates the β-glucosidase enzyme (Cho *et al.*, 2013; Massey *et al.*, 2007b). Cyanide ingested by release from a plant containing cyanogenic glycosides, either prior to or following consumption, follows the known cyanide metabolic pathway for humans and animals.

2.4 Cyanogenic Glycosides-Environment Interaction

It is usual to find cyanogenic and acyanogenic plants within the same species, where the function of cyanogenesis is revealed through their phenotypic characteristics. This is due to the variation in factors such as edaphic, climate and intensity of defoliation. Cyanide can be present in environmental matrices and waste streams in various simple cyanides (e.g. HCN, CN, NaCN), metal cyanide complexes, cyanates and nitriles (Ebbs, 2004). Soil as a weathered structure does not naturally generate cyanides nor does it contain cyanides, it only supports the growth of plants, microorganisms, and other intimate soil life. Cyanide may enter to the soil through anthropogenic activities such as use of fertilizers and dumping of industrial products. The loading rate in soil is the chief factor determining toxicity to microorganisms or hazard for movement into groundwater and food chain in plant and animal life (Francisco and Pinotti, 2000). Mobility of cyanide in soils is mostly influenced by volatilization and distribution. However, the rate of volatilization from soils is complex and depends on many factors

Aquatic grasses showed small increases of cyanide in areas where HCN was present in soil; however, the same stage of grass maturity was not always available, nor were stream conditions always the same; this may confound resulting data, as (Gleadow *et al.,* 2009) has shown that arrow grass growing on drier sites is higher in cyanide than that which grows in wetlands. In the research done by Howe and Noble (2005) on vegetation bordering a black hills stream South Dakota, the data show that a relationship may exist between cyanide in soil and its uptake by certain ambient plants. Dandelions growing along the creek, and to some extent *Tanacetum vulgare*, increased in HCN potential when HCN increased in soil. Dandelions seem to indicate the presence of cyanide in soil (Table 1) and they do this more effectively than do grasses or *Tanacetum vulgare*. Dandelions taken from the control site did not have detectable levels of HCN, but those from cyanogenic soil produced it. Since statistical evidence does not show high correlation, follow-up studies were done in the laboratory (Howe and Noble, 2005). Young plants were harvested from non-cyanogenic soil and their roots placed in cyanide solutions of varying concentration; the leaves were tested for HCN three days later and results are as shown in Table 1. The results shows that there were more levels of cyanogenic glycosides in plants as NaCN increase in soils.

Table 1: Concentration of cyanide in soil and plants

Concentration of NaCN solution, ppm	Average Total Cyanides in Dandelions, ppm
0.5	0.2
5.0	0.2
500.0	11.5
5000.0	373.9

Source: (Howe and Noble, 2005)

There is evidence that the use of certain fertilizers increases cyanide producing potential in sorghum grass; however, when HCN was given to such plants, it was metabolized, but not into cyanogenic compound (Jonathan, 2009). Production of cyanide is thought to be due to the presence of cyanogenic glycosides that release HCN (hydrogen cyanide) when acted upon by enzymes found within plant cells (Ramirez and Barry, 2005). Thus, it appears that HCN does not naturally occur in the free state, but is produced after the plant cell is crushed or disrupted, allowing one or more specific enzymes to make contact with the cyanogenic compound and release HCN.

In general, plants supplied with high levels of nitrogenous fertilizers (ammonia or nitrate) have an increased content of CNglcs. Highly fertilized fields of forage sorghum, for example, can sometimes become toxic to livestock (HCNp > 600 ppm) (Wheeler *et al.,* 1990). A link between nitrogen supply and CNglc deployment has also been observed in legumes, where the rate of colonization by nitrogen-fixing rhizobia has been associated with higher concentrations of linamarin and lotaustralin and decreased herbivory in both clover (Rose *et al.,* 1996) and lima beans (Ballhorn *et al.,* 2013). Not all plants respond to nitrogen in this way. In a study by Busk & Møller, (2002), dhurrin concentration did not increase in very young seedlings grown at high levels of potassium nitrate. Although cyanide is ubiquitous in the environment, the highest environmental levels are found in the vicinity of combustion sources (automotive exhaust, fires, cigarette smoke and solid waste incineration); in waste waters from water treatment facilities, iron and steel plants, and organic chemicals industries; in landfills and associated ground water; and in areas of road salt applications and run off (Field *et al.,* 2006).

According to Ebbs, (2004) cyanide can be present in environmental matrices and waste streams as simple cyanides (e.g. HCN, CN-, NaCN), metal cyanide complexes, cyanates and nitriles. Moreover, Ebbs (2004) found out that the variation in concentrations of cyanogenic glycosides is as a result of genetic and environmental factors, location, season, and soil type. Climatic conditions and soil properties play an important role in determining concentration and residual effects of various biochemical for example transports of cyanide in soils are mostly influenced by volatilization and soil density and porosity (Ubalua, 2010). Accordingly, high volatility of cyanide and the action of soil microbes ensure that high levels of cyanide do not persist or accumulate in soil under natural conditions (Ganjewala *et al.,* 2010)

Climate variables play a role in formation of chemical complexes as well as intake by plants. The study done by Ebbs (2004) showed that arrow grass growing on drier sites is higher in cyanide than that which grows in wetlands. This illustrates that precipitation is facilitating the dissolution of chemicals and leaching process. Though cyanides may be absorbed by several materials, including clays and biological solids (Chatwin and Trepanowski, 1987 and Chatwin, 1989), existing data indicates that the rate of hydrogen and metal cyanide absorption in soils is not significant when compared with rates of volatilization and biodegradation (Callahan *et al.,* 1979). Cyanide must be present as hydrogen cyanide as in surface waters in order to volatilize from soils

(Higgs, 1992). However, the rate of volatilization from soils is complex and depends on many factors, including pH, cyanide solubility, hydrogen cyanide vapour pressure, free cyanide concentration, soil water content, soil absorptive properties, soil porosity, organic matter content, density and clay content and atmospheric conditions such as barometric pressure, humidity, and temperature (Chatwin and Trepanowski, 1987; Chatwin, 1989).

Cyanogenic plants frequently have a bitter taste that is known to deter herbivores (Nahrstedt, 1985) however, flavor may be modified by the presence of various organic acids (King and Bradbury, 1995) in the soil. Soil pH consequently influence the plant test and cyanogenic plants may taste bitter or less bitter in different soil pH (Kursar, 2003)). This claim is supported by Bernays *et al.*, (1977) who concluded that a particular plant may be innocuous under one set of conditions such as environmental factors (pH, soil porosity, density as well as moisture), but lethal under another conditions. Some cyanide may be released to the atmosphere and dispersed depending on the pH and redox of the soil environment. Cyanide (CN-), up to 200 ppm at least, is readily converted to fertilizer nitrogen in the soil (Ganjewala *et al.*, 2010).

Moreover, the mobility of cyanide compounds in soil depends on stability and dissociation characteristics of the compound, soil type, soil permeability, soil chemistry, and the presence of aerobic and anaerobic microorganisms (Ganjewala *et al.*, 2010; Higgs, 1992). Additionally, soils conditions that increase the mobility of cyanide include low pH, high negative soil charges, and low clay content. Neutral to alkaline pH, high clay content, high positive soil charges, and the presence of organic matter and iron or other metal oxides appear to increase the attenuation of cyanide in soils (Agrawal, 2006). Acetone cyanohydrin spontaneously decomposes to acetone and HCN at pH 5.0 or temperatures 35°C and can be broken down enzymatically by HNL (Chatwin and Trepanowski, 1987 and Chatwin, 1989). These environmental parameter as temperature and soil pH conversely influence the rapid production of Hydrogen Cyanide when plants containing these glycosides (linamari) is damaged or disturbed e.g. during grazing. Cyanides that enter the soil from the low-level natural sources are rapidly biodegraded and quickly metabolized by soil microorganisms. One of the important aspects of biodegradation is the optimization of the growth of the microorganisms involved in the process, in terms of pH, temperature, nutrient status, oxygen availability, population density and the presence of interacting inorganic and organic compounds. For example, strains of Alcaligens sp (Ganjewala *et al.*, 2010)

Temperature is an important parameter in the determination of the rate of biodegradation and different soil communities may have dissimilar temperature optima (Baxter and Cummings, 2006). Populations in the upper layers of soil are exposed to varying temperatures, due to fluctuations throughout the day and seasonal changes, whereas populations in the soil subsurface are subjected to low temperatures with less fluctuation. The ability to degrade cyanides has been demonstrated by both eukaryotes and prokaryotes from a diverse range of taxa across a wide range of metabolic pathways (Baxter and Cummings, 2006).

2.5 Grazing Intensities and Cyanogenesis

Many natural products do not appear to participate directly in plant growth and development, and in many circumstances their roles are not well understood. Conventionally, these compounds are referred to as secondary plant products (SPPs) and certain families of plants exhibit great metabolic specificity in the SPPs they produce. Primary metabolites are generally found in all plants and are thought to play a vital roles in essential plant metabolic functions (Croteau *et al.,* 2000; Field and Jordan, 2006; Massey *et al.,* 2007b; Weston *et al.,* 2013) whereas SPPs play roles in chemical signaling and defense against grazing herbivores, insects, pests and even other plants (Field, *et al.,* 2006; Wink, 1999; Weston and Mathesius, 2013).

Grasses have been considered to primarily employ tolerance in lieu of defense in mitigating damage caused by herbivory. Weston and Hildebrand (2013) compiled evidence that demonstrated that after defoliation of some grasses by army worm and moths (*Spodoptera exempta and Lepidoptera spp)* levels of cyanide in leaves of *Cynodon plectostachyus* were sufficient to kill cattle, but larvae of *S. exempta* are not affected by cyanide. A reduced variance in cyanide concentration was found in the plants on highly grazed pasture (Cao *et al.,* 2013). Plants use chemical synthesis as reciprocal co-adaptation against herbivory that cause defoliation (Vulink *et al.,* 2000). This is possible from the taste derived; from precursor amino acid, that may be aromatic, aliphatic in nature and avoided by predators for bitter taste (Blaedal and Easty, 1971) Cyanogenesis may not necessarily be used for plant survival; it may take part in metabolic and excretory processes but there certainly is a characteristic of value for these species (Cao *et al.,* 2013; Jane *et al.,* 2013)

Given the well documented toxicity of HCN, a role in plant protection against herbivores, pathogens, and competitors is appealing. Much evidence, indeed, favors a defense function for cyanogenic against certain animals including insects (Seigler *et al.*, 2002). Species showing cyanogenic polymorphism have frequently been exploited to investigate such plant-herbivore interactions. In critically examining the literature, however, Mali and Borges, (2003) found few studies incorporating adequate statistical analyses or having addressed herbivore feeding specificity. A smaller subset of SPPs is associated with interactions between plants and their herbivores, which include grazing animals as well as insects and arthropods. Some of these compounds are critically important in the attraction and deterrence of herbivores, one form of chemical communication or signaling (Wink, 1999; Pickett, 2012; Berenbaum and Feeny, 1981)

Cyanogenic glycosides are a group of nitrile-containing plant secondary compounds that yields cyanide (cyanogenesis) following their enzymatic break down. The functions of cyanogenic glycosides remain to be fully determined in many plants; although in some plants they have been implicated as herbivore deterrents and as transportable forms of reduced nitrogen (Coley, 2003; Mali and Borges, 2003). Recent experiments have further accentuated the possibility that cyanogenic glycosides and cyanolipids might serve as nitrogen storage compounds. In *Hevea brasiliensis* seeds, the endosperm represents almost 85% of the seed dry matter and contains more than 90% of the cyanogenic glycoside, linamarin. During germination and plantlet development, the cyanogenic potential of the entire seedling declines by 85% as cyanogenic compounds are metabolized to non-cyanogenic substances and negligible amounts of gaseous HCN are liberated during this process

Normally, vegetation biomass, vegetation height and canopy cover percentage are reduced with increasing the grazing intensity (Milchunas and Lauenroth, 1993). However, the light and moderate grazing intensities can cause an increase in species diversity and plant production in comparison with rangelands under heavy grazing intensity (Briske and Richards, 1994). Whereas Massey, Ennos, and Hartley, (2007b) expressed that although light grazing increases the above-ground biomass, canopy cover and height of the species but from a long-term perspective, moderate grazing can help balance the production of different species and livestock production.

