

## **Grazing Intensity Contributes to Cyanogenic Toxicity in Savannah Grasses in Baringo County**

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## ABSTRACT

*The objective of this study was to quantify the influence of grazing intensity on cyanogenic glycosides in Lake Bogoria, Kenya. Field experiments were carried out in ten enclosures. Grazing intensity was varied using simulated grazing method. Grasses were categorized into two age classes; young and old. Cyanogenic glycosides were tested using impregnated picrate paper and concentration determined by hydrolysis and trapping in 1M NaOH. Five of 16 sampled species produce cyanogenic glycosides. There was an inverse relation between Cyanide concentration and age of the plants. Young cuttings yield more Hydrogen Cyanide than older cuttings. Grazing intensity had a significant effect on the concentration of cyanogenic content in some grass species; *C. dactylon* ( $P=0.024$ ) and *S. laevigatus* ( $P=0.003$ ). This study imply that grazing regime of managed pastures should consider the age of forage while allowing utilization of pastures preferably grazed on mature pastures with low levels of cyanogenic glycosides.*

**Keywords: Cyanide, grazing, intensity, grasses and glycosides**

## 1.1 INTRODUCTION

Cyanogenic glycosides (CNgls) are bioactive plant products derived from amino acids. They are a group of plant secondary compounds that contain nitrogen and yield cyanide (cyanogenesis) following their enzymatic breakdown (Møller, 2010). Natural sources of cyanide, include bacteria, plants, and fungi which synthesize and secrete cyanide but the most common sources of cyanide in the environment are from industrial wastes which enter the soil through the solution with rain water and infiltration (Woodrow et al., 2002).

According to Zagrobelny, Bak, & Møller, (2008), Cyanogenesis; the evolution of toxic hydrogen cyanide from endogenous CNgls—is an effective defense against generalist herbivores but less effective against fungal pathogens). Plants have evolved a plethora of different protection chemicals that covers almost all classes of (secondary) metabolites that represent a significant defense to herbivory: Some are constitutive; others are induced after an attack (Mithöfer & Boland, 2012). According to (Ballhorn Kautz & Lieberei, 2010) many compounds act directly on the herbivore, whereas others act indirectly via the attraction of organisms from other trophic levels that, in turn, protect the plant. An enormous diversity of plant (bio) chemicals is toxic, repellent, or anti-nutritive for herbivores of all types. Examples include cyanogenic glycosides, glucosinolates, alkaloids, and terpenoids; others are macromolecules and comprise latex or proteinase inhibitors. Grasses are also known to produce an array of secondary metabolites, such as hydroxamic acids (Pentzold et al. 2014) and alkaloids, albeit at levels much lower than dicotyledons (Zagrobelny, Bak, & Møller, 2008). Their modes of action include membrane disruption, inhibition of nutrient and ion transport, inhibition of signal transduction processes, inhibition of metabolism, or disruption of the hormonal control of physiological processes.

The level of cyanogenic glycosides produced is dependent on the age and variety of the plant, as well as environmental factors (Møller, 2010; Ubalua, 2010). 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). Certain plant species synthesize cyanogenic glycosides and cyano lipids which when disrupted by grazing are hydrolyzed and in the process liberate Hydrogen Cyanide (HCN). Hydrogen cyanide produced has a potential to cause health concerns which include the arrest of the

ATP production and cell death by blocking cytochrome oxidase (Sirikantaramas, Yamazaki & Saito, 2008). Recognizing the herbivore challenge and precise timing of plant activities as well as the adaptive modulation of the plants' metabolism is important so that metabolites and energy may be efficiently allocated to defensive activities. This study seeks to identify savanna the grasses which synthesize cyanide and attempt to elucidate the biological pathways that link mammalian grazing disturbance with cyanogen toxicity associated with these grasses in Kenya. This study also sought to explain on the concentration of cyanogenic glycosides as affected by the age of grasses.

