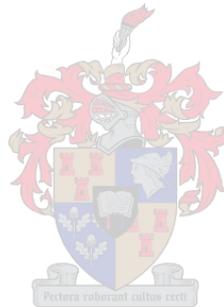


Interspecific and temporal variation of condensed tannins and cyanide concentrations in potential dietary sources of extralimital giraffes (*Giraffa camelopardalis*) in the Karoo

by

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof, that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Eugene Marais

April 2019

Abstract

During 2016 and 2018 four giraffe (*Giraffa camelopardalis*) mortalities occurred in the Karoo potentially caused by acute hydrogen cyanide poisoning. Plants have various defence mechanisms to protect themselves against herbivory, including the production of secondary metabolites such as condensed tannins and hydrogen cyanide. This study quantified condensed tannin and hydrogen cyanide production in selected Karoo plant species that giraffe may browse, to assess the possibility of acute hydrogen cyanide poisoning and condensed tannin intoxicification. Condensed tannins and hydrogen cyanide concentrations were explored in both spatial and temporal scales. The spatial assessment was performed at macro-scales (different locations within the Karoo), whereas temporal assessment was performed at seasonal scale. The effect of water availability and herbivory on condensed tannin production in *Vachellia karroo* trees was also investigated. Condensed tannin concentrations were high throughout seasons and did not differ significantly among the study sites in plant species giraffe primarily browse. In winter, *V. karroo* leaves were unavailable and secondary plant species increased in dietary importance. The most preferred plant species in the giraffes' diet, *V. karroo*, contained high levels of condensed tannins in mature leaves as well as in new-growth plant tissue. Condensed tannin concentrations increased significantly in several evergreen tree species during winter, including *Schotia afra* var. *afra* and species of the *Rhus* genus, which may indicate an increase in dietary importance during winter season. *Schotia afra* var. *afra* contained lower condensed tannin concentrations than *V. karroo* throughout the study. Condensed tannin production increased significantly in three of the four treatment groups of *V. karroo* trees that received simulated herbivory regardless of the browsing intensity. Both treatment groups which received water, increased in nitrogen contentment, whereas trees from the browsed and not watered treatment decreased in nitrogen content value and palatability. The high condensed tannin concentrations seem to be a fixed defence response by Karoo plants to browsing, or a response when sufficient water is available. The high condensed tannin concentrations may reduce the available browse as giraffe and other herbivores may reject leaves high in condensed tannins. However, giraffe have the ability to partially degrade condensed tannins and will therefore not be as susceptible to tannin intoxicification than other herbivores. The higher browsing pressure caused by giraffe may therefore be detrimental to other herbivores utilising the same plant species in the Karoo, that do not have the ability to

degrade condensed tannins. Therefore, careful considerations should be taken when introducing large game species into the Karoo. Only focusing on vegetation composition and abundance may be insufficient in predicting carrying capacities in semi-arid environments such as the Karoo without taking chemical composition into account. None of the plant species, except for one *Eucalyptus cladocalyx* tree, contained any measurable hydrogen cyanide, therefore making the probability of acute hydrogen cyanide poisoning highly unlikely. However, various other poisonous plants occur in the Karoo, these plants need to be investigated to determine whether they form part of the giraffe diet during times of limited browse, and how these plants may respond to browsing.

Uittreksel

Plante het verskeie verdedigingsmeganismes om hulself teen herbivore te beskerm, insluitende die produksie van sekondêre metaboliete soos gekondenseerde tanniene en waterstofsianied. In hierdie studie is gekondenseerde tannien- en waterstofsianied produksie in geselekteerde Karoo-plantspesies wat kameelperde (*Giraffa camelopardalis*) kan ineem/eet gekwantifiseer, om die moontlikheid van akute waterstofsianied vergiftiging en gekondenseerde tannien vergiftiging te bepaal. Gekondenseerde tanniene en waterstofsianied is ondersoek in beide ruimtelike en temporale skale. Die ruimtelike assessering is op makro-skaal (verskillende lokaliteite binne die Karoo) uitgevoer, terwyl tydelike assessering op seisoenale skaal uitgevoer is. Gekondenseerde tannienproduksie is ook ondersoek as 'n reaksie op verskillende omgewingstoestande en blaar verlies as gevolg van herbivore. Gekondenseerde tannien konsentrasies was hoog gedurende al die seisoene en het nie beduidend verskil tussen die studie areas nie. Plantspesies uit die *Rhus*-genus het gedurende die winter toegeneem in benutting en het aansienlik toegeneem in gekondenseerde tannienproduksie by al die studie areas. Die belangrikste plantspesie in die kameelperde se dieet, *V. karroo* het hoë vlakke van gekondenseerde tannienkonsentrasies in volwasse blare asook in nuwe groei plantweefsel bevat. Gedurende die winter was *V. karroo* blare onbeskikbaar en het sekondêre plant spesies toegeneem in benutting. Die gunsteling plant spesies in die kameelperd se dieet, *V. karroo*, het hoë vlakke gekondenseerde tanniene bevat in beide ou en nuwe blare. Immergroen plante soos *Schotia afra* var. *afra* en spesies van die *Rhus* genus was dus meer benut tydens die winter, en het hoër gekondenseerde tannien konsentrasies bevat tydens winter. Gekondenseerde tannienproduksie het toegeneem in drie uit die vier behandelingsgroepe van *V. karroo* bome wat gestimuleerde beweiding ontvang het, ongeag van die beweidingsintensiteit, aangesien geen beduidende verskille tussen die behandelingsgroepe geïdentifiseer kon word nie. Beide behandelingsgroepe wat water ontvang het, het egter toegeneem in voedingswaarde, terwyl bome in die behandelingsgroep wat hoë beweiding ontvang het, maar geen water nie, verminder het in voedingswaarde en smaaklikheid. Die hoë gekondenseerde tannienkonsentrasies blyk om 'n stabiele verdediging meganisme te wees in Karoo plante teen beweiding, maar ook tydens fases wanneer water beskikbaar is. Daarom wanneer dit oorweeg word om groot-wildspesies in die Karoo aan te hou, moet verskeie aspekte rakende chemiese verdediging deur plante in ag geneem word, en moet daar nie slegs net gefokus word op plantegroei samestelling en voorkeur nie. Dit kan onvoldoende wees om drakragte in semi-ariëde omgewings soos die Karoo te voorspel sonder om chemiese samestelling in ag te neem.