Additionally, Watkinson and Ormerod, (2001) illustrated that light grazing promotes the succession from grassland to woodland, while cessation of grazing has been found to lead to a

decline in species diversity in pasture lands. On the other hand, selective grazing, as well as heavy stocking rate, may alter floristic composition and result in a shift from long-lived perennials to annuals and forbs, with a concomitant decrease in production (Fuhlendorf *et al.,* 2001). Vegetation response to the different grazing intensities have been investigated in several studies in which indicate that overgrazing has caused changes in vegetation structure through increasing non-palatable species (Wink, 1999). Moreover, the study done by Pfister, (1999) reported that intensive grazing of livestock reduced the vegetation cover and changes species composition.

According to Wink (1999), grazing animals can use both behavioral and physiological adaptations to reduce the risk of irritation and potential poisoning over time. In grazing livestock, the occurrence of continued irritation or toxicity can result in conditioned learning or avoidance of selected plants, as demonstrated by choice preference tests or trials (Pfister, 1999; Provenza, 1992). Some of the effects of grazing on plants is the removal of biomass (browsing and grazing) which is the main biotic factor affecting vegetation structure and dynamics (Belsky, 1992; Briske and Richards, 1994; Diaz, Noy-Meir and Cabido, 2001; Bakker *et al.,* 2006). Rook *et al.,* (2004) found out that grazing changes the arrangement of photosynthetic structure of communities according to the type of animal involved. The hooves of grazing animals affect the vegetation by detaching or destroying plant material and by influencing the water regime as a result of soil compaction (Abdel-Magid, Trlica and Hart, 1987; Hansson, 2004).

According to Zagrobelny *et al.*, (2008) cyanogenic glucosides are phytoanticipins known to be present in more than 2500 plant species. They are regarded as having an important role in plant defense against herbivores due to bitter taste and release of toxic hydrogen cyanide upon tissue disruption, but recent investigations demonstrate additional roles as storage compounds of reduced nitrogen and sugar that may be mobilized when demanded for use in primary metabolism. Some specialized herbivores, especially insects, preferentially feed on cyanogenic plants. Such herbivores have acquired the ability to metabolize cyanogenic glucosides or to sequester them for use in their own defense against predators (Zagrobelny *et al*., 2008). Cyanogenesis is the process by which hydrogen cyanide is released from endogenous cyanide containing compounds.

Many cyanogenic plants release HCN in sufficient quantities to be toxic and, as a result, tend to be avoided by herbivores. However, there are many exceptions with some herbivores either immune to the cyanogenic status of the plant, or in some cases attracted to cyanogenic plants. This

has led to a certain degree of scepticism regarding the role of cyanogenic glycosides as defense compounds (Gleadow and Woodrow, 2002)

2.6 Research gaps

More than 60 different CNglcs are known to be present in more than 2,500 plant species including ferns, gymnosperms, and angiosperms and it is not uncommon to find cyanogenic and acyanogenic plants within the same species, where the function of cyanogenesis is revealed through their phenoltypic characteristics. The literature does not however illustrate the variation of cyanogenic glycosides as a result of age of grasses. Also as a result of comprehensive survey, there is no studies done to simulate the effect of grazing intensity on concentration of cyanogenic glycosides in savanna grasses. Although there are studies conducted on vegetation bordering a black hills stream South Dakota on absorptivity of cyanogenic glycosides, no studies have been done to simulate the soil properties associated with cyanogenesis in savanna grasses (Huitu *et al.,* 2014). Moreover, literature surveyed illustrate functional groups that are toxic to wide spectrum of organisms that are adaptive to chemical poisoning. However research using less adaptive herbivores such as domestic animals and cyanogenic grasses have not been explored. Most species documented with cyanide are higher plants distributed throughout 110 different families of ferns, gymnosperms, and both monocotyledonous and dicotyledonous angiosperms.

Cyanogenesis in grasses have not been explored exclusively. Vegetation response to the different grazing intensities have been investigated in several studies in which the results indicate that overgrazing has caused changes in vegetation structure through increasing non-palatable species (Increaser species). However, vegetation chemo-physiology with respect to grazing intensities have not been elucidated in most studies. Most of those previous studies focus on the effects of medium and long-term grazing on total soil organic carbon (TOC). Most studies have failed to identify significant effects of grazing on levels of defensive secondary metabolites in grasses (Jane *et al.,* 2013; Huitu *et al.,* 2014). Nonetheless, graminivorous herbivores are highly selective feeders both within and between plant species (Massey *et al.,* 2007b). As this behavior is to a great extent governed also by the secondary chemistry of their food plants variation in grass secondary metabolite concentrations holds the potential to influence grazing mammals and their population demography

2.7 Conceptual Framework

The conceptual framework below puts cyanogenesis on the centre on focus. Interrelationship between elements from environment leads to formation of cyanogenic glycosides. Species of grass primarily determines the formation of cyanogenic glycosides as well as other plant secondary compounds such as plant alkaloids. Also environmental factors such as soil properties influence the formation of cyanide as well as other plant alkaloids. Age of grasses and grazing intensity influences the concentration of cyanide. Production of cyanide is thought to be due to the presence of cyanogenic glycosides that release HCN (hydrogen cyanide) when acted upon by enzymes found within plant cells. Livestock poisoning is by the action of cyanogenic glycosides as well as other secondary plant compounds.

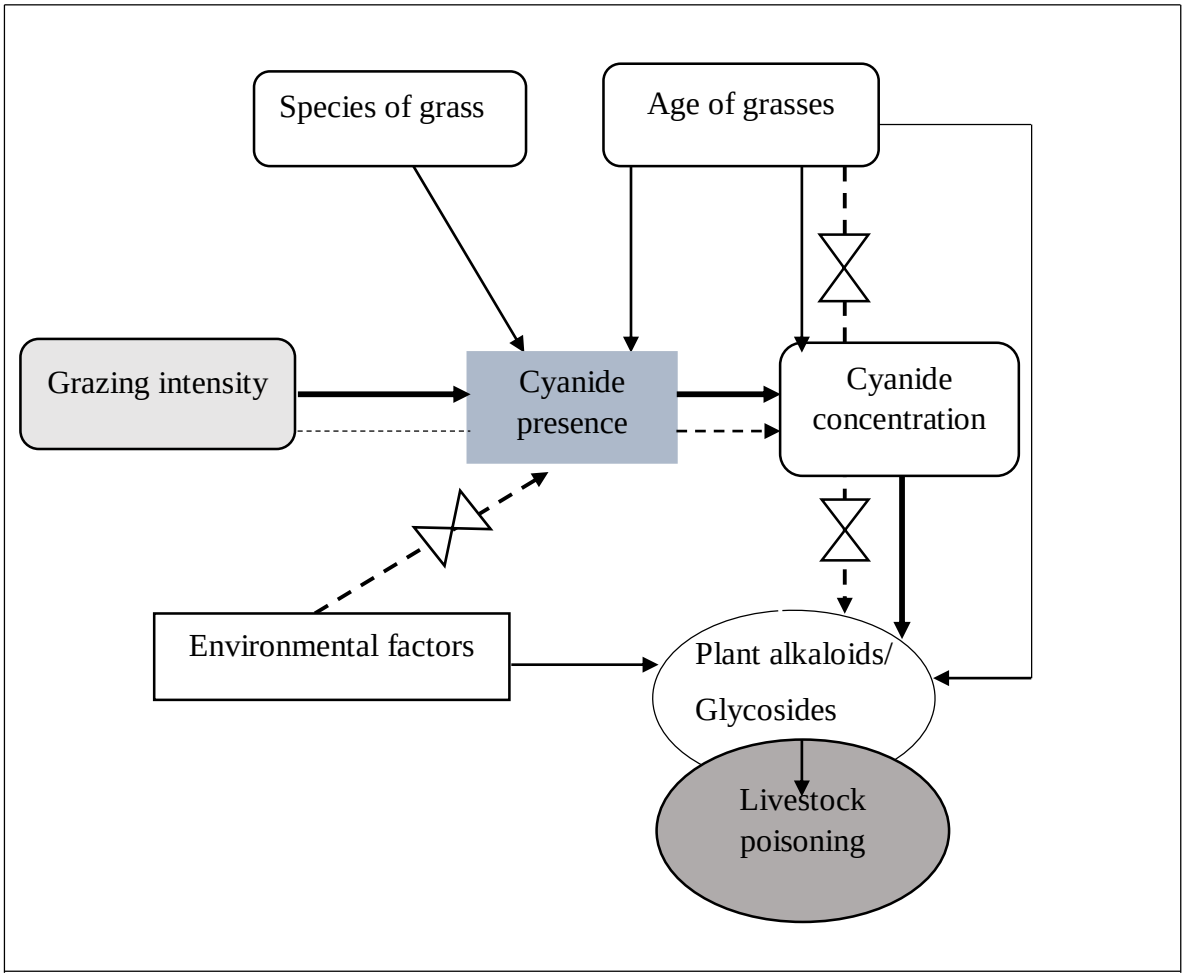

Figure 2: Conceptual framework showing the relationship of variables

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

3.1.1 Geographical location, climate and geology

This study was carried out in Marigat, Baringo County, one of Kenya's semi-arid ecosystems. The study sites were located in between latitude 00°28' N and longitude 35° 59'E (Fig 3) This ecosystem was selected to simulate the effects of edaphic factors, age of grasses and concentration of cyanide in savanna grasses because dominant vegetation and activity is grasses and pastoralism respectively. The study was specifically conducted in Lake Bogoria National Reserve. The area is hot and dry throughout most of the year with average annual temperature of about 24.6 ℃ and rainfall is highly variable with annual mean of between 635mm and 671mm, with weak bimodal peaks recorded from March to May and June to August. The area is characterized by bare ground and loose sandy loam soil with traces of stones on the surface but clayey soil is dominant in waterlogged and marshy areas. The altitude varies from 1000m to 2600m above sea level.

3.1.2 Land uses

The area is occupied by Tugen (Samor) mainly from upper region i.e. south, southeast and southwest, Pokot at the western and Njemps (Ilchamus) mainly from low regions towards northwards. Their main preoccupation is pastoralism, bee keeping and small scale farming of drought tolerant crops such as melons, onions, cassava, millet and potatoes. Their main food crops are maize, pigeon peas, beans, Irish potatoes, sweet potatoes, sorghum, cassava, onions, melons and finger millet. Marketing of locally produced products such as goat meat, honey, water and cultural artefacts such as braided calabash, Ilchamus sheet and Tugen Bow and arrows is facilitated by surging local and international tourists who visit the vicinity tourism destinations such as L. Baringo and L. Bogoria.