## **1.2 MATERIALS AND METHODS**

The study was conducted in fenced enclosures in Lake Bogoria (00°20'N, 35°59'E) during the months of June-September, 2015. The enclosures (10 in total each 50m×10m) were established in June 2015 and enclosed for one month. In the first experiment, however, grasses were sampled outside enclosures on a random basis and tested for cyanogenic glycosides using picrate-impregnated paper. After identifying the grasses with cyanogenic content, enclosures 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 (LG + Plot1, p2, p3...), heavy grazing (Hg), and no grazing (Ng) in each plot. Two levels of grazing intensity were applied; light (15cm height) and heavy (5cm). The grazing treatment was begun in late June 2015 by first clipping then second clipping. One control experiment in each sample was established (where no consideration to variation in grazing intensity was established). Sampling units defined by quadrat measuring 0.25m radius was distributed on purposive sampling. Age of grasses was classified into two; young and old pastures using characteristics such as; fluorescence, and leaf blade length. The concentration of Cyanogenic glycosides in grass extracts was measured by hydrolyzing the glycosides and trapping the evolved cyanide in 1M NaOH well a modification of method by Gleadow *et al.*, (2006). Freeze-dried grounded grass tissues (10-15g) was incubated 20h at a temperature of 37<sup>0</sup>c with 1ml of 0.1M citrate buffer-HCL pH 5.5, a condition which allowed for the complete conversion of cyanogenic glycosides to cyanide. The cyanide detected using this method is directly proportional to the concentration of cyanogenic glycosides, for example, 1mg CN is equivalent to 11.35mg glycoside prunasin (Gleadow and Woodrow, 2002)

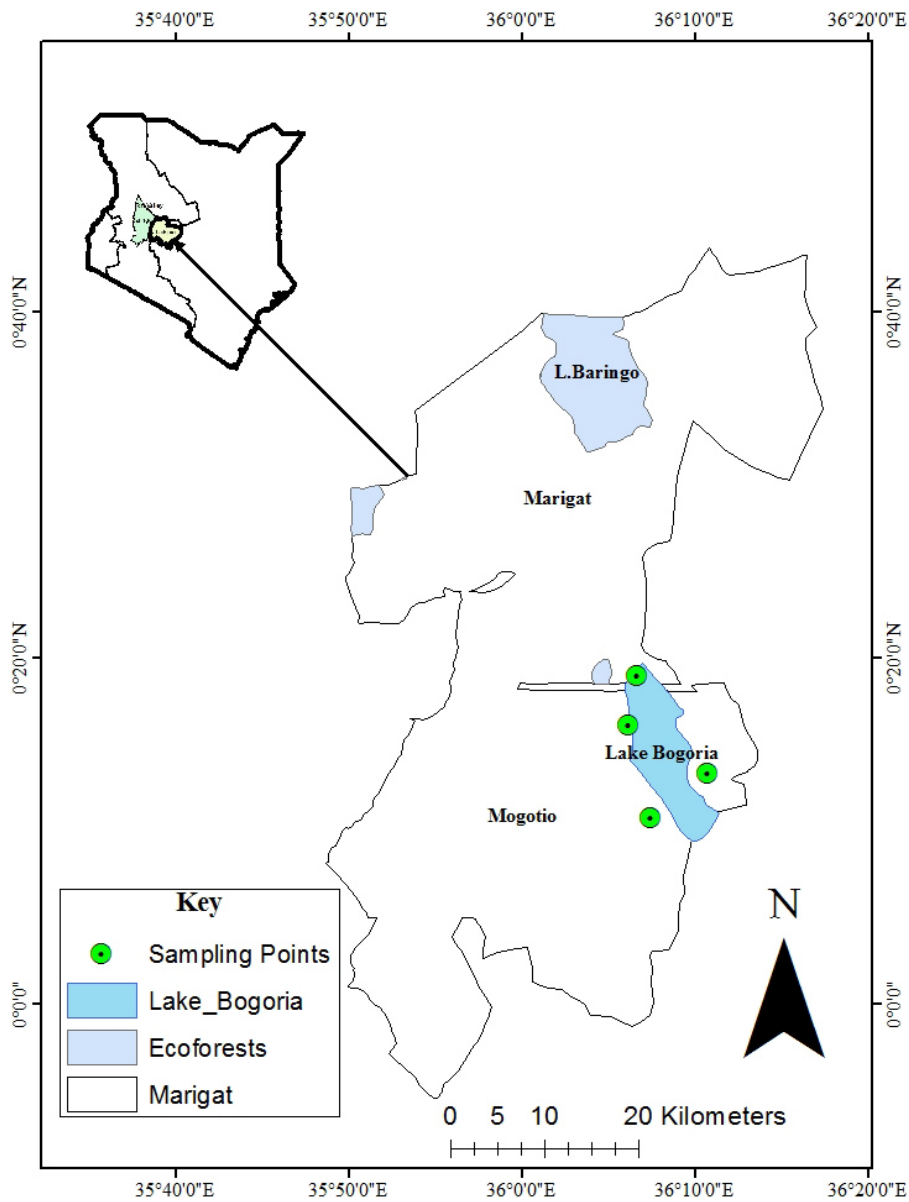


Figure 1: Map of study area

### 1.3 RESULTS

The results of Experiment 1 showed out of 16 species sampled and tested only five species indicated active on impregnated picrate paper test while other eleven species had no effect on picrate paper illustrating non-cyanogenic. These species which change color on impregnated paper include; *Cynodon dactylon*, *Cynodon plectostachyus*, *Digitaria scalarum*, *Sporobolus spicatus* and *Cyperus laevigatus*.