Geen van die plantspesies wat in hierdie studie ingesluit was, behalwe vir een *Eucalyptus cladocalyx* boom, het enige meetbare waterstofsianied geproduseer nie, dus is die waarskynlikheid van akute waterstofsianied vergiftiging hoogs onwaarskynlik. Daar is egter verskeie ander giftige plante in die Karoo, wat ondersoek moet word om te bepaal of hulle deel vorm van die kameelperde se dieet gedurende tye van beperkte plantegroei, en hoe hierdie plante potensieël kan reageer ten opsigte van chemiese produksie teen beweiding.

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Chapter 1

General introduction

1.1. Historical giraffe distribution and native feeding behaviours

Historically the highest concentrations of giraffe populations occurred in the savanna biome, which ranges from the northern parts of South Africa, to the Oranje River (Plug, 2001; Theron, 2005). The Limpopo Province in South Africa contains the largest giraffe populations as it falls directly into the savanna biome, making the habitat the most suited for giraffe due to the largest part of the savanna biome receiving summer rainfall, leading to prolonged summers, favouring *Acacia* (now referred to as *Vachellia*) tree species (deciduous trees) (Hall-Martin, 1974; Hall-Martin & Basson, 1975; Van Aarde & Skinner, 1975; Kok & Opperman, 1980; Sauer, 1983; Furstenburg & Van Hoven, 1994).

Giraffes generally browse from 1.8 to 4.6m above the ground, where they either strip or tip branches and require relatively large quantities of plant foliage to sustain their reproductive and metabolic requirements (Parker & Bernard, 2005). A sub-adult giraffe is known to consume 20-30kg, a cow up to 45kg, and a bull up to 48kg of fresh plant matter per day (Bothma & Du Toit, 2010). Trying to meet these energy needs, may require giraffes to be less selective during non-optimal conditions, which ultimately could result in generalist browsing by adding secondary plant species (up to 20 species) (Parker & Bernard, 2005).

Sauer et al. (1977) studied the feeding behaviour of giraffes in the Limpopo Province, to determine which plant species giraffe utilize the most. The results showed seasonal variation in the diet of giraffes as some plant species are deciduous. In a similar study, Frustenburg & Van Hoven (1994) studied the feeding behaviour of giraffes in the Kruger National Park (KNP), over a period of 12 months. In the KNP the diet of giraffes included 32 tree and shrub species, with only five species forming 83% of the total consumed plant species (*Acacia nigrescens*, *Acacia tortilis*, *Acacia welwitschii*, *Combretum imberbe* and *Dichrostachys cinereal*). However, during the cold dry season (winter) when the availability of leaf foliage of the preferred species declined, the giraffes changed their dietary preference to other plant species (*Acacia erubescens*, *Lonchocarpus capassa*, *Acacia robusta*, *Acacia xanthophloea*, *Acacia exuvailis* and *Maytenus heterophylla*) (Frustenburg & Van Hoven, 1994).

1.2 Feeding behaviour of extralimital giraffe populations

Several other feeding behaviour studies have been done in other provinces, to assess if vegetation outside of the Savanna biome could provide sufficient browse to extralimital giraffes. In the Free State, giraffes were observed browsing on 28 plant species, but only three plant species made up 74% of their diet (*V. karroo*, *Asparagus lariginus* and *Ziziphus mucronata*) (Theron, 2005). *Vachellia karroo* had a higher utilization value during the wet season (44%) than in the dry season (33%), *A. lariginus* had a constant utilization value throughout the year (16%) and *Z. mucronata* also showed a constant utilization value throughout the year. For the remaining plant species, the utilization value increased during the transition from the wet season (20%) to the dry season (32%) (Theron, 2005). Therefore, the utilization of less dominant plant species during the dry season increased in importance to fulfil dietary requirements as primary dietary plant species decreased (Theron, 2005).

Similar studies done by Parker & Bernard (2003, 2005) in the Eastern Cape, South Africa, identified 14 plant species forming the major component of the giraffes' diet, with the most important species being *Rhus longispina* (47.9%), *V. karroo* (25.7%) and *Euclea undulata* (17.6%). The importance of *R. longispina*, *V. karroo* and *Tarchonanthus camphoratus* fluctuated seasonally, with *R. longispina* being more important in the winter (61.1%) than in the summer (34.7%) and *V. karroo* were more important during the summer (39.6%) than in the winter (12.9%). *Tarchonanthus camphoratus* was only utilized during summer (18.2%).

1.2.1 Feeding behaviour of giraffes in the Karoo

Giraffes introduced into the Little Karoo have been known to mainly feed on *V. karroo*, *E. undulata*, *R. lancea*, and *Schotia afra* var. *afra* tree species found in and around river catchments (Gordon et al., 2016). *Vachellia karroo* are the most abundant tree species in the river catchments, but being a deciduous tree, only provides sufficient browse during the summer. The change in available browse (driven by seasonality and drought-precipitation cycles) have been linked to a shift in giraffe browsing to other evergreen plant species (Owen-Smith, 1992; Parker & Bernard, 2003, 2005; Gordon et al., 2016). In the winter months, Gordon et al. (2016) observed giraffes moving out of the main river of a particular region (Brak River) into smaller tributaries, searching for alternative browse, mainly on *Schotia afra* var. *afra* (Karoo Boer Bean) and *E. undulata*. Furthermore, Gordon et al. (2016) also noted giraffe regularly feeding below a height of 1.5m.