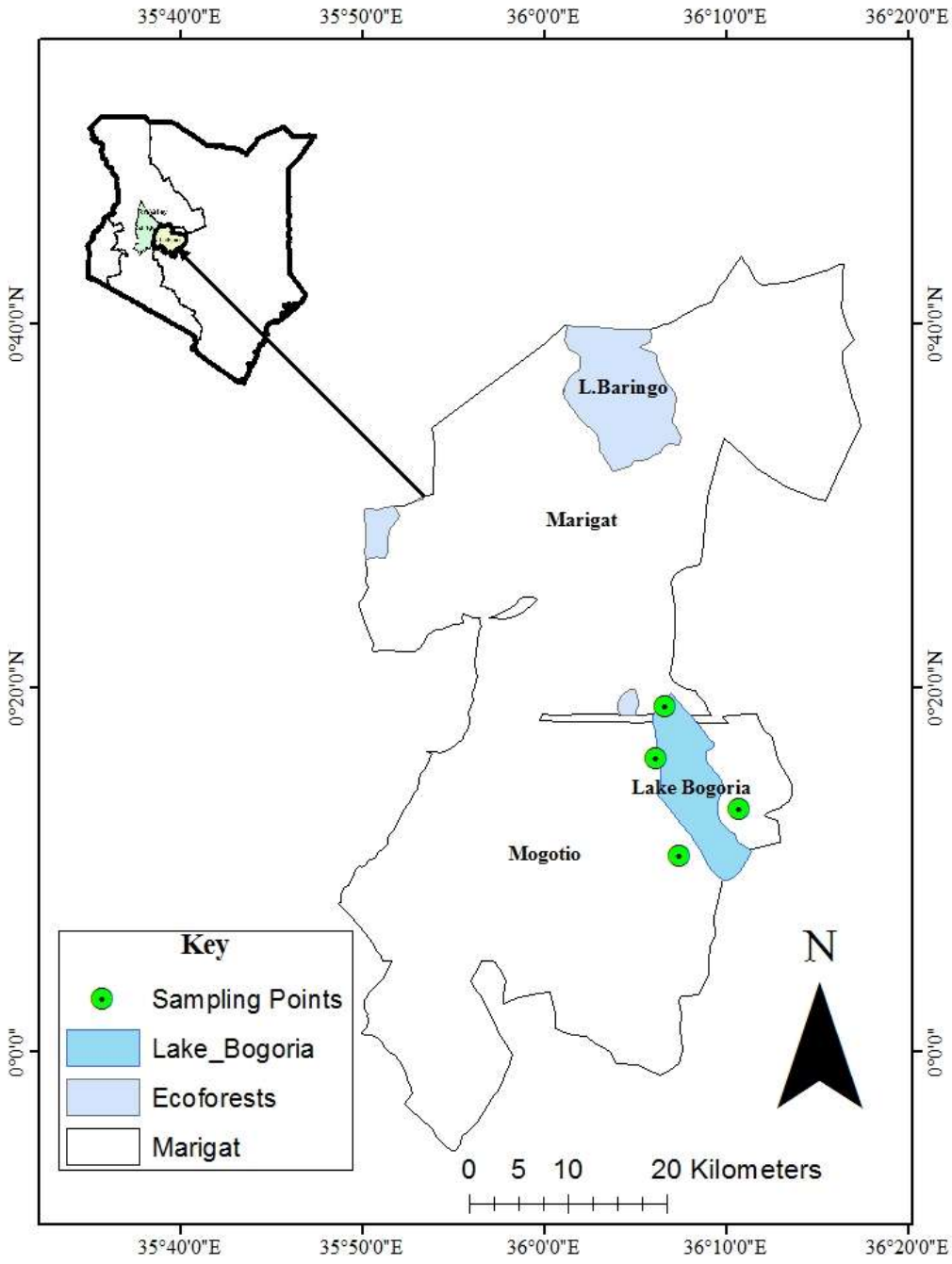

Figure 3: Map of study area, (Modified from ESRI 2014 data)

3.1.3 Flora and fauna

The dominant vegetation in this area is grasses such as *Acrachne racemosa, Gloris gayana, Agrostis keniensis, Cynodon dactylon, Cymbopogon nardus, Digitaria scalarum, Hyparrhenia hirta* and *Hyparrhenia schimperi* and acacia woodland which includes; *Acacia tortilis, Acacia reficiens* and *Boscia corriacea*. Other common plant species include *Balanites aegyptiaca, Cordia sinensis, Salvadora persica* and *Maerua anglolensis*. Thickets are also found in isolation in open grasslands but are not dominant vegetation. Most fauna (grazers and browsers) found in the area are domestic animals such as sheep, goats and cattle. In addition many people also rear many birds in free range system such as chicken, turkeys, Guinea fowl etc. Most of these birds are feeding on natural vegetation including grasses and small insects and vertebrates that are also interdepend on grass. Ostrich and tortoise are common wild animals found in the study area.

3.2 Research Design

The study was conducted in fenced exclosures in Lake Bogoria (00°28', 35°59'E) during months of April to August 2015. The exclosures (10 in total each 50m×10m) were established in June 2015. In the first experiment however, grasses were sampled outside exclosures in random basis and tested for cyanogenic glycosides using picrate-impregnated paper which changes colour from yellow to brown-red in presence of cyanide. After identifying the grasses with cyanogenic content, exclosures in sampling points were made to determine the influence of age and grazing intensity on cyanogenic concentration. In total, the experiment consisted of 10 replicates of each of the following factorial treatments: Light grazing, heavy grazing (Hg), and no grazing (Ng) in each plots.

Individual grasses which were found to have cyanide for example *C. dactylon* were experimentally treated to simulate the effect of grazing on cyanide concentration. Two grazing intensities were classified as light (LG) and heavy (HG), according to estimations of differences in ground coverage and heights. Light grazing was defined by 15cm height above the ground while heavy grazing intensity was defined by 5cm above the ground. Simulated grazing (Hand clipping) was used for these classifications. Samples of heavily defoliated grass samples and less defoliated identified from survey on the selected plots as a result of livestock herbivory was observed after the experiments with livestock densities relative to size of hectares as defined in the design. The

pastures were assumed to be small enough and the number of sheep large enough to permit the lapse of only 1 day between the before-and after-grazing clippings. Five species which tested positive to cyanogenic test were purposively sampled for use in the experiment. Samples for test was collected after a lapse of 1 day to enable the grasses to respond to grazing intensity. Second clipping was done 2 week after first clipping and extracted plant material at magnitude relatively higher than first clipping. Plant material clipped in first round was purposively sampled for second clipping. One control experiment in each sample where no consideration to variation in grazing intensity was established. Sampling units was defined by circular quadrat measuring 0.25m radius distributed on purposive sampling.

Age classes of grasses were considered in assessments of presence and relative concentration of cyanide in grass extracts using grass height, foliage succulence, inflorescence, leaf longevity and flower formation where grasses with flowers were classified as old and grass not yet blossomed were treated as young. For purpose of this study, two classification schemes were used based on leaf characteristics; young grasses and old grasses based on the combination of the five elements; grass height, foliage succulence, inflorescence, leaf longevity and flower formation. Young (y; >2cm in length, soft, not fully expanded without flower formation) and old (O; 2cm> fully expanded, toughened leaves, dark, brittle, with some senescence at the tip of the leaf with flower formation). The levels of cyanogenic glycosides in grass extracts was measured by hydrolyzing the glycosides and trapping the evolved cyanide in 1M NaOH, a modification of method by Gleadow *et al.,* (1998); Brinker and Seigler, (1989).

3.3 Sampling Procedure

Plots for sampling were selected on simple random basis while considering the topography and at least a distance of 200m from one transect to the next since the distribution of grazing areas in the study area assume ecological islands. A total of ten plots each measuring 50m by 10m were established in the study area. Sampling units were defined by the quadrats measuring 0.25m radius distributed on purposive sampling protocol based on selected species availability. All samples were collected at the same time (duration of two weeks) and treatment done simultaneously using impregnated picrate paper after a lapse of 1 day in the laboratory. A total of 20 quadrat samples were collected in each plot; total of 200 samples. Samples were transported to laboratory using ice box to reduce the deterioration of plant content which subsequently could lead to loss of cyanogenic content in grasses.

Plate 1: Quadrat sampling

A total of 16 grass species were sampled in sampling points distributed within the reserve and community private lands as the diversity varied between the two sites as well as grazing intensities. However, in the subsequent experiments, only species exhibiting cyanogenic glycosides were purposively sampled in plots. These species were distributed over 12 genera and majority were stoloniferous perennial or rhizomatous annual species. Most grasses sampled within the reserve were distributed near the lake (Bogoria) (0-200 m) in marshes and dry ecological islands that supports the growth of vegetation. The sample size collected was based on the amount enclosed in a quadrat but essentially, a handful bunch equivalent to 15g was collected in each quadrat.

3.4 Chemical Test for Cyanogenic Glycosides

3.4.1 Picrate acid method

All savanna species of grasses in sampling units in the study area were sampled and tested using picric acid paper, which changes colour in the presence of cyanide, to determine whether a cyanogenic capacity was a common trait among the sampled grass species. A modified method of Harbone (1972) was used. Cyanogenic glycosides was detected using the technique of the picrate impregnated paper which changes colour from yellow to brown-red in presence of cyanogenic content.

3.4.2 Picrate paper and plant material preparation

For picrate paper preparation, 100ml of 0.05M picric acid was prepared, by dissolving 1.146g of crystalline solid in 100ml of distill water (Plate 3). The acid was neutralized by adding sodium bicarbonate solid and filter the solid residue. Strips of filter paper (5.0 x 1.0 cm) was soaked in that aqueous solution of 0.05M picric acid. For plant material preparation, fresh plant material was cut into small pieces and crush with pestle and mortar and placed in a test tube with 1.5 ml of distilled water (Plate 2). Drops of chloroform was added followed by briefly crushing the material with a glass rod.

Plate 3: Picric acid preparation

Plate 2: Paste and mortar in use

Plate 4: Samples incubation for observations

The tube is then stoppered with a cork containing a strip of picrate impregnated paper hanging down from the stopper, and incubated at ambient temperature for 30 minutes and make observation (Plate 4). Observations were made also after 2h, 6h, 12h, 24h and 48h if there is no colour change in preceding step. For the purpose of replication, plant tissues was frozen in liquid nitrogen and ground in a mortar and pestle with 100 mm sodium phosphate, pH 7.0, and 500 mm NaCl for extraction of total soluble proteins in second replicate for investigation of low levels of cyanide.

3.4.3 Observations and inference

A colour change of the paper, from yellow to brown-red, indicated the release of HCN by the plant. A brown-red coloration within 2 hours indicate the presence of cyanogenic glycoside and the respective hydrolytic enzyme, and the plants was considered highly cyanogenic. If there is no release of HCN within 2 hours, indicating a negative test, the tube was left at ambient temperature for 24 and 48 hours, for a later re-examination. A brown-red color appearing within 48 hours indicated that the cyanogenic glycoside spontaneously released HCN without the action of

enzyme. If there is no color change after 48 hours showed that the test was negative for cyanogenic glycoside

Concentration of the cyanogenic glycosides was determined by hydrolyzing the glycosides and trapping the cyanide evolved in 1M NaOH. Freeze-dried grounded grass tissues (10-15mg) was incubated 20h at temperature of 37^0c with 1ml of 0.1M citrate buffer-HCL pH 5.5, condition which allowed for complete conversion of cyanogenic glycosides to cyanide. Cyanide in NaOH was determine using the method of Gleadow and Woodrow (2002) adapted from Brinker and Seigler, (1989). The cyanide detected using this method is directly proportional to the concentration of cyanogenic glycosides, with for example, 1mg CN is equivalent to 11.35mg glycoside prunasin (Gleadow and Woodrow, 2002)

3.5 Data Analysis

All statistical analyses were carried out using Minitab 14.0 as summarized in Table 2 below. Two sample t-tests and one-way unstacked ANOVA with 95% confidence interval was used to test the hypothesis. Tukey's individual error rate (Post Hoc tests) were used to separate means.

Table 2: Summary of data analysis

Hypotheses	Variables	Statistical tool and procedures
The levels of cyanogenic glycosides do not vary within species in savanna grasses	Grass species, soil samples, cyanide test	One-way unstacked ANOVA
The levels of cyanogenic glycosides do not vary with age of grasses	Grass samples under two classification schemes, cyanide presence and concentration	Two sample t-test Bivariate correlation analysis-to examine relationship between age and cyanide presence in grass
Grazing intensity has no effect on the concentration of cyanogenic glycosides in grasses	Heights of grasses, defoliation rates/grazing intensities	Two-sample t-test and, One-way unstacked ANOVA- to examine relationship between two grazing intensities and controlled experiment

CHAPTER FOUR

RESULTS

4.1 Occurrence of Cyanogenic Glycosides in Savanna Grasses

The results in the experiment 1 showed that out of 16 species sampled and tested, only five species indicated positive test on impregnated picrate paper test while other eleven species had no effect on picrate paper illustrating non-cyanogenic. These species which change colour on impregnated paper include; *Cynodon dactylon, Cynodon plectostachyus, Digitaria scalarum, Sporobolous spicatus and Cyperus laevigatus* (Table 3). *Cynodon dactylon* was recognized as a mat-forming slander perennial with underground rhizomes and slander surface stolons; with culms between 7-30cm high and 0.5-1mm across the base. In contrast *Cynodon plectostachyus* is perennial without rhizomes but with stout and woody arching surface stolons; culms robust, 35-90 cm high, 1-4 mm across at the base. Most habitat for *C. dactlon* in study area was roadsides and unsettled areas while *C. plectostachyus* was common in deciduous bush land where disturbances through grazing was evident.