The results of age experiment show that younger cuttings of grass had relatively more concentration of cyanogenic content than older cuttings (Table 1). However in some species (*C. laevigatus*) older cuttings had more cyanide content than younger cuttings (1.470Mg CN g<sup>-1</sup> DW and 1.240 Mg CN g<sup>-1</sup> DW, respectively). There were higher levels of cyanide in *C. dactylon* (1.89 Mg CN g<sup>-1</sup> DW–young and 1.74 Mg CN g<sup>-1</sup> DW–old) than all other species while *D. scalarum* had the lowest level of cyanogenic content(1.210 Mg CN g<sup>-1</sup> DW young cuttings and 1.130 Mg CN g<sup>-1</sup> DW older cuttings). However, there was no significant difference in cyanide concentration relative to age of grasses in all species (P>0.05)

**Table 1: Cyanide levels and Age of grasses**

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

In experiment 3 the general trend shows that cyanogenic content in grasses increases with increase in grazing intensity (Fig 2-5). Two sample t-test, however, showed that there was no significant difference between the two grazing intensities in all species (P>0.05). Nevertheless, one way unstacked-ANOVA test with two grazing intensity and one control experiment showed there was a significant difference in concentration of cyanide as a result of grazing intensity in two species *C. dactylon* (P=0.024) and *C. plectostachyus* (P=0.003) while the other three species, there was no significant difference in levels of cyanogenic glycosides under the two grazing regimes and control.