1.2.2 Impact of giraffe introduction on vegetation

Bond & Loffell (2001) studied the impacts of introduced giraffe on *Acacia* species at Ithala Game Reserve, KZN. The results showed that *Acacia davayi* largely disappeared from areas densely populated by giraffes. *Vachellia karroo* also showed high mortality in heavily browsed areas, although many trees continued to produce foliage on heavily browsed branches. Bond & Loffell (2001) concluded the constant intense browsing pressure is likely to weaken the trees, and predicted increased mortality during stressful situations, like drought and disease in the following years. Similar results were found by Viljoen (2013) who examined the effect of extralimital giraffe on the vegetation structure in the south-western region of the Kgalagadi Transfrontier Park. These results indicated that *Acacia haematoxylon* could suffer selective tree mortality through the impacts of giraffe browsing due to only a few potential food sources and the weaker thorns of *A. haematoxylon*, resulting in a loss of species and vegetation structural diversity.

However, browsing may also be beneficial to plants and herbivores. For example, Du Toit et al. (1990) reported *Acacia* (*Vachellia*) species compensated in shoot regrowth in heavily browsed areas compared to lightly browsed areas. Furthermore, Du Toit et al. (1990) reported foliage of heavily browsed *Acacia nigrescens* to be lower in defence chemicals such as condensed tannins (CT) and higher in nutrients than that of lightly browsed trees. Du Toit et al. (1990) proposed that severe pruning by browsers reduces the inter-shoot competition for nutrients, promoting rapid shoot regrowth. Carbohydrate demands of rapid regrowth reduce carbon-based secondary synthesis, resulting in patches of highly palatable browse that attract further browsing, creating a browsing regrowth feedback loop. Such patches may be considered analogous to grazing lawns.

1.2.3 Possible impact on Karoo vegetation

Due to giraffes being historically absent in the Karoo, native flora evolved in the absence of such browsing (trees browsed at higher heights, increasing the browsing intensity). Introducing giraffes may therefore potentially pose a risk to the native vegetation (over-browsing) as well as ecosystem structure (tree mortalities) (Bond & Loffell, 2001; Zinn et al., 2007; Viljoen, 2013) on the one hand, but may also threaten the introduced megaherbivore browsers on the other hand (food supply and plant response to increased browsing) (Parker & Bernard, 2005). However, most of the dietary studies regarding introduced giraffes, reported on browsing preferences or the effect of increased browsing on local vegetation, but few studies refer to

potential health effects (potentially leading to death) related to secondary metabolite plant responses linked to grazing or overgrazing that giraffes may experience.

1.3 Plant defence response to browsing

Plants have various defence mechanisms, including physical defences (thorns production; Myers & Bazely, 1991; Gowda, 1996), chemical deterrent defences (Du Toit et al., 1990), growth strategies (fast growth rates, growing too tall for leaves to be browsed; Milewski et al., 1991), and the inhibition of nutrient uptake (condensed tannins decreases palatability and nutrient absorption; Du Toit et al., 1990; Lundberg & Astrom, 1990; Rhoner & Ward, 1997; Stamp, 2003; Zinn et al., 2007; Idamokoro et al., 2016).

The production of secondary metabolites as a defence mechanism, either continuously or as a reactive response, acting directly or indirectly to manipulate/distract the herbivore is no new concept (Mithöfer & Boland, 2012). Moreover, differential defence responses against herbivory may be associated with different phenological stages (Matsuki et al., 2004) or environmental stress situations (for example drought, high temperature and solar radiation) (Mithöfer & Boland, 2012; Pavarini et al., 2012). Condensed tannins (CT) (proanthocyanidins) and hydrogen cyanide (HCN) are well-known secondary metabolites associated with herbivory defence in plants. Both are primarily produced by plants as an herbivore repellent by decreasing plant palatability (Schofield et al., 2001; Haque & Bradbury, 2002; Mithöfer & Boland, 2012).

1.3.1 Condensed tannins (CT)

Tannins are water-soluble polyphenolics with molecular weights usually ranging from 1000 to 3000 (Swain, 1979). In vascular plants, there are two main chemically distinct groups of tannins namely: hydrolysable tannins, which are further divided into ellagitannins and gallotannins, and condensed tannins (proanthocyanidins) which cannot be hydrolysed (Waterman & Mole, 1994, Hättenschwiler & Vitousek, 2000). A diversity of condensed tannins exists with distinct molecular structures. These compounds can be branched or linear polymers and flavan-3-ols such as epicatechin, catechin, epigallocatechin and galocatechin are the primary monomers (Gessner & Steiner, 2005).

Condensed tannins have ability to bind with dietary, microbial and enzymatic proteins thus creating insoluble complexes that are not degraded in the rumen of animals, resulting in reduced intake and digestibility of plant material. Condensed tannins can therefore lead to poisoning in wildlife (Cooper & Owen-Smith, 1985). Makkar (1995b) showed that rumen microbes are not capable of degrading condensed tannins, by exposing rumen microbes to

small amounts of quebracho tannins for 8 days, using a rumen simulation technique (Makkar et al., 1995b). Makkar (2003) concluded, that under situations of intestinal damage due to consumption of high levels of tannins or other intestinal membrane irritants, condensed tannins may get absorbed and can cause organ damages, eventually leading to death. For example, *Tragelaphus strepsiceros* (Kudu) mortalities were recorded during the dry winter periods in the savanna bushveld (game ranches in the Limpopo Province), from 1981 to 1986, and again during a drought in 2002 (Van Hoven, 1991; Hooimeijer et al., 2005). Significant correlations were found between average rainfall (decreased), density of kudu, tannin content of the browse and percentage mortality (Van Hoven, 1991). Frustenburg & Van Hoven (1994) investigated condensed tannin production by plants forming part of the diet of giraffes in the Kruger National Park. These results showed that condensed tannins negatively influenced the nutritional value of plants giraffe browsed on. Furthermore, plant species containing high levels of condensed tannins were found to be largely avoided by giraffes.