Sporobolus spicatus was recognized as tufted perennial with wiry culms 10-70cm high depending on rate of disturbance, often arching over and rooting to form looping stolons with fascicles of shoot at the nodes. Its leaf blades are 2-30 cm long, usually convoculate, at some cases are flat and 1-4 mm wide and pungent at the tip with ability to prick a bare foot. In the study area, the distribution of this species include grassland and open bush land on saline or alkaline soil of lake shores, flood plains, pans and stream beds; 0-2600m a.s.l.

Other eleven species tested and showed negative test to cyanogenic glycosides include *Chloris gayana, Themeda triandra, Brachiaria dictyoneura, Eragrostis tenuifolia, Sporobolous pyramidalis, Sporobolous agrostoides, Seteria verticillata, Pennisetum clandestinum, Hiperhenia hirta, Cyperus rotundus* and *Cyperus papyrus* (Table 3). In the subsequent experiment these species were not sampled as they do not exhibit cyanogenic capacity.

Table 3: Picrate paper treatment observations

Family name	Botanical name	Common name	Observation	Inference
Poaceae	*Chloris gayana*	Rhodes grass	No colour change	0
Poaceae	*Cynodon dactylon*	Couch grass	Brown-red colour	1
Poaceae	*Cynodon plectostachyus*	Star grass	Brown-red colour	1
Poaceae	*Themeda triandra*	Kangaroo grass	No colour change	0
Graminae	*Brachiaria dictyoneura*	Dictyoneura	No colour change	0
Poaceae	*Digitaria scalarum*	Blue Couch	Brown-red colour	1
Poaceae	*Eragrostis tenuifolia*	Love grass	No colour change	0
Poaceae	*Sporobolous pyramidalis*	Giant rats tail	No colour change	0
Poaceae	*Sporobolous agrostoides*	Sporobolous	No colour change	0
Poaceae	*Seteria verticillata*	Bristle grass	No colour change	0
Poaceae	*Sporobolous spicatus*	Salt grass	Brown-red colour	1
Cyperaceae	*Cyperus laevigatus*	Smooth flatsedge	Brown-red colour	1
Poaceae	*Pennisetum clandestinum*	Kukuyu grass	No colour change	0
Poaceae	*Hiperhenia hirta*	Thatching grass	No colour change	0
Cyperaceae	*Cyperus rotundus*	Nut grass	No colour change	0
Cyperaceae	*Cyperus papyrus*	Paper reed	No colour change	0

Legend: 0=Negative test (No Release of Hydrogen cyanide) 1=Positive test (plant is cyanogenic)

4.2 Analysis of Levels of Cyanide and Age of Grasses

Table 4: Concentration of cyanogenic glycosides relative to ages of grass

	Cyanogenic glycosides concentration (Mg CN g^{-1} dw)		
Species	**Young cuttings**	**Old cuttings**	**p-value**
C. dactylon	1.890 (±0.16)	1.740(±0.15)	0.503
C. plectostachyus	1.420 (±0.09)	1.320(±0.11)	0.483
S. spicatus	1.260(±0.09)	1.170(±0.11)	0.538
C. laevigatus	1.240(0±0.06)	1.470(±0.12)	0.103
D. scalarum	1.210(0±0.08)	1.130(±0.07)	0.447

The results shows that younger cuttings of grass had relatively higher concentration of cyanogenic content than older cuttings (Table 4). However in some species (*C. laevigatus)* older cuttings had more cyanide content than younger cuttings (1.470Mg CN g^{-1} dw and 1.240Mg CN g^{-1} dw, respectively). There were higher levels of cyanide in *Cynodon dactylon* (1.89Mg CN g^{-1} dw – young and 1.74Mg CN g^{-1} dw-old) while *Digitaria scalarum* had the lowest level of cyanogenic content (1.210 Mg CN g^{-1} dw younger cuttings and 1.130Mg CN g^{-1} dw older cuttings) (Fig 4). However there were no significant difference in mean concentration of cyanide relative to age of grasses ($P>0.05$)

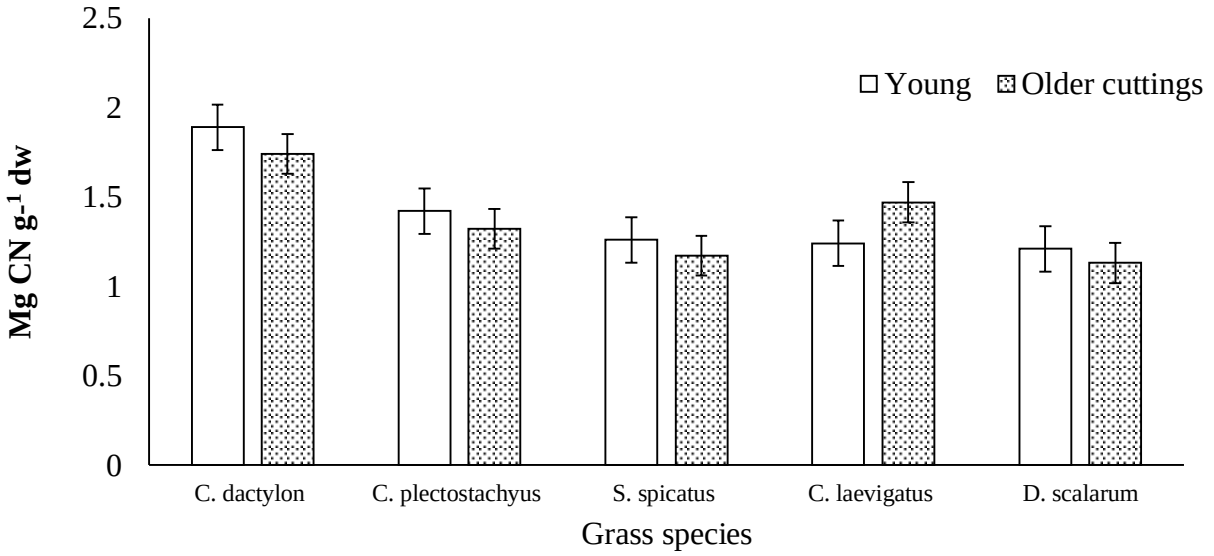

Figure 4: Cyanogenic concentration in grass species relative to age

In this experiment to determine if there is significant difference in concentration of cyanide with increase in age of grasses, two-sample t-test was used with 95% confidence interval. The null hypothesis in this experiment was; the age of grasses do not affect the concentration levels of cyanide in grasses. The general trend was that there was a consistent decrease in concentration of cyanide with increase in age of the grasses. However the difference in both age classes is not statistically significant ($P>0.05$). Based on the results, the null hypothesis was not rejected, and concluded that age do not significantly affect the levels of cyanide in grasses. While there was a general trend of lower levels of cyanogenic glycosides with increase in age of most grasses, in other species vice versa was true such as *C. laevigatus.* There were more concentration of cyanide in mature than younger plants in this species (Fig 4).

4.3 Effect of Grazing Intensity on CNglc. Concentration in Savanna Grasses

Table 5: CNglc. concentration relative to grazing intensity (t-test)

	Cyanogenic glycosides concentration (Mg CN g^{-1} dw)		
Species	**1st clipping**	**2nd clipping**	**P-value**
C. dactylon	1.25 (±0.09)	1.50 (±0.11)	0.092
C. plectostachyus	1.23(±0.08)	1.42 (±0.09)	0.148
S. spicatus	1.34 (±0.07)	1.30 (±0.07)	0.954
C. laevigatus	1.32 (±0.08)	1.58 (±0.06)	0.082
D. scalarum	1.22 (±0.07)	1.28 (±0.06)	0.716

A two-sample t-test (Table 5) of two grazing intensities showed that there were no statistical difference in means of both intensities (P>0.05). Results were compared to control experiment that was not treated with clipping (grazing variation) and one-way analysis of variance (One-way ANOVA) with 95% CI was performed to show if grazing intensity significantly affect the levels of cyanide in selected grasses. Results (Table 6) showed that grazing intensity significantly influenced the concentration levels of cyanogenic glycosides in *Cynodon dactylon* (p-value=0.024) and *Cyperus laevigatus* (P-value=0.003).

Table 6: Concentration of CNglc. in control and two grazing intensities

Cyanogenic glycosides concentration (Mg CN g^{-1} dw)				
Species	Control	1st clipping	2nd clipping	P-value
C. dactylon	1.14 (±0.06)	1.25 (±0.09)	1.50 (±0.11)	0.024
C. plectostachyus	1.26 (±0.05)	1.23 (±0.08)	1.42 (±0.09)	0.202
S. spicatus	1.22 (±0.05)	1.34 (±0.07)	1.30 (±0.07)	0.431
D. scalarum	1.20 (±0.07)	1.22 (±0.07)	1.28 (±0.06)	0.661
C. laevigatus	1.27 (±0.05)	1.32 (±0.08)	1.58 (±0.06)	0.003

While there was general trend that cyanogenic content in grasses increase with increase in grazing intensity in some species the trend was indifferent such as in *Sporobolus spicatus* (1.320 Mg CN g^{-1} dw and 1.330 Mg CN g^{-1} dw respectively**)**. In *Cynodon plectostachyus*, the grazing intensities did not significantly affect the concentration of cyanogenic glycosides (p-value=0.202). In the

successive experiments for the remaining species the variation in the CNglc. was not statistically different as influenced by grazing <0.05 (Table 5).

Table 7: Tukey's pairwise mean comparisons

Species	**1st Clipping** ($\overline{X}$ 1- $\overline{X}$ 2)	**2nd Clipping** ($\overline{X}$ 2- $\overline{X}$ 3)	**Control** ($\overline{X}$ 3- $\overline{X}$ 1)	**P-value**
C. dactylon	0.25 (±0.09) ^{Aa}	-0.36(±0.11)^{Bb}	-0.11(±0.064)^{Da}	**0.024**
C. plectostachyus	0.19(±0.08)^{Aa}	-0.16(±0.09)^{Ba}	0.03(±0.052)^{Da}	0.202
S. spicatus	-0.04(±0.07)^{Aa}	-0.08(±0.07)^{Ba}	-0.12(±0.059)^{Da}	0.431
D. scalarum	0.06(±0.07)^{Aa}	-0.08(±0.06)^{Ba}	-0.02(±0.067)^{Da}	0.661
C. laevigatus	0.26 (±0.08)^{Aa}	-0.31(±0.06)^{Cb}	-0.05(±0.054)^{Dc}	**0.003**

(Similar small letters (a a) shows no significant difference within species while similar upper case (AA) shows no significant difference between species and vice versa shows significant difference)

Post Hoc analysis using Tukey's family error rate (honestly significant difference test) was used to separate means of groups. The hypothesis in this objective was H_O: $\mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5$ and a multiple comparison test was carried out to determine between which population mean difference exist. The means of groups were subtracted from each subsequent mean i.e. first clipping subtracted from second clipping to test if there were relationship between means. In the first species, *C. dactylon*, there was no difference between first clipping and second clipping but the mean of second clipping differed from that of control experiment. In the three subsequent species *C. plectostachyus, S. spicatus and D. scalarum*, there was no significant difference among the group means. In *C. laevigatus*, the mean was significantly different between the two clippings and control experiment (P=0.003). Between species there was no significant difference in concentration of cyanogenic glycosides except in *C. laevigatus* (Table 7). From this results it is evident that only *C. laevigatus*, is most susceptible to variation with disturbance (stress)

From illustration in Fig 5-9, there was evidence of increase in concentration of cyanogenic glycosides with increase in disturbance. Light grazing for instance had almost the same concentration of glycosides with control experiment while heavy grazing intensity had relatively higher levels of cyanogenic content than the later (Fig 6, 7and 8). In some species however grazing pressure do not significantly affect the level of cyanogenic capacity of grasses as seen in *C. Plectostachyus.*