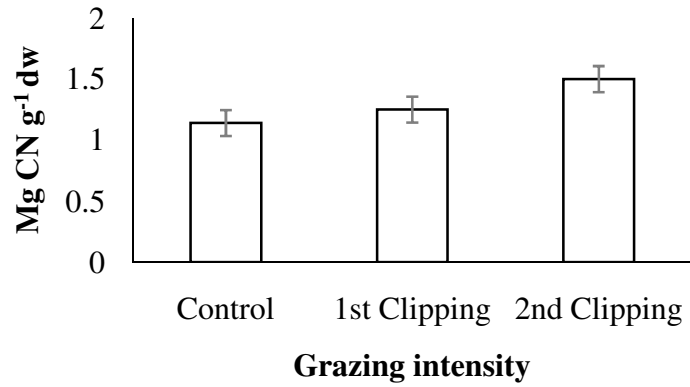


Figure 2: *Cynodondactylon*

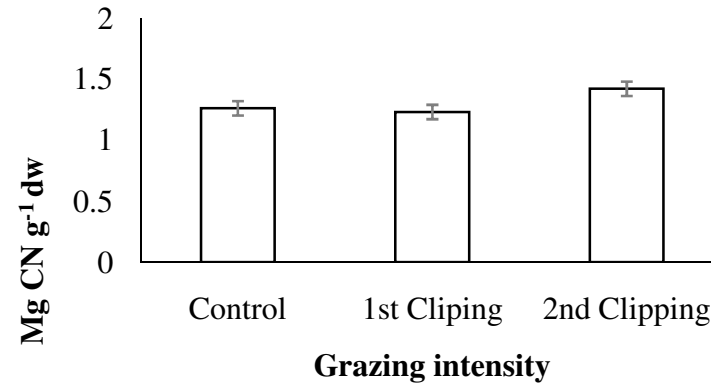


Figure 3: *Cynodonplectostachyus*

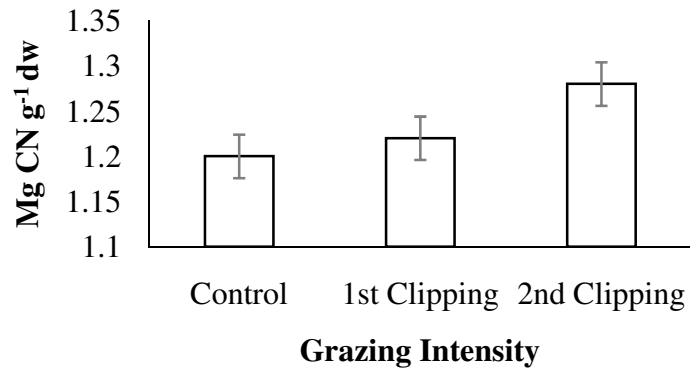


Figure 4: *Digitariascalorum*

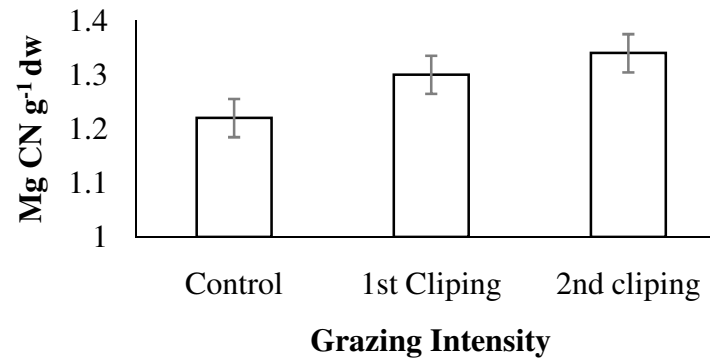


Figure 5: *Sporobolusspicatus*

By Tukey's comparison, null hypothesis  $H_0: \mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5$  that grazing intensity has no effect on the concentration of cyanide was rejected and concluded that grazing intensity influence the level of cyanogenic content in grasses. The result also showed there was no significant difference in levels of cyanide across all species except *C. laevigatus*. It illustrated that *C. laevigatus* was most susceptible to grazing pressure as compared to other species.

**Table 2: Turkey's Pairwise Comparisons of two Grazing Intensities and control treatment**

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

## 1.4 DISCUSSION

The results of the second experiment showed that generally, younger plants had a higher concentration of cyanide as compared to older. This result compares favorably with the result in the study done by Ebbs (2004) which showed that cyanide concentrations in tansy were variable, but the plant appeared to concentrate cyanide where soil concentration increased. In his study, Ebbs concluded that the ability to concentrate cyanide may be related to plant age; i.e., younger cuttings tended to yield more HCN than older plants that were taken from the same cyanogenic soil. Also, the higher concentration of cyanide in younger plants has been particularly well documented in sorghum, which is highly toxic to grazing stock when young, but becomes suitable for pasture as plants mature (Ganjewala et al. 2010).

According to Ballhorn et al (2011), CNglc 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. 2008). 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 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) (Ganjewala et al. 2010; 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 (Kempel, Brandl and Schadler, 2009) and lima beans (Ballhorn, Kautz and Schadler, 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.

In 2014, Miller, Gleadow, and Cavagnaro found out that the higher concentration of cyanide in young plants as compared to the older plants is related to enzymatic activity and adaptive mechanism. They 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. However, since highest levels of the cyanide detoxifying enzyme  $\beta$ -cyanoalanine synthase occur in young seedling tissues, (Webber and Woodrow, 2009), proposed that linamarin is transported from the endosperm via the apoplast to the young, growing tissues for further catabolism.

In further support of this, Ballhorn, Lieberei and Ganzhorn (2005) found out that young leaves exhibit a higher HCNp and HCNc than mature leaves. They 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 and Woodrow, 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, 2008), 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, 2008). 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 CYP79 genes involved, as in sorghum, where the CYP79A1 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 (CYP79D3 and CYP736A2) is highest in the apical leaves (Tako et al. 2010).



In further support of this finding, Gleadow & 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, 2008) 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 (Schappert and Shore, 1999). The often-observed location of CNglcs and their catabolic enzymes at the periphery or other entrance sites of plant tissues (peel, epidermis, and vascular bundles) and in young, soft tissues is consistent with a defensive role.

In the third experiment, as predicted, grazing intensity influence the concentration of cyanide, the concentration of cyanide across the species tested varied considerably with some species. 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 was a significant difference in cyanogenic glycosides concentration of two grass species, *Cynodon dactylon* (p-value=0.024) and *Cyperus laevigatus* (P-value=0.003). Cyanogenic glycosides are not toxic and are stored intracellularly in the vacuole, whereas the related glycosidase is present in the cytoplasm. However, upon cell destruction by a feeding herbivore, cleaving off the aglycone moiety is no longer preventable via separation of the enzyme from the substrate. Subsequently, acetone cyanohydrin is released, which can be converted into HCN and acetone either spontaneously or by a *hydroxynitrile lyase* (Ballhorn et al. 2008)

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 Mg CN g<sup>-1</sup> DW) and lowest in *D. scalarum* (1.250Mg CN g<sup>-1</sup> DW). In animals, the lethal doses of HCN are reported to be between 1.66 and 15 mg/kg body weight (BW) for various species (Ernesto et al., 2002). These varieties, however, could be toxic to grazers if feed exclusively on particular species. In the study area, resources for grazing were limited and depleted, and grazers were considered generalize because all species of grasses were consumed and was the basis for grazers escaping poisoning. Because plants, animals, and fungi all have mechanisms to detoxify and excrete HCN, poisoning occurs only when the rate of intake is greater than the rate of detoxification.