In addition, there was a positive correlation between condensed tannin production and browsing intensity, forcing giraffes to switch their diet among seasons to avoid plants responding by producing high tannin content. Frustenburg & Van Hoven (1994) also suggested that, when fencing off small land units consisting of homogeneous vegetation, the animals will be forced to utilize the same individual dietary plants more frequently (overgrazing preferred plant species). This may cause browsers to suffer from inadequate protein uptake, reduced digestion and tannin intoxication.

1.3.2 Cyanogenic glycosides (HCN)

In the plant, hydrogen cyanide is stored as inactive conjugates (mostly as glycosides) in the central vacuole (Mithöfer & Boland, 2012). In general, β -glucosidase hydrolyses glycosides, generating sugars and a cyanohydrin that naturally break-down to HCN and an aldehyde or ketone. When the plant is intact, the cyanogenic glycoside and β -glucosidase (in cytoplasm) remain separated, but when the plant tissue is damaged (chewing, wilting or some other cause), enzyme mediated hydrolyses can occur and HCN is released (Haque & Bradbury, 2002). Hydrogen cyanide (HCN), due to its capability of binding with metals (Cu^{++} , Fe^{++} , Mn^{++} and Zn^{++}), that form part of the catalytic centres of catalytic enzymes, may be exceptionally toxic (in some cases lethal) to many organisms (Vet Manual; Mithöfer & Boland, 2012). Although the HCN absorbed by the alimentary tract will be subjected to detoxification in the liver, high doses may exceed the capacity of this pathway allowing HCN to enter the cardiovascular system, eventually inhibiting mitochondrial metabolism, preventing oxygen use

by cells (McKenzie et al., 2009). This phenomenon may be linked to differential detoxification capacity in different herbivore species (McBarron, 1972). If the attractiveness and palatability of plant material is not affected (or the browser have no choice), increased feeding on such plant species may therefore pose a risk to herbivores.

Various studies have reported an increase in HCN production during drought, even more so following drought breaks, when new plant growth occur (Haque & Bradburry, 2002; Hayden & Parker, 2002; Robson, 2007). Fowler (1983) reported mortalities of several animal species linked to HCN poisoning. McKenzie et al. (2007), suggested that HCN poisoning was responsible for the deaths of 40 cows in Queensland, Australia, during, and after a drought period. Ruminants (cattle and wildlife, including giraffes) that digest their food through four rumination stages, and are known to be more susceptible to HCN poisoning than monogastric animals (for example, horses), due to the lower pH in the stomach of the monogastric animals, which helps to denature glycosidase (Bate-Smith & Swain, 1962; Robson, 2007). McKenzie et al. (2007) confirmed the increase sensitivity, of ruminants to HCN poisoning, reporting a minimum lethal dose ranging from 2-2.4mg/kg. Sudden death incidents by HCN poisoning have been reported in Alpacas in Australia that fed on the introduced South African daisy, *Dimorphotheca cuneata* (Karoo bietou,) (McKenzie et al., 2009). Several cases in South Africa, where *D. cuneata*, *Brabejum stellatifolium* (wild almond) and *Eucalyptus cladocalyx* were responsible for human, livestock and wildlife HCN poisoning have been reported (Steyn, 1949; Watt & Breyer-Brandwijk, 1962; Gleadow & Woodrow, 2000).

1.4 Giraffe mortalities

Although giraffe mortalities in the Little Karoo occurred on two private game reserves, unconfirmed reports suggested that this may be a more wide-spread phenomenon among extralimital giraffe populations. Moreover, confirmed mortalities occurred during April 2016, after a summer drought spell, followed by rains in March, and subsequent new plant growth (positive HCN production). It could therefore be hypothesized that as preferred plant species become unavailable, giraffes switched their food preference, resulting in increased browsing of secondary food species, that subsequently responded with variation in HCN and/or tannin production. Whether the severity of a prior drought period affects the magnitude of such defence response in affected plants remains unknown. Moreover, few studies have, in addition to condensed tannins, examined variation in HCN production in plants browsed on by giraffes.

The cross-talk pathways and interplay between these two well-known response pathways is not well-understood. Therefore, it is not surprising that the combined effects of these compounds have not been studied for extralimital giraffes in the Karoo.

1.5 Study Aims

In this study plant species forming the majority of the giraffes' diet in the Karoo was assessed for condensed tannin and hydrogen cyanide production, in both spatial and temporal scales. The spatial assessment was performed at macro-scale (at different locations), whereas temporal assessment was performed at seasonal scale. Condensed tannin production was furthermore explored as a response to different environmental conditions and browsing.

The main objectives of the current study were:

1. To test whether hydrogen cyanide and condensed tannins production varies seasonally, related to drought and different phenology stages, in the most important plant species in the giraffes' diet in the Karoo.
2. To explore whether the risk of hydrogen cyanide and condensed tannin poisoning increase during spring time (September), when there is higher species diversity providing new browse.
3. Evaluate the association between water content and condensed tannins and therefore the effect of drought stress.
4. To assess how the effect of herbivory and drought stress, and the combination of these factors may influence condensed tannin production as well as the nitrogen and carbon content of *V. karroo*.

Chapter 2

Seasonal variation of condensed tannins and hydrogen cyanide in plant species giraffe primarily browse in the Karoo

2.1 Introduction

Meeting metabolic and reproductive requirements is one of the most important determinants of animal fitness (Parker et al., 2009). In semi-arid landscapes seasonal changes affect the availability of plant species as well as the quality of the foliage (Parker & Bernard, 2003, 2005; Gordon et al., 2016). The change in plant species availability may have different consequences for different ungulate species, preferred tree species may be deciduous and thus cause a shift in browsing preference to evergreen trees or other plants. Herbivores may therefore exert high browsing pressure on selected plant species depending on the plant species phenology, as well as the evolutionary defence mechanisms.