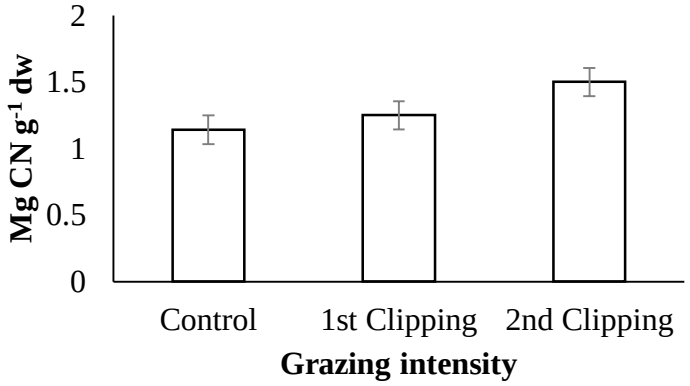

Figure 5: ***Cynodon dactylon***

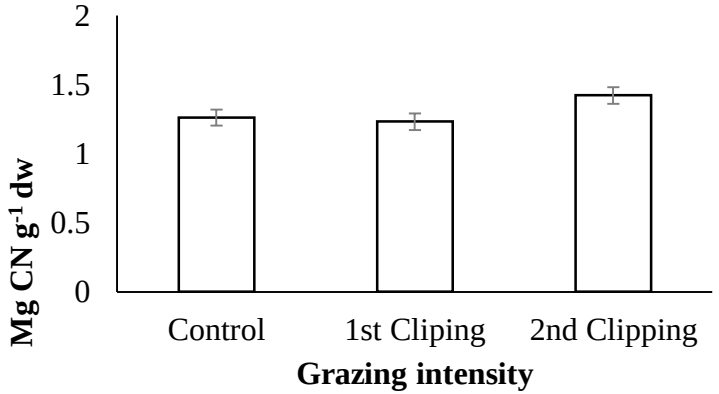

Figure 6: ***Cynodon plectostachyus***

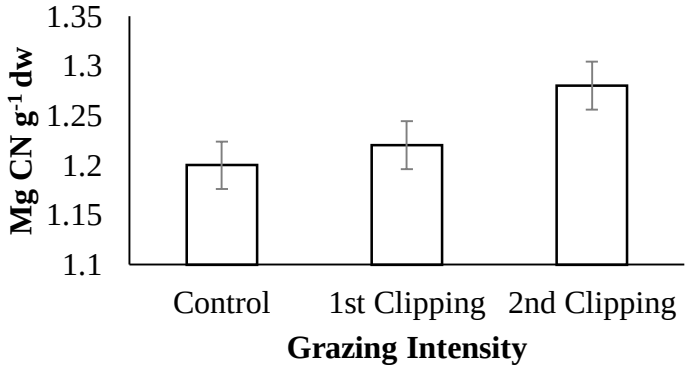

Figure 8*: Digitaria scalarum*

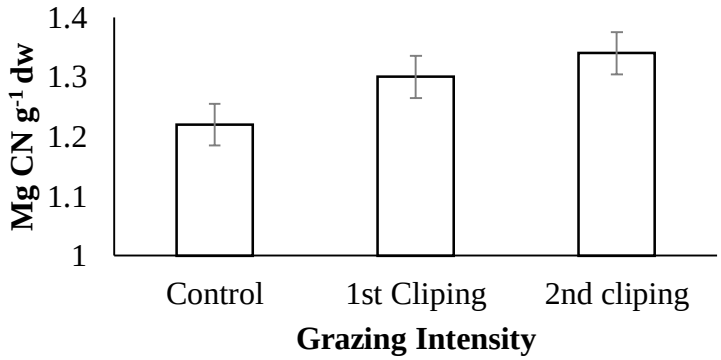

Figure 7: ***Sporobolus spicatus***

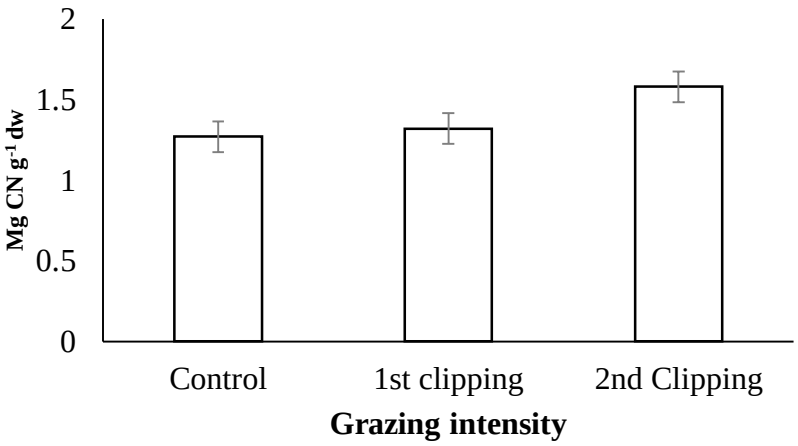

Figure 9: ***C. laevigatus***

CHAPTER FIVE

DISCUSSION

5.1 Cyanogenic Glycosides in Savanna Grasses Species

A total of 16 grass species were sampled distributed within the reserve and community private lands as the diversity varied between the two sites as well as grazing intensities. All the species of grass enclosed in the quadrat was sampled excluding herbaceous plants not related to grass family. These species are distributed over 12 genera and majority were stoloniferous perennial or rhizomatous annual species. Most grasses sampled within the reserve were distributed near the lake (Bogoria) (0-200 m) in marshes and dry ecological islands that supports the growth of vegetation.

As compared to previous studies, Francisco and Pinotti, (2000) found out that cyanogenic glycosides are widely distributed among 100 families of flowering plants. They are also found in some species of ferns, fungi and bacteria. There are many economical important plants highly cyanogenic, including white clover, linum, almond, sorghum, forage grasses, the rubber tree and cassava (Cheeke, 1995). In their study (Francisco and Pinotti, 2000) some they found out that some plants release HCN within 2 h, and were considered cyanogenic in the field. They included *Manihot esculenta* (Euphorbiaceae), *Passiflora edulis* (Passifloraceae), *Macadamia ternifolia* (Proteaceae) and *Prunus persica* (Rosaceae). *Beleperone sp* (Acanthaceae) released HCN within 24h; although it was cyanogenic it is not cyanogenic in the field because the evolution of HCN is very slow; most likely non-enzymic. In this study cyanide test experiment was carried out for a duration of between 30 mins to 48 h. All species released HCN after 2 h and the cyanogenic content were relatively low. Cyanogenesis is widespread in plants, but relatively few cyanogenic compounds have been isolated and characterized.

Moreover, several plants, primarily from the Brassicaceae (Cruciferae), have been reported to contain both cyanogenic materials and glucosinolates. Cyanogenic glycosides are widely distributed in more than 1000 species of food plants (notably cassava, peas, beans, and kernels of almonds) (Dahler *et al.*, 1995) but the identity of the cyanogenic compound(s) has been established for less than 100 of these species.

Ballhorn *et al.,* (2006) carried out an experiment to test the presence of cyanogenic glycosides (dhurrin) in grasses. In their results, 72 entries of grasses representing 39 species, 14 genera, and two tribes were tested for the presence of dhurrin by examination of the absorption spectrum in the range 250 to400 nm. Only the 13 *Sorghastrum* entries had well defined peaks in the vicinity of 330 nm, indicating the presence of dhurrin in the seeding leaves. The *Euchlaena* (teosinte) entry and several of the *Zea* (corn) entries yielded extracts with plateaus or small peaks near 330 nm, but ether extraction, which readily removed p-HB from the *Sorghastrum* extracts, did not remove the 330 nm-absorbing material from the *Euchlaena* and *Zea* aqueous extracts (Ballhorn *et al.,* 2006). Also, an extract of young *Zea* leaves, prepared by heating the leaves in 95% ethanol, failed to develop a 330 nm peak when diluted in 0.lM NaOH at room temperature showing non-cyanogenic. This behavior is in contrast to that of dhurrin-containing alcoholic extracts of Sorghum or *Sorghastrum* seedlings.

Apart from grasses, a variety of complex cyclopentenoid cyanogenic glycosides, as well as glycosides apparently derived from valine and isoleucine, occur in members of the Passifloraceae (Olafsdottir *et al.,* 1989a, b). Other cyanogenic glycosides that appear to be derived from phenylalanine also occur in the family (Buhrmester *et al.,* 2000). Additionally, several plants, primarily from the Brassicaceae family (Cruciferae), have been reported to contain both cyanogenic materials and glucosinolates (Jonathan, 2009). Some of these reports are due to false positive results from both Feigl–Anger and picrate tests.

5.2 Relationship between Cyanogenic Glycosides and Age of Grasses

The results in this study showed that younger plants had higher concentration of cyanide as compared to older. However some varieties were consequently showing that older plants had higher concentration than younger counterparts (*C. laevigatus*). The difference in concentration of cyanide however was not significant (p-value <0.05). These results compares favorably to the result in study done by Ebbs (2004) which showed that cyanide concentrations in *Tanacetum vulgare* were variable, but the plant appeared to concentrate cyanide where soil concentration increased and the ability to concentrate cyanide may be related to plant age; i.e., younger cuttings tended to yield more HCN than older plants taken from the same cyanogenic soil.

The higher concentration of cyanide in young plants as compared to the older plants is related to defense mechanism. Selmar *et al.,* (2013) found out that during germination and plantlet development, the cyanogenic potential of the entire seedling declines by 85% as cyanogenic compounds are metabolized to non-cyanogenic substances and negligible amounts of gaseous HCN are liberated during this process. Moreover Ballhorn *et al.* (2005) found out that young leaves exhibit a higher HCNp and HCNc than mature leaves. This ontogenetic variability of cyanogenesis was valid for all accessions studied. According to Ballhorn *et al.,* (2006) they found phenotypic plasticity of cyanogenesis in young leaves of lima bean *Phaseolus lunatus* based on increased activity of the beta-glucosidase in response to herbivore attack. Concurrently, Jonathan, (2009) found out that cyanogenic glycosides are synthesized but reach levels equal to only one-fourth of the original cyanolipid content as plant matures. This large decrease in cyanogenic potential points to major utilization of cyanolipids for synthesis of non-cyanogenic compounds. Selmar *et al.* (1988) further proposed that linamarin is transported from the endosperm via the apoplast to the young, growing tissues for further catabolism.

Young leaves have been found to be the most defended and the cyanogenic defense chemicals decrease with age. Additionally this greater commitment to defense in young leaves has been found for terpenes Crankshaw and Langenheim, phenolics and tannins (Coley, 1986) as well as for alkaloids (Ganjewala *et al.,* 2010). Similarly, studies report a decrease in foliar cyanogenic capacity with leaf age across a range of species including species of tropical *Macadamia* (Dahler *et al.,* 1995) as well as *Eucalyptus* and *Prunus* (Gleadow and Woodrow, 2000)

This pattern of levels of CNglc decreasing with plant age is consistent with optimal allocation theory (Agrawal, 2011), which predicts that the most vulnerable (most likely to be attacked) and valuable part of the plant in terms of fitness value, will be most highly defended. Younger plants were most preferred by grazers as opposed to dryer older plants. In this optimal allocation theory, young expanding leaves which suffers high herbivory and which have yet to provide return on investment in terms of carbon gain for plant, are well defended. More specifically, in young expanding leaves, the deployment of mobile, low molecular weight compounds (e.g. Cyanogenic glycosides), which can be re-metabolized, is assumed to be of greater advantage (Agrawal, 2011). By contrast, the cost of associated with the ongoing turnover of these compounds, is predicted to be prohibitive for maintenance of high levels in old leaves (Coley, 1986; 2003). The results also

compares favorably with studies done Francisco and Pinotti, (2000) who concluded that the level of cyanogenic glycosides produced is dependent upon the age and variety of the plant, as well as environmental factors

In the study done by Ballhorn *et al.* (2006) extracts of roots from 8 and 19-day seedlings were scanned, and each had a definite absorption peak at 330 nm. Thus, it appeared that dhurrin was present in the seedling roots as well as the shoots, but concentrations in the roots were relatively low, ranging from 100 to 200 ppm as compared to shoots. HCN-p values for the various shoot portions indicated that first leaves were relatively high in HCN-p and that, as previously observed for Sorghum seedlings (Francisco and Pinotti, 2000) values for first leaves remained quite uniform over a considerable span of seedling age. The HCN-p for second leaves dropped appreciably during early expansion of these leaves, but after about day 9, values were uniformly about half as great as those for first leaves. In this study the levels of cyanogenic glycosides was measured in leaves. It was consistent with this findings that levels decline with increase in plant age.