CNglcs are only one of many defenses at a plant's disposal. Defense strategies are likely to vary with different selective pressures (magnitude and type) and with developmental stage (Agrawal, 2011; Ballhorn et al. 2008). CNglcs are effective deterrents to generalist herbivores (Ballhorn et al. 2008; Gleadow and Woodrow, 2002; Zagrobelny et al. 2004), and this is most likely the main evolutionary driver in their occurrence across the plant kingdom (Neilson et al. 2014). CNglcs may also serve as transport forms of carbon and nitrogen (Agrawal, 2011), and endogenous turnover processes may release the nitrogen from CNglcs in the form of ammonia (Neilson et al. 2014). More recently, it has been proposed that CNglcs may also function in modulating oxidative stress (Neilson et al. 2014).

In an extensive survey of the shrub *T. ulmifolia*, Mithöfer & Boland, (2012) found out that in naturalized populations in Jamaica, an inverse correlation was found between mean HCNp and the number of herbivore taxa visiting the plant (Schappert and Shore, 1999). Moreover, 40% of the most highly cyanogenic individuals

were not visited by insects at all. Similarly, only one insect (*Leucopodoptera eumundii*) has ever been found feeding on *Ryparosa kurrangii* (sensu *R. javanica*), a long-lived, highly cyanogenic understory tree from tropical Australia (Webber et al. 2003).

Several studies have detected a correlation between bitterness and HCNp (Lee et al. 2013), as recognized in the common names of highly cyanogenic varieties of Prunus, such as bitter and sweet cherries (*P. emarginata* and *P. avium*, respectively) and almonds (*P. amygdalus* syn *P. dulcius*). The levels of plant defense chemicals are further influenced by damage (Kadow et al. 2012). Several specialist herbivores not only tolerate CNgls but also actually sequester them for use in their arsenal of defense compounds against predators (Nishida, 2002; Zagrobelny et al. 2007). For example, larvae of *Euptoieta hegesia* (Lepidoptera) that sequester CNgls from their host (*T. ulmifolia* L.) are more distasteful to their *Anolis* predators (Zagrobelny et al. 2007; Lee et al. 2013). Larvae of the Burnet moth (*Z. filipendulae*; Lepidoptera) can sequester the CNgls linamarin and lotaustralin from their cyanogenic host plants, typically bird's-foot trefoil (Zagrobelny, Bak, and Møller, 2008; Zagrobelny and Møller, 2011)

In further support of these, Gleadow & Møller, (2014) documented evidence that demonstrates that factors affecting CNgc concentration can be explained in terms of a resource-based trade-off between plant growth and defense. The difficulty in calculating such costs may arise because the production costs are low and because CNgls have secondarily acquired important roles in nitrogen transport and storage and offer improved tolerance to oxidative stress, offsetting the direct costs of production. They further postulate that cyanogenesis is an effective defense against generalist herbivores but is not particularly effective against fungal pathogens. Many fungi efficiently convert HCN into ammonia and carbon dioxide. Some insect specialists have evolved mechanisms to sequester or denovo synthesize CNgls and use them as their defense against predators and as a source of reduced carbon and nitrogen (Webber and Woodrow, 2009).

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 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  $\beta$ -glucosidases and water.

## 1.5 CONCLUSION

The level of Cyanogenic glycosides varies phenologically, ontogenetically and chronologically. Cyanogenic content decrease with age of plants as influenced by increased activity of the beta-glucosidase; as cyanogenic compounds are metabolized to non-cyanogenic substances as the plant matures. Moreover, the highest levels of the cyanide detoxifying enzyme  $\beta$ -cyanoalanine synthase occur in young seedling tissues a response to higher levels of cyanogenic content. Based on this findings, it is recommended that managed pastures ought to be utilized preferably at mature stages with low levels of cyanogenic content. Additionally, levels of nitrogenous fertilizers (ammonia or nitrate) should be kept low as it increases the content of CNgls in pastures. Highly fertilized fields of forage should be avoided for grazing at succulent and immature stages. On the subject of grazing pressure, plants respond to herbivory by increasing the defensive chemicals; a proxy of

susceptibility to browsing. Defense strategies are likely to vary with different selective pressures (magnitude and type) and with developmental stage thus grazing regimes should consider intensities as well as grazing frequency.

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