Plants have various defence mechanisms, such as chemical deterrent defences (Du Toit et al., 1990), physical defences such as thorns (Myers & Bazely, 1991; Gowda, 1996), growth strategies (fast growth rates, growing too tall for leaves to be browsed, Milewski et al., 1991), and the inhibition of nutrient uptake (condensed tannins decreases palatability and nutrient absorption, Du Toit et al., 1990; Lundberg & Astrom, 1990; Rhoner & Ward, 1997; Stamp, 2003; Zinn et al., 2007; Idamokoro et al., 2016). While thorns may deter herbivores to graze foliage (Sebata, 2016), chemical defence compounds (secondary metabolites) may create resistance against herbivory to achieve the same result. Either strategy require plants to invest resources, potentially at the expense of growth and reproduction (Du Toit et al., 1990; Rohner & Ward, 1997; Viljoen, 2013). Moreover, to reduce costs allocated to defence, several authors suggested that there may be a trade-off between different types of defence mechanisms (and within) (Coley et al., 1985; Rohner & Ward, 1997; Sebata, 2016), although not mutually exclusive (Hanley et al., 2007, Sebata, 2016). It is also generally accepted that plants that use chemical defence strategies rely less on thorn defences (length and density) (Rohner & Ward, 1997; Zinn et al., 2007).

Since the aim of the larger investigation relates to the potential role of chemical defence compounds in recent mortalities reported in introduced giraffe (*Giraffa camelopardalis*) populations in the Western Cape the focus of the present study was on chemical defence

strategies. It is well documented that various plant species occurring in the Western Cape, may rely on chemical defence strategies (Van Wyk et al., 2002).

One chemical defence response in plants is the production of condensed tannins (CT, proanthocyanidins) (Rohner & Ward, 1999; Ward & Young, 2002; Scogings et al., 2004). Condensed tannins bind to protein molecules, reducing nutrient availability through decreased fibre digestibility, thereby affecting food and diet consumption of herbivores, which eventually could lead to starvation (reduced nutrient availability) (Ward & Young, 2002; Scogings et al., 2004; Zinn et al., 2007). Condensed tannins therefore could function as an effective anti-herbivory mechanism, although low concentrations may be beneficial, improving digestibility and alleviating parasite infection (Idamokoro et al., 2016; Rhodes et al., 2018).

Another general chemical defence response in plants are the production of cyanogenic glycosides, capable of liberating hydrogen cyanide (HCN, prussic acid) known to both affect palatability and exhibit acute toxicity when ingested (Harborne, 1993; Ngwa et al., 2004). In several reported cases high levels of HCN were associated with livestock mortalities during drought years, suggesting a link between climatic variation and the risk of HCN poisoning (Conn, 1979; Belovsky & Schmitz 1991; Van Wyk et al., 2002; McKenzie et al., 2007, 2009).

It is generally assumed that investment in defence mechanisms are costly since it will be offset against growth and reproduction (Briggs & Schultz, 1990; Rohner & Ward, 1997). Furthermore, different strategies and traits of herbivore deterrence may be negatively correlated, as few plants can invest in more than one of the above-mentioned defence mechanisms simultaneously (Rosenthal & Kotanen, 1994). In general, woody plants growing on nutrient-poor soils are slow-growing and contain relatively high levels of secondary metabolites such as CT, making them less palatable to browsers, than faster growing plants on nutrient-rich soils (Bernays et al., 1989; Du Toit, 1995). Plants with long-lived leaves, will have a fixed defence budget, and investment in defence mechanisms such as tannins is expected (Zinn et al., 2007). New plant growth however exhibits a high induction of defences, investing in more mobile defences such as HCN (Zinn et al., 2007). Various studies have reported an increase in HCN production during drought, even more so, following drought breaks, when new plant growth occur (Haque & Bradbury, 2002; Hayden & Parker, 2002; Robson, 2007).

The Karoo is a semi-desert environment where both water availability and soil nutrient status are low (Esler et al., 2006; Mucina et al., 2006). Various Karoo plants exhibit high levels of

chemical and mechanical defence mechanisms to protect them from browsing (Esler et al., 2006; Van Wyk et al., 2002). In recent years many agricultural farms in the Karoo have been converted to game farms due to the profitability associated with the hunting and tourism industry (Castley et al., 2001; Parker & Bernard, 2005; Skidmore, 2014). As a result, large charismatic herbivore species such as giraffe are frequently introduced into the Karoo. The introduction of these extralimital megaherbivore species may occur in spite of limited impact assessment, mostly without extensive scientific consideration of secondary metabolite responses by plants, which evolved in the absence of such browsing (trees browsed at higher heights, increasing the browsing intensity). Furthermore, *V. karroo* is one of the main plants in the giraffes' diet but are deciduous trees, only providing sufficient browse during the summer. The change in available browse (driven by seasonality and drought-precipitation cycles) have been linked to a shift in giraffe browsing to other evergreen plant species (Owen-Smith, 1992; Parker & Bernard, 2003, 2005; Gordon et al., 2016).

Two independent cases of giraffe mortalities occurred on a farm in the Karoo, where-after the autopsy stated the deaths may have been caused by HCN poisoning. Therefore, in this paper I examine two secondary metabolites, HCN and CT, and possible defence trade-offs in plants giraffe primarily browse in the Karoo. Furthermore, I examined whether HCN and CT production varies seasonally, related to drought and different phenology stages, in selected plant species.

2.2 Materials and methods

2.2.1 Study sites

The study was conducted at three sites in the Western Cape Province of South Africa. The three sites were selected based on their geographical position, plant diversity and the presence of giraffe populations. Doornrivier Private Game Farm (hereafter referred to as Doornrivier) lies at 33°39'26.8"S and 21°01'26.9"E. Touwsberg Private Game Reserve (Touwsberg) is situated at 33°37'59.7"S and 20°59'10.0"E, and Klipkraal Private Game Farm (Klipkraal) is positioned at 32°48' 18" S and 20°13'00" E (Figure 1).