The results in this study also compares favorably with studies done by Ballhorn *et al.,* (2006) who found out that HCN-p values for the entire shoot declined with advancing seedling age. Additional calculations revealed that young leaves consistently accounted for a greater percentage of the shoot's HCN than of its fresh weight, whereas the SR consistently contributed a lower percentage of HCN than of weight. Second and third leaves contributed roughly the same percentage of HCN as of fresh weight in this experiment. According to Ballhorn et al (2011), CNglcs concentrations are higher when growth is limited by environmental factors such as light, temperature, or drought. The study area classified as ASAL is hot and dry throughout most of the year with an average annual mean temperature of about 26.6°C and rainfall is highly variable with a yearly mean of between 635mm. Three explanations are often presented to account for this: (a) CNglcs are concentrated in a smaller amount of plant tissue (Selmar and Kleinwachter, 2013), (b) the plants are phenologically younger owing to delayed growth (Miller *et al.*, 2014), or (c) there is active upregulation at the transcriptional level (Busk & Møller, 2002; Zhu-Salzman *et al.,* 2004). The magnitude of the increase in HCNp in response to low soil moisture depends on the severity and duration of the stress, the ontogenic stage, and the availability of other resources (Gleadow and Woodrow, 2002; O'Donnell *et al.*, 2013; Vandegeer *et al.*, 2013). In cassava, drought-stressed

tubers may become more toxic because of a direct increase in concentration and relocation of linamarin from leaves to tubers. This increased HCNp in drought-stressed cassava is not permanent and decreases after plants are re-watered (Vandegeer *et al.*, 2013).

In other species such as *C. laevigatus* there were more concentration of cyanide in mature than younger plants in this species. This was explained by increased activity of beta-clucosidase with increase in plant as well as increase in susceptibility for browsing with increase in age as opposed to previous species. Ballhorn *et al.* (2005) concluded that phenotypic plasticity of cyanogenesis in young leaves of lima bean *Phaseolus lunatus* was based on increased activity of the beta-glucosidase in response to herbivore attack. Similarly, Gleadow & Møller (2014) found out that HCNp varies ontogenetically, phenologically, and chronologically. HCNp is highest in seedlings and decreases with plant age (Gleadow *et al.*, 2008; Webber and Woodrow, 2009). For example, in *E. cladocalyx*, in the series Sejunctae, seedlings have a high HCNp (Goodger *et al.*, 2006). A similar pattern occurs in lima beans, where only secondary leaves are cyanogenic (Goodger *et al.*, 2006). Newly formed tissues are also nearly always more cyanogenic than older tissues (Gleadow and Woodrow, 2002), as in *E. cladocalyx*, where HCNp is as high in newly formed shoots and young reproductive organs of adult plants as it is in seedlings (Gleadow and Woodrow, 2002). On the contrary, notable exceptions to the pattern described above are the cyanogenic Eucalyptus species from the series *Maidenaria*. They are essentially acyanogenic as seedlings (<10 ppm HCN), becoming cyanogenic only after 6–12 months (Goodger *et al.*, 2006).

Similarly, Webber and Woodrow (2008) concluded that the higher HCNp in younger plants and plant parts is consistent with the optimal allocation theory of plant defense, but as leaves expand, there may simply be a trade-off with leaf toughness and other forms of chemical defense. This may correlate with the transcript levels of the genes involved, as in sorghum, where the transcript levels are higher in young seedlings (Busk and Møller, 2002) and in *L. japonicus*, where expression of the two CYP genes governing the synthesis of lotaustralin and linamarin is highest in the apical leaves (Takos *et al.*, 2010).

The higher levels of CNglc. in younger plant materials is also relative to nitrogen supply and later metabolism. In support of this finding, Gleadow and Møller, (2014) found out that CNglc concentration is usually higher in young plants when nitrogen is in ready supply, or when growth is constrained by non-optimal growth conditions. All plants produce tiny amounts of HCN as an

additional product in the biosynthesis of ethylene, but some plant species can release large amounts from endogenously stored cyanogenic glycosides (CNglcs). CNglcs may accumulate in all parts of a plant [e.g., as in cassava (Wheeler *et al.*, 1990)], only in the aboveground parts [e.g., as in Eucalyptus (Gleadow and Woodrow, 2002) and white clover (Olsen *et al*, 2013)], or only in vegetative tissues [e.g., as in sorghum (Wheeler *et al.*, 1990)]. This pattern may vary with the reproductive stage as well. Some *T. ulmifolia* populations, for example, lose their cyanogenic capacity around flowering, whereas others do not (Wheeler *et al.*, 1990).

5.3 Influence of Grazing Intensity on Cyanogenic Glycosides Concentration

As predicted, grazing intensity influence the concentration of cyanide, the concentration of cyanide across the species tested varied greatly within some species as proxy of different grazing intensity. Two sample t-test showed that there was no significant difference in cyanogenic concentration in all the species when subjected to both grazing intensities. However, one-way ANOVA shows there were significant difference in cyanogenic glycosides concentration in two grass species when compared to control treatment, C*ynodon dactylon* (p=0.024) and *Cyperus laevigatus* (P=0.003). However other three species showed no significant difference in concentration of cyanogenic capacity with influence of grazing intensities. This results compares favorably with the studies of Rook *et al.,* 2004; Schappert and Shore, (1999a, b); Buhrmester *et al.,* (2000); Gleadow and Woodrow, (2000b), Goodger *et al.,* (2002), who found out that natural populations of plants, vary widely in cyanogenic glycoside concentration which in turn, impacts on the degree of herbivory (Feeny, 1976). The results also compares favorably with Huitu *et al.* (2014) study who found a reduced variance in cyanide concentration in the plants on less grazed pasture as compared to heaviliy grazed pastures dominated by the same plant species. Gleadow and Woodrow (2000a), for example, detected a significant inverse correlation between the concentrations of cyanogenic glycosides in the young leaves of *Eucalyptus cladocalyx* and the amount of damage by herbivores.

In the plots that was not enclosed, there was no outstanding species with less grazing effect (all species were consumed relative to their availability) as the grazing material is limited in the area which was compounded by limited precipitation. The level of grazing on individual grasses was not measured, but visual inspection indicated that while heavy grazing did have a substantial effect on vegetation, grasses were at no point during the experiment entirely depleted within the plots.

As a result domestic animals were grazing on all materials uniformly despite the cyanogenic capacity of some grasses (Gleadow and Møller, 2014). This results supports the claim by Tattersall *et al.*, (2001) who examine studies of cyanogenesis in relation to herbivory and argue that conflicting data can generally be reconciled when the morphology, physiology, and behavior of the herbivores, together with the concentration of cyanogenic glycosides in the host plant, are taken into account. This claim is also supported by study done by Calatayud *et al.,* (1994) and Quinn *et al.*, (2014) who found out that in some cases, plant cyanide production actually acts as a phagostimulant rather than an inhibitor and glycosides are shown to have little or no effect on herbivores.

Moreover, Schappert and Shore (1999c) studied the abundance and diversity of herbivores on populations of *Turnera ulmifolia* in detail in Jamaica. They found that the number of herbivore taxa in any particular population was inversely proportional to the cyanogenic glycoside content of the leaves. However, the amount of herbivory was similar in all populations (1–9% leaf area lost), and most of the damage to cyanogenic plants came from a relatively small suite of insects. Thus, in *T. ulmifolia*, cyanogenesis appears to deter generalist herbivores, but not specialists (Schappert and Shore, 1999b). In this study the herbivores under consideration were generalist and feed exclusively on all available forage items. The levels of cyanogenic content in the grasses studied did not deter the cattle and could not subsequently poisned them.

On average the five species that shows cyanogenic trait had relatively low levels of cyanide to be considered toxic (which was highest in *C. laevigatus* (1.580 CN g^{-1} dw) and lowest in *D. scalarum* (1.250 CN g^{-1} dw). In animals, the lethal doses of HCN are generally reported to be between 0.66 and 15 mg/kg body weight (bw) for various species (Ernesto *et al*, 2002). Sheep for example, can tolerate about 2.5 mg/kg (Jones, 1972). These varieties however could be toxic to grazers if feed exclusively on particular species. The dose required to cause acute poisoning varies with body size and species. The rate of ingestion is also important, as animals can detoxify the cyanide over time (Rook *et al.,* 2004). In the study area, resources for grazing were limited and depleted and grazers were considered generalist because all species of grasses were consumed and was basis for grazers escaping poisoning. Plant cyanogenesis means the release of gaseous hydrogen cyanide (HCN) in response to cell damage (Ballhorn *et al,* 2007) and is considered as an effective defense against

generalist herbivores. In contrast, specialists are generally believed not to be affected negatively by this trait since they specialize on particular plants.

Cyanogenic glycosides increased with increase in grazing intensity pastures. Plant cyanogenesis occurs after tissue damage, which brings specific beta-glucosidases in contact with their substrates, cyanogenic glycosides, from which they are separated by compartmentation in the intact plant tissue. Plants commonly store toxic compounds in an inactive form which are activated during grazing by herbivores. According to Ballhorn *et al.,* (2007) in all cyanogenic plants surveyed to date it is apparent that the cyanogenic glycoside and its corresponding cyanogenic enzymes are localized in different cellular compartments or tissues. This compartmentalization prevents cyanogenesis until the tissue is disrupted. In some cyanogenic plants the separation of substrate and cyanogenic enzyme(s) is at a tissue level. In sorghum (sorghum bicolor) leaves, the cyanogenic glycoside dhurrin is located in vacuoles of leaf epidermal cells, whereas the b-glucosidase are localized in the cytoplasm and plastids of mesophyll cells

Although the concentration of cyanogenic content was not measured in experiment 1, the species that showed no cyanogenic glycosides were more palatable as illustrated by *Pennisetum clandestinum* and *Chloris gayana.* However due to scarcity of forage items, livestock were forced to feed exclusively on all available forage items. However in the exclosure used in experiment 2 and 3 for age and grazing intensity, after cattle were released to feed on enclosure, several species such as *Sporobolus spicatus* and *Cyperus laevigatus* were still most dominant with spikelets the most distinct. This illustrates the low palatability of the species as their selection of these species by grazers was low. However after a duration of more than two weeks, the cattle turned to all available species as preferred alternatives got exhausted. This results supports the findings by Scriber, 1978; Ferriera *et al.,* 1997; Glander *et al.,* 1989 who concluded that despite evidence documenting the deterrent effect of cyanogenic glycosides, glycosides are shown to have little or no effect on herbivores rather plant cyanogenic glycosides perform the role of phagostimulant rather than an inhibitor.

Animals in the study however were not showing signs of distraction from any specific species under the study as a result of the cyanogenic capacity. According to Feeny, (1976), specialist herbivores appear to have evolved mechanisms to tolerate cyanogenic glycosides. Some even use them as a source of nitrogen, as do some fungi. Larvae of the neotropical butterfly, *Heliconius*

sara, do this by sequestering the cyanogens from the leaves of the host plant (*Passiflora auriculata*), preventing the release of cyanide. The cyanogen is then metabolized within the insect, and the nitrogen is recovered and used in protein synthesis (Engler *et al.*, 2000). Other specialist herbivores retain the sequestered cyanogenic glycosides and employ them in defense against predators (Nahrstedt, 1985, 1988; Hughes, 1991; Engler *et al.*, 2000). For example, larvae of *Eupitoiea hegesia* take up cyanogenic glycosides from their host plant (*Turnera ulmifolia*) and become distasteful to common predators such as *Anolis* lizards (Schappert and Shore, 1999b). Specialist herbivores gain a competitive advantage by feeding on plants that are unpalatable to other animals.

Nearly all of the variability in the effectiveness of cyanogenic glycosides in defense can be explained by four confounding factors. First, the concentration of the cyanogenic glycosides may be below the threshold toxicity (the concentration are well below the capacity to cause poisoning). Second, the animal feeding on the species under examination may be a specialist that has evolved mechanisms to cope with high levels of HCN in the diet. Third, the cyanogenic plant might normally be consumed as part of a mixed diet and, therefore, might not be toxic. Fourth, the mode of feeding may be such that the animal does minimal damage to the leaf, thereby limiting the mixing of the cyanogenic glycoside with the degradative β-glucosidases and water. According to Nahrstedt, (1985) cyanogenic plants frequently have a bitter taste that is known to deter herbivores.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

- Not all savanna grasses have ability to absorb and metabolize cyanogenic content. This was illustrated by first experiment that showed only five species (*Cynodon dactylon, Cynodon plectostachyus, Digitaria scalarum, Sporobolous spicatus and Cyperus laevigatus)* out of 16 species that was tested. Ability of the plant to absorb cyanide is depended of the species of the plant as influenced by other physical conditions such as disturbance (Grazing, defoliation or herbivory).
- It was generally concluded that the cyanogenic glycosides and their catabolic enzymes decrease with increase in age of plants. Young cuttings were found to yield more HCN than older cuttings of the same grasses. However plant age does not significantly influence the level of cyanogenic glycosides.
- Heavily grazed pastures exhibit more cyanogen content as plant response to stress and improve adaptive mechanisms. There were evidence that grazing intensity significantly influenced the concentration of CNglc in the plants.
- The cyanide levels in the 5 species that showed cyanogenic trait was below the lethal level of between 1.66 and 15 mg/kg body weight (bw).
- Although it was evident in unenclosed pastures that cattle were generalist and feed exclusively on all available pastures, it was evident in exclosures that cattle avoided prickly *Sporobolus spicatus* in first grazing encounter. However cattle after depletion of other major pastures consume the species.

6.2 Recommendations of the Study

- Grazing regime of managed pastures should consider the age of pastures while allowing utilization of pastures. Since young grasses have higher cyanogenic content as opposed to mature grasses, pastures should be utilized for grazing at mature stage based on phonological characteristics of the grasses to lower potential risks to toxicity
- This finding support the grazing regime that avoids overstocking and that compels the animal to feed on former avoided pastures and even pastures previously used with potentially more levels of CNglc.

6.3 Recommendations for Further Studies

- It is recommended that a study be conducted on how soil fertility such as use of commercial fertilizers, pH and soil properties influences the concentration of cyanogenic glycosides in plants.
- Further studies is needed in seasonal variation of cyanogenic glycosides concentration as well as concentration in plants in below ground and above ground tissues.

REFERENCES

Abdel-Magid, A.H., Trlica, M.J. and Hart, R.H. (1987). Soil and vegetation response to simulated trampling. *Journal of Range Management* 40, 303-306.

Agrawal A.A., (2006). Plant defense syndromes. *Journal of Ecology, 87*, 132-149.

Bak, S. R., Paquette, S. M., Morant, M., Morant, A. V., Saito, S., Bjarnholt, N., (2006). Cyanogenic glycosides: a case study for evolution and application of cytochromes P450. *Phytochemistry Reviews, 5(2-3), 309-329.*

Bakker, E.S., Ritchie, M.E., Olff, H., Milchunas, D.G. and Knops, J.M. (2006). Herbivore impact on grassland plant diversity depends on habitat productivity and herbivore size. *Ecology Letters* 9, 780-788.

Ballhorn D.J, Lieberei R, and Ganzhorn J.U (2005) Plant cyanogenesis of Phaseolus lunatus and its relevance for herbivore-plant interaction: the importance of quantitative data. *Journal of Chemical Ecology*. Jul; 31(7):1445-73. PubMed PMID: 16222786.

Ballhorn, D.J, Heil, M, and Lieberei, R. (2006). Phenotypic plasticity of cyanogenesis in lima bean Phaseolus lunatus-activity and activation of beta-glucosidase. *Journal of Chemical Ecology*. Feb;32 (2):261-75. Epub 2006 Mar 16. PubMed PMID: 16541336.

Ballhorn, D.J., Heil M, Pietrowski, A, and Lieberei R. (2007).Quantitative effects of cyanogenesis on an adapted herbivore. *Journal of Chemical Ecology*. Dec; 33 (12):2195-208. Epub Nov 7. PubMed PMID: 17987336.

Ballhorn, D.J, Kautz, S, Schadler, M . (2013). Induced plant defense via volatile production is dependent on rhizobial symbiosis. *Oecologia* 172:833–46

Baxter, J and Cummings, S.P (2006). The current and future applications of microorganism in the bioremediation of cyanide contamination. Antonie van Leeuwenhoek 90: 1-17

Bazely, D. R., Vicari, M., Emmerich, S., Filip, L., Lin, D., and Inman, A. (1997). Interactions between herbivores and endophyte- infected Festucarubra from theScottish islands of St Kilda, Benbecula and Rum. *Journal of Applied Ecology*. 34, 847–860.

Belsky, A.J. (1990). Tree/grass ratios in East African savannas: a comparison of existing models.

Berenbaum, M., and Feeny, P. (1981) Toxicity of angular furanocoumarins to swallowtail butterflies—Escalation in a co-evolutionary arms-race. *Science* 1981, 212, 927–929.

Bernays, E. A., Cooper-Driver, G.A., and Bilgener, M., (1989). Herbivores and plant tannins. Advance *Ecology. Res*. 19, 263–303.

Blaedal, W. J., and Easty, D. B. (1971). "Potentiometric determination of cyanide with an ion selective electrode Application to cyanogenic glycosides in Sudan grasses." *Analytical chemistry* 43 (7): 890-894

Briske, D.D and Richards J.H., (1994). Physiological responses of individual plants to grazing: current status and ecological significance, p. 147-176. In: M. Vavra, W.A. Laycock, and R.D. Pieper, (eds.), *Ecological implications of herbivory in the West*. Society for Range Management, Denver.

Buhrmester, R. A., Ebinger, J. E., and Seigler, D. S. (2000). Sambunigrin and cyanogenic variability in populations of *Sambucus canadensis*. (Caprifoliaceae). *Biochemical Systematics and Ecology, 28(7), 689-695.*

Burns, A. E., Gleadow, R. M., and Woodrow, I. E. (2002). Light alters the allocation of nitrogen to cyanogenic glycosides in Eucalyptus cladocalyx. *Oecologia*, *133*(3), 288-294.

Busk PK, Møller B L. (2002). Dhurrin synthesis in sorghum is regulated at the transcriptional level and induced by nitrogen fertilization in older plants. *Journal of Plant Physiol.* 129:1222–31

Cao, J., Wang, X., Sun, X., Zhang, L., and Tian, Y. (2013). Effects of grazing intensity on soil labile organic carbon fractions in a desert steppe area in Inner Mongolia. *SpringerPlus*, *2*(Suppl 1).

Calatayud, P.A. and LE Ru, B. (1996). Study of the nutritional relationships between the cassava mealybug and its host plant. *Bull. Soc. Zool. Fr. Evol. Zool*. 121:391–398 (in French).

Chatwin T.D (1989) Cyanide attenuation/degradation in soil. Resource recovery and conservation company (R2C2), Salt Lake City, UT

Chatwin T.D, and Trepanowski, J.J (1987) Utilization of soils to mitigate cyanide releases. In: Han KN, Kliche CA (eds) Proceedings of the 3rd Western Regional Conference on precious metals, coal and the environment. Black Hill Section, A.I.M.E., Rapid City, SD

Cheeke, P.R. (1995), Endogenous toxins and mycotoxin in forage grasses and their effects on livestock. Journal of Animal Science., 73, 909-918

Cho, H.J., Do, B.K., Shim, S.M., Kwon, H., Lee, D.H., and Nah, A.H., (2013). Determination of Cyanogenic Compounds in Edible Plants by Ion Chromatography. *Official Journal of Korean Society of Toxicology 29*(*2), 143-147.*

Coley, P.D. (1986.) Costs and benefits of defense by tannins in a neotropical plant. Oecologia 70:238–41

Coley, K. A. (2003). Convergence in defense syndromes of young leaves in tropical rainforests. *Biochemistry Syst Ecology*, *31*(2), 929-949.

Conn, E. E. (1998) "Cyanogenesis, the Production of Hydrogen Cyanide by Plants," Effects of Poisonous Plants on Livestock, 1st ed Academic press, New York, pp. 301-310.

Coughenour, M. B., (1985). Graminoid responses to grazing by large herbivores: adaptations, exaptations, and interacting processes. *Ann. Mo. Bot. Gard*. 72, 852–863.

Croteau, R., Kutchman, T.M., and Lewis, N.G. (2000) Natural products (secondary metabolites). In Biochemistry and Molecular Biology of Plants; *American Society of Plant Biologists: Rockville, MD, USA*

Dahler, J.M, McConchie, C, Turnbull, CGN. (1995). Quantification of cyanogenic glycosides in seedlings of three Macadamia (Proteaceae) species. Aust. *Journal of Botany. 43:619–28*

Diaz, S., Noy-Meir, I. and Cabido, M. (2001). Can grazing response of herbaceous plants be predicted from simple vegetative traits? *Journal of Applied Ecology 38, 497-508.*

Dyer, M.I., Acra, M.A., Wang, G.M., Coleman, D.C., Freckman, D.W., and McNaughton, S.J., (1991). Source-sink carbon relations in two *Panicum coloratum* ecotypes in response to herbivory. *Journal of Ecology 72*, 1472–1483.

Ebbs. (2004). Biological degradation of cyanide compounds. *Curr Opin Biotechnol, 15*, 231-236.

Engler, H. S., Spencer, K. C., and Gilbert, L. E., (2000). Insect metabolism: Preventing cyanide release from leaves. *Nature,* 406 (6792), 144-145.

Ernesto, M., Cardoso, A.P., Nicala, D., Mirione, E., Massaza, F., Cliff, J., Haque, M.R. and Bradbury, J.H. (2002) Persistent konzo and cyanogens toxicity from cassava in northern Mozambique. Acta Tropica 82, pp 357-362.

Feeny, P. (1976). Plant apparency and chemical defense. Recent Adv. *Phytochem. 10:1–40.*

Ferreira, C., Parra, R. P., and Terra, W. R. (1997). The effect of dietary plant glycosides on larval-glucosidases from Spodoptera frugiperda and Diatraea saccaralis. *Insect Biochem. Mol. Biol*. 27:55–59.

Field, B, Jordan, F., and Osbourn, A. (2006) First encounters-Deployment of defense related natural products by plants. N. Phytol., 172, 193–207.

Francisco, I. A., and Pinotti, M. H., (2000). Cyanogenic glycosides in plants. *Brazilian Archives of Biology and Technology, 43(5), 487-492.*

Fuhlendorf, S.D., Briske, D.D. and Smeins, F.E. (2001). Herbaceous vegetation change in variable rangeland environments: The relative contribution of grazing and climatic variability. *Applied Vegetation Science 4, 177-188*

Ganjewala, D., Kumar, S., Devi, S. A., & Ambika, K. (2010). Advances in cyanogenic glycosides biosynthesis and analyses in plants: A review. *Acta Biologica Szegediensis*, *54*(1), 1-14.

Georgiadis, N. J., and McNaughton, S. J., (1988). "Interactions between grazers and a cyanogenic grass *Cynodon plectostachyus*." 343-350

Gleadow, R. M., and Møller, B. L. (2014). Cyanogenic glycosides: synthesis, physiology, and phenotypic plasticity. *Annual review of plant biology*, *65*, 155-185.

Gleadow, R. M., and Woodrow, I. E. (2002). Defense chemistry of cyanogenic Eucalyptus cladocalyx seedlings is affected by water supply. *Tree Physiology*, *22*(13), 939-945.

Gleadow, R. M., Edwards, E. J., and Evans, J. R. (2009). Changes in nutritional value of cyanogenic Trifolium Repens grown at elevated atmospheric CO2. *Journal of chemical Ecology, 35(4), 476-478.*

Gleadow, R. M., Haburjak, J., Dunn, J. E., Conn, M. E., and Conn, E. E. (2008). Frequency and distribution of cyanogenic glycosides in Eucalyptus. *Phytochemistry, 69*(9), 1870-1874.

Glander, K. E., Wright, P. C., Seigler, D. S., Randrianasol, V., and Randrianasol, B. (1989). Consumption of cyanogenic bamboo by a newly discovered species of bamboo lemur. Am. *Journal of Primatol*. 19:119–124.

Goodger, J.D, Gleadow, R.M, Woodrow, I.E. (2006). Growth cost and ontogenetic expression patterns of defense in cyanogenic Eucalyptus spp. *Trees 20*:757–65

Guilloton, G. S, and Espie, P. M., (2002). What is the role of cyanase in plants? *Rev. Plant Biochem Biotechnol, 1*, 57-79.