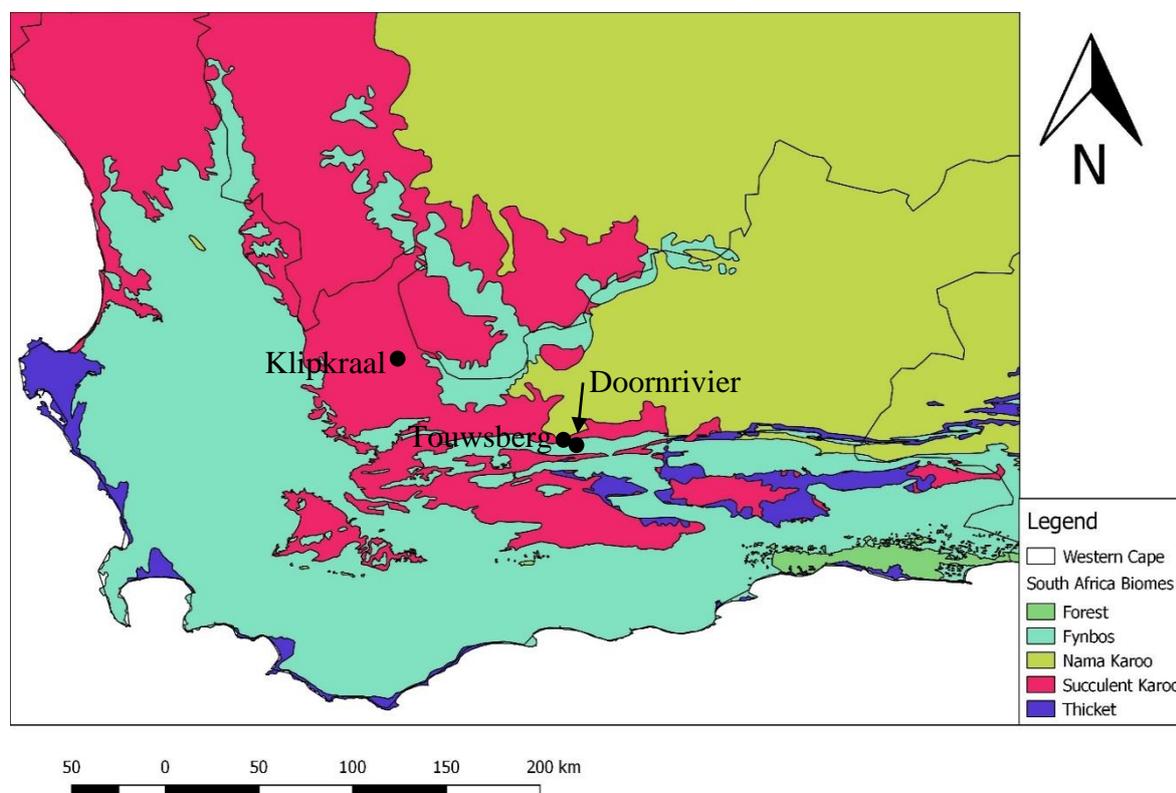


Figure 1: The map indicates the different biomes in which Klipkraal, Doornrivier and Touwsberg private game reserves are located, as well as their proximity to each other. The map was created using QGIS 2.18

Doornrivier: - This farm is the largest of the three study sites being ~ 17000 ha. The dominant geological formations of the reserve are derived from Devonian sandstone and parent materials of the Table Mountain Group, and the soil consists of saline, lithosol and loamy soils (Vlok et al., 2005). The non-perennial Doorn River flows through the reserve, entering on the south-western corner, and exiting on the north-eastern part of the reserve. Doornrivier falls within the

winter rainfall region of the province, but rainfall is sporadic, with minor peaks in March and November (Figure 2). The sporadic rainfall causes fluctuations in the amount of precipitation annually, and therefore rainfall can be as low as 80mm per year and as high as 230mm per year (Figure 2). The mean ambient temperature ranges from 16 to 40 °C, frost may occur for up to 14 days of the year, during the winter months (Gird, 2013). The vegetation of Doornrivier comprises of the Western Little Karoo and Muscadel Riverine vegetation types. The Western Little Karoo vegetation type are characterised by mosaics of Karoo shrublands of low and medium height non-succulents (*Chrysocoma*, *Pentzia*, *Rhigozum*) and succulent plant species (*Crassula*, *Euphobia*) (Mucina & Rutherford, 2011). The Muscadel River vegetation type consists out of several complex riverine thickets dominated by *V. karroo*, *S. afra* var. *afra* and *R. lancea* along with succulents such as *Salsola* species, and low vygie shrubland (Mucina & Rutherford, 2011). Five giraffe were initially introduced in 2014, however due to mortalities the total number of giraffes introduced to the property until 2018 were ten, of which two remain alive in 2018.

Touwsberg: - This game farm is ~ 8000 ha and lies adjacent, just north-west of Doornrivier (8,7km from Doornrivier to Touwsberg via road). The dominant geological formations of the reserve are derived from Devonian sandstone and parent materials of the Table Mountain Group, the soil consists of saline, lithosol and loamy soils (Vlok et al., 2005). The Touwsberg mountain occurs on the property, accumulating most of the rainfall on the reserve due to its elevation, creating a micro climate and allowing a variety of plant species including fynbos to grow on the mountain slopes. The mountain slopes downwards into the non-perennial Touws River which flows through the property. Like the Doornrivier farm, the Touwsberg farm is located within the winter rainfall region of the province, but rainfall is sporadic, with minor peaks in March and November (Figure 2). The sporadic rainfall causes fluctuations in the amount of precipitation annually, and therefore rainfall can be as low as 80mm per year and as high as 230mm per year (Figure 2). Most of the vegetation can be classified into the Little Karoo Broken Veld, which includes: Zorgvliet Apronveld, Kareebosch Ranteveld, Zorgvliet Pruiinveld and Zorgvliet Fynbos Gwarrieveld. The Muscadel Rivere vegetation type consists out of several complex riverine thickets dominated by *V. karroo* and *S. afra* var. *afra* along with several other *Rhus* and succulent species such as *Salsola* and low vygie shrubland (Mucina & Rutherford, 2011). In 2015 the farms giraffe population increased from five to eight since 2015, with no mortalities reported.

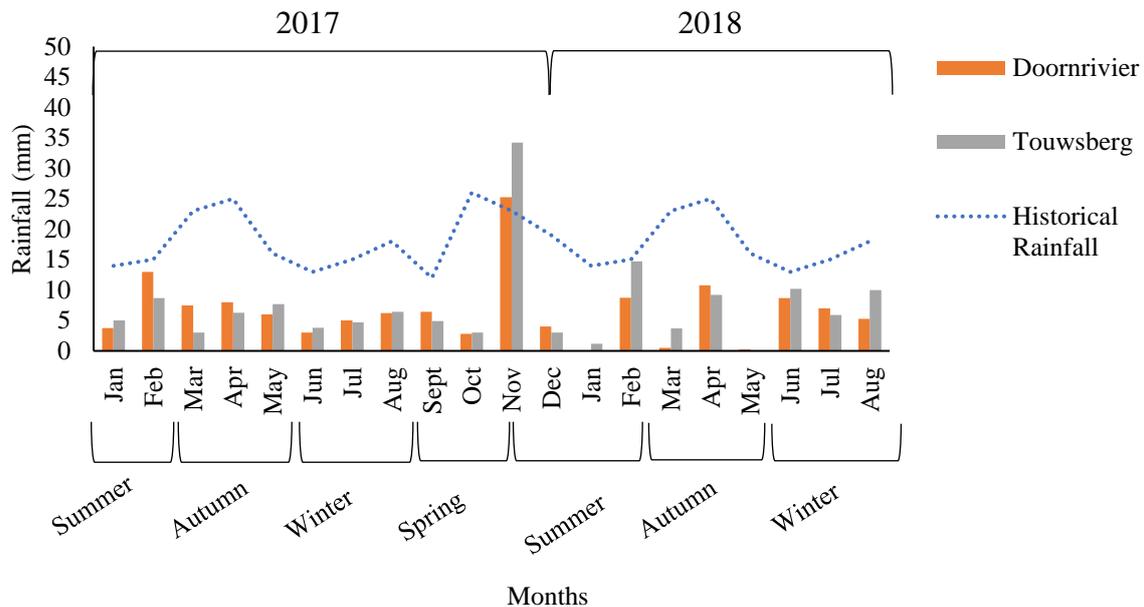


Figure 2: Annual historical rainfall (blue) from the Doornrivier and Touwsberg area, as well as measured rainfall at Doornrivier (orange) and Touwsberg (grey) during 2017 and 2018. *Note.* (2018). Meteoblue [online]. Available at: https://www.meteoblue.com/en/weather/forecast/week/touwsberg_south-africa_948297 (Accessed: 28 August 2018).

Klipkraal: - This private game farm is the smallest of the three study sites being only ~ 1885 ha and falling in the Greater Karoo, approximately 300km North-East of the other study sites (Figure 1). The dominant geological formations of the reserve include soils that consist mainly out of mudstone, sandstone and shale of the Adelaide Subgroup, together with sandstone, shale and mudstone of the Permian Waterford Formation and shale sandstone of other Ecca Group Formations as well as Dwyka Group diamictites (Mucina & Rutherford, 2006). The non-perennial Brak River flows through Klipkraal, with floodplains up to 400m wide. Klipkraal falls within the winter rainfall region of the province, with slight rainfall optima in June and July (Figure 3). Rainfall fluctuates annually, and can be as low as 100mm per year, and as high as 400mm per year. The mean ambient temperature is 15.8°C, with frost occurring approximately 30 days of the year during the winter months. The vegetation of Klipkraal comprises of SKv6 Koedoesberg-Moordenaars Karoo, and FRs5 Central Mountain Shale Renosterveld, with the most dominant shrubs being *Pteronia*, *Drosanthemum* and *Galenia*. (Mucina & Rutherford, 2006), and the floodplains of the river are characterised by thick stands

of *V. karroo* and *R. lancea* plants. Four giraffes were introduced to the reserve in 2013, and from a nucleus of one male and three females the population had grown to six in 2018, one calf did however die in 2018 when it fell from a rock ledge.