Hansson, M., (2004). The effects of cattle grazing and fire on infiltration in the savanna woodland of Burkina Faso. Department of Forest Ecology and Management, Swedish University of Agricultural Sciences. Msc Thesis. 26 pp

Harborne, J.B. (1972), Cyanogenic glucosides and their function. In: *Phytochemical ecology. Academic Press, London, 104-123*

Higgs, T.W. (1992). Technical guide for the environmental management of cyanide in mining. British Colombia technical and research committee on cyanide reclamation sub-committee

Howe, M., and Noble, D. (2005). Effect of cyanide residue on vegetation bordering a black hills stream. *Journal of science 64*(8).

Hughes, M. A. (1991). The cyanogenic polymorphism in Trifolium repens L. (white clover). Heredity 66:105–115.

Huitu, O., Forbes, K. M., Helander, M., Julkunen-Tiitto, R., Lambin, X., Saikkonen, K., and Hartley, S. (2014). Silicon, endophytes and secondary metabolites as grass defenses against cattle herbivores. *Frontiers in Plant Science*, *5*, 478. *J. Biogeogr. 17:483–489.*

Jane, C. Quinn, A.K., and Weston, L.A., (2013) Secondary Plant Products Causing Photosensitization in Grazing Herbivores: Their Structure, Activity and Regulation *International Journal of Molecular Sciences*

Jonathan, E. (2009). Cyanogenesis in Plants Department of Botany, *University of Iowa*:

Jones, D. A., (1998). Why are so many food plants cyanogenic? Phytochemistry 47,155–162.

Kamal, M.I and Christine, H.S (1987). An Illustrated manual of Kenya Grasses, *FAO 1987*

Karban, R., and Baldwin, I. T. (2007). Induced Responses to Herbivory. Chicago, IL:

Kursar T.A, (2003). Convergence in defense syndromes of young leaves in tropical rainforests. *Biochem Syst Ecol, 31*(2), 929-949. Launchbaugh, K.L. Biochemical Aspects of Grazing Behavior; CAB International: Wallingford, Oxfordshire, UK, 1996; pp. 159–184.

Li, H., Nakashima, T., Tanaka, T., Zhang, Y.-J., Yang, C.-R., and Kouno, I. (2008). Two new maltol glycosides and cyanogenic glycosides from Elsholtzia rugulosa Hemsl. *Journal of natural medicines, 62*(1), 75-78.

Lindroth, R. L., and Batzli, G. O., (1984). Plant phenolics as chemical defenses: effects of natural phenolics on survival and growth of prairie voles (Microtus ochrogaster). *Journal of Chemical Ecology.* 10, 229–244.

Mali, S., and Borges, R. M. (2003). Phenolics, fibre, alkaloids, saponins, and cyanogenic glycosides in a seasonal cloud forest in India. *Biochemical Systematics and Ecology, 31*(11), 1221-1246.

Marten, G. C., Barnes, R. F., Simons, A. B., and Wooding, F. J. (1973). Alkaloids and palatability of *Phalaris arundinacea* L. grown in diverse environments. *Agron. J.65, 199–201.*

Massey, F. P., Ennos, A. R., and Hartley, S. E. (2007a). Herbivore specific induction of silica-based plant defenses. *Oecologia* 152, 677–683. doi: 10.1007/s00442-007-0703-5

Massey, F. P., Ennos, R., and Hartley, S. E. (2007b). Grasses and the resource availability hypothesis: the importance of silica-based defenses. *Journal of Ecology*. 95, 414–442.

McMahon J.M, White W.B, Sayre R.T. (1995). Cyanogenesis in cassava (Manihot esculenta Crantz). Bot. 46:731–41

McNaughton, S. J., and Tarrants, J. L., (1983). Grass leaf silicification: natural selection for an inducible defense against herbivores. Proc. *Nat. Acad. Sci*. U.S.A. 80, 790–791

Milchunas, D.G., and Lauenroth, W.K., (1993). Quantitative effects of grazing on vegetation and soils over a global range of environments. *Ecological Monographs 63, 327-366*

Miller, R.E, Gleadow, R.M and Cavagnaro, T.R. (2014). Age versus stage: Does ontogeny modify the effect of phosphorus and arbuscular mycorrhizas on above- and below-ground defense in forage sorghum? *Plant Cell Environ. 37*:929–42

Nahrstedt, A. (1985). Cyanogenesis and the role of cyanogenic compounds in insects Plant Syst. Evol. 150:35–47.

Nahrstedt, A. (1988). Cyanogenic compounds as protecting agents for organisms, pp. 131–150, in D. Evered and S. Harnett (eds.). Cyanide Compounds in Biology. John Wiley & Sons, Chichester, United Kingdom.

Olafsdottir, E.S., Andersen, J., Jaroszewski, J.W., (1989a). Cyclopente-noid cyanohydrin glycosides. Part 9: Cyanohydrin glycosides of Passifloraceae. *Phytochemistry* 28, 127–132.

Olafsdottir, E.S., Cornett, C., Jaroszewski, J.W., (1989b). Cyclopente-noid cyanohydrin glycosides with unusual sugar residues. Acta Chemica Scandinavica 43, 51–55.

Olsen, K.M, Kooyers, N.J, & Small, L.L., (2013). Recurrent gene deletions and the evolution of adaptive cyanogenesis polymorphisms in white clover (*Trifolium repens L*.). *Journal of Mol.. Ecology*. 22:724–38

Niemeyer, H. M., (1988). Hydroxamic acids (4-hydroxy-1, 4-benzoxazin-3-ones), defense chemicals in the Gramineae. *Phyto-chemistry* 27, 3349–3358.

Peter H., and Marcus H., (1999). Cyanogenesis in savanna grasses, University College London, UK and University of Zurich, UK

Pfister, J.A., (1999). Behavioral Strategies for Coping with Poisonous Plants; University of Idaho: Moscow, ID, USA

Pickett, J.A. (2012). New synthesis: Chemical ecology and sustainable food production. *Journal of Chemical Ecology. 38, 1071–1071.*

Provenza, F.D., Pfister, J.A., Cheney, C.D. (1992). Mechanisms of learning in diet selection with reference to phytotoxicosis in herbivores. *Journal of Range Management, 45, 36–45.*

Quinn, J. C., Kessell, A., and Weston, L.A., (2014). Secondary Plant Products Causing Photosensitization in Grazing Herbivores: Their Structure, Activity and Regulation. *International Journal of Molecular Sciences, 15(1), 1441–1465.*

Ramirez, C. A., and Barry, T. N., (2005). Alternative temperate forages containing secondary compounds for improving sustainable productivity in grazing ruminants. *Animal Feed Science and Technology, 120(3), 179-201.*

Rook, A.J., Dumont, B., Isselstein, J., Osoro, K., WallisDeVries, M.F., Parente, G. and Mills, J. (2004). Matching type of livestock to desired biodiversity outcomes in pastures - a review. *Biological Conservation 119, 137-150.*

Rose U.S.R, Manukian, A, Heath R.R, Tumlinson J.H. (1996). Volatile semi-chemicals released from undam-aged cotton leaves: asystemic response of living plants to caterpillar damage. *Plant Physiol*. 111:487–9

Seigler, D. S., Pauli, G. F., Nahrstedt, A., and Leen, R. (2002). Cyanogenic allosides and glucosides from *Passiflora edulis* and *Carica papaya*. *Phytochemistry, 60*(8), 873-882.

Selmar D, Lieberei R, and Biehl B (1988). Mobilization and utilization of cyanogenic glycosides. The linustatin pathway. *Journal of Plant Physiol* 86: 711-716

Selmar, L. R, and Biehl B, (2004). Hydroxynitrile lyase in Hevea brasiliensis and its significance for rapid cyanogenesis. *Physiology of the Plant 79, 97-101.*

Scriber, J. M. (1978). Cyanogenic glycosides in Lotus corniculatus. Their effect upon growth, energy budget, and nitrogen utilization of the southern armyworm, Spodoptera eridania. Oecologia 34:143–155.

Schappert, P.J. and Shore, J.S. (1999a). Cyanogenesis in *Turnera ulmifolia L. (Turneraceae*). I. Phenotypic distribution and genetic variation for cyanogenesis on Jamaica. *Heredity* 74:392–404.

Schappert,P.J.and Shore, J. S. (1999b). Effects of cyanogenesis polymorphism in Turnera ulmifolia on Euptoieta hegesia and potential Anolis predators. *Journal of Chemical Ecology*. 25:1455–1479.

Selmar, D, and Kleinwachter, M., (2013). Stress enhances the synthesis of secondary plant products: the impact of stress-related over-reduction on the accumulation of natural products. *Plant Cell Physiol. 54:*817–26

Simons, A. B., and Marten, G. C. (1971). Relationship of indole alkaloids to palatability of *Phalaris arundinacea L. Journal of Agronomy. 63, 915–919.*

Siritunga, D., and Sayre, R. (2004). Engineering cyanogen synthesis and turnover in cassava (Manihot esculenta). *Plant molecular biology, 56(4), 661-669.*

Spencer, K.C., Seigler, D.S., (1983). Cyanogenesis of Passiflora edulis. *Journal of Agricultural and Food Chemistry* 31, 794–796

Stochmal, A, Oleszek, W. (1997). Changes o f cyanogenic g lucosides in white clover (Trifolium repens L.) during the growing season. *Journal of Agriculture*. 45:4333–36

Takos, A M, Lai D , Mikkelsen, L, Maher, A H, Shelton, D, (2010). Genetic s creening identifies cyanogenesis-deficient mutants of *Lotus japonicus* and reveals enzymatic specificity in hydroxynitrile glucoside metabolism. Plant Cell 22:1605–19

Ubalua, A. O. (2010). Cyanogenic Glycosides and the fate of cyanide in soil *Australian journal of crop Science, 4(4), 223-237.* University of Chicago Press.

Vandegeer R, Miller R.E, Bain M, Gleadow R.M, Cavagnaro T.R., (2013). Drought adversely affects tuber development and nutritional quality of the staple crop cassava. *Funct. Plant Biol.* 40:195–200

Vetter, J. N. (2000). Plant cyanogenic glycosides. *Toxicon, 38(1), 11-36.*

Vicari, M., and Bazely, D. R., (1993). Do grasses fight back? The case for antiherbivore defenses. *Trends Ecol. Evol. 8, 137–141.*

Vulink, J. T., Drost, H. J., and Jans, L. (2000). The influence of different grazing regimes on Phragmites and shrub vegetation in the well-drained zone of a eutrophic wetland. *Applied Vegetation Science, 3*(1), 73-80.

Watkinson, A.R. and Ormerod, S.J. (2001). Grasslands, grazing and biodiversity: Editors' introduction. *Journal of Applied Ecology 38, 233-237.*

Webber, B.L, Woodrow, I.E., (2009). Chemical and physical plant defense across multiple ontogenetic stages in a tropical rain forest understorey tree. *Journal of Ecology.* 97:761–71

Weston, L.A., and Mathesius, U. (2013) Flavonoids: Their structure, biosynthesis and role in the rhizosphere, including allelopathy. *Journal of Chemical Ecology, 39, 283–297*

Weston, P.A., Weston, L.A, and Hildebrand, P.A. (2013). Metabolic profiling in Echium plantagineum: Presence of bioactive pyrrolizidine alkaloids and naphthoquinones from accenssions across Australia. *Phytochem. Rev*. 2013, 12, 831–837.

Wheeler, J.L, Mulcahy, C, Walcott, J.J, Rapp, G.G. (1990). Factors affecting the hydrogen cyanide potential of forage sorghum. Aust. *Journal of Agriculture*. Res. 41:1093–100

Wink, M. (1999). Functions and biotechnology of plant secondary metabolites. *Ann. Plant Rev*. 1999, 39, 1–16.

Woodrow, I. E., Slocum, D. J., and Gleadow, R. M. (2002). Influence of water stress on cyanogenic capacity in Eucalyptus cladocalyx. *Functional Plant Biology, 29*(1), 103-110.

Zagrobelny, M, Bak S, and Møller, B.L (2008). Cyanogenesis in plants and arthropods. Phytochemistry. May; 69(7):1457-68.

Zhu-Salzman, K., Salzman, R.A, Ahn, J.E, Koiwa, H., (2004). Transcriptional regulation of sorghum defense determinants against a phloem-feeding aphid. *Plant Physiol. 134*:420–31