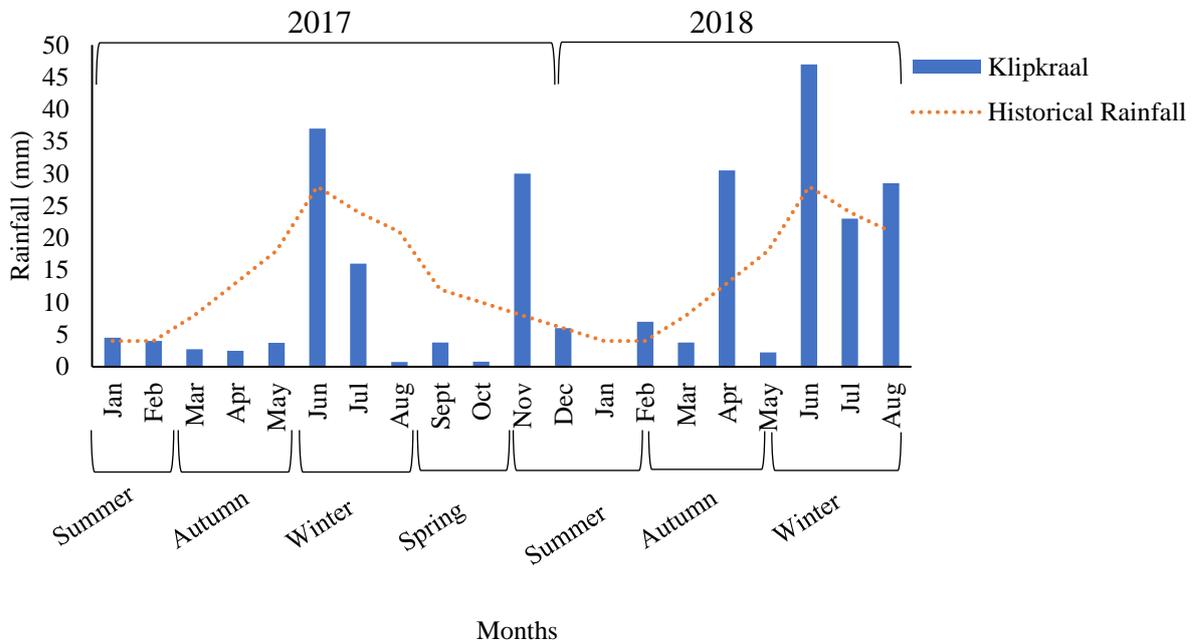


Figure 3: Annual historical rainfall (orange) for Klipkraal, as well as measured rainfall at Klipkraal during 2017 and 2018. *Note.* (2018). Meteoblue [online]. Available at: https://www.meteoblue.com/en/weather/forecast/week/gifkop_south-africa_1001907 (Accessed: 28 August 2018).

2.2.2 Study species

The plant species were selected based on published dietary reports (Parker & Bernard, 2003, 2005; Theron, 2005; Skidmore, 2014; Gordon, 2016) and impact assessments (Gird, 2012; Martin, 2012) to determine the HCN and CT concentrations in the primary food sources of giraffes' (Detailed list in Appendix A, section 2.1).

Table 1: Plant species sampled at Doornrivier, Touwsberg and Klipkraal during winter (July 2017), spring (September 2017), summer (December, only *V.karoo* and *S. afra* var. *afra*) and autumn (April 2018).

Family	Species	Location
Fabaceae	<i>Vachellia karroo</i>	All three study sites
Amaranthaceae	<i>Salsola aphylla</i>	All three study sites
Solanaceae	<i>Lycium oxycarpum</i>	All three study sites
Fabaceae	<i>Schotia afra</i> var. <i>afra</i>	Doornrivier & Touwsberg
Bignoniaceae	<i>Rhigozum obovatum</i>	Doornrivier & Touwsberg
Ebenaceae	<i>Euclea undulata</i>	Doornrivier & Touwsberg
Anacardiaceae	<i>Rhus burchellii</i>	Klipkraal
Sapindaceae	<i>Pappea capensis</i>	Doornrivier & Touwsberg
Anacardiaceae	<i>Rhus pallens</i>	Touwsberg
Celastraceae	<i>Gloveria integrifolia</i>	Touwsberg
Anacardiaceae	<i>Rhus lancea</i>	Klipkraal
Anacardiaceae	<i>Rhus longispina</i>	Doornrivier

2.2.3 Sample collection

Samples were collected in 2017-2018 during winter (July 2017: cold and dry climatic conditions, rainy season for Klipkraal), spring (September 2017: new growth), summer (December 2017: new-growth leaves of *V. karroo* and *S. afra* var. *afra*) and autumn (April 2018: Rainy season for Doornrivier and Touwsberg). All measurements of a type were taken at the same time of day, to avoid errors related to sampling at different times (circadian rhythm associated flux in secondary metabolites). Six plants were sampled per species, by clipping the end parts of the twigs and branches, where most of the leaves occur, from the top part of the tree (higher than 50% of the tree height), and from the lower half of the tree. Samples from these two height categories were taken from all four wind-directions around the tree (NESW), ensuring a good representation of the whole tree. The samples were placed in labelled plastic bags, and stored frozen at -18°C. At the laboratory the leaf samples were grinded into a powder

by using liquid nitrogen and a mortar and pestle. Thereafter, powder samples were stored in 50ml screw cap plastic centrifuge tubes at -80°C , until used for HCN and CT analyses.

2.2.4 Chemical analyses

Condensed tannins (CT): - All the methods used for CT analysis can be found in the Tannin Handbook written by Ann Hagerman (Hagerman, 2002). Condensed tannins were extracted from the grinded plant material, where-after aliquots of the samples were used in the acid-butanol assay (Hagerman, 2002) to determine CT concentrations. Quebracho tannin was purified and used as standard reference material and all the results were converted from grams of quebracho equivalents per millilitre (gQE/ml), to grams in quebracho equivalents per kilogram wet plant material (gQE/kgWM). The samples were loaded into a 96-well microtiter plate with the standard reference solution, and the absorbance was measured at 540nm using a spectrophotometer (Figure 4A) (detailed method description in Appendix A, section 1.2.1). Although several studies suggested that this assay may not account for certain insoluble and hydrolysable tannins, and therefore potentially underestimate the total tanniferous capacity of plant tissue (Waterman & Mole, 1994; Schofield et al., 2001; Heil et al., 2002), this assay is still commonly used as the most reliable method for condensed tannin determinations (Schofield et al., 2001; Scogings et al., 2013).

Hydrogen cyanide (HCN); - Feigl-Anger test papers as described by Kakes (1991) were used to test for total HCN production. Indicator paper-strips were prepared in-house using the methods described by Narval et al. (2011). Paper-strips were placed inside sealed test tubes containing individual grinded plant samples (Detailed description in Appendix A, section 1.1.1 and 1.1.2). The test tubes were incubated at room temperature for 24h, where-after paper strips were removed and photographed. The colour saturation of the photographed test papers was quantified using ImageJ software (6th edition, Version 1.52f) (Refer to Appendix A, section 1.1.2, for a detailed description). A standard curve was constructed using a certified reference standard HCN (Sigma-Aldrich: 90157) ranging from 1.995mg/l to 998mg/l (doubling dilution) (Figure 4B). By regressing the log of the mean saturation of the standard solutions on standard concentrations, the regression function obtained was used to estimate HCN concentrations in samples (Hayden & Parker, 2002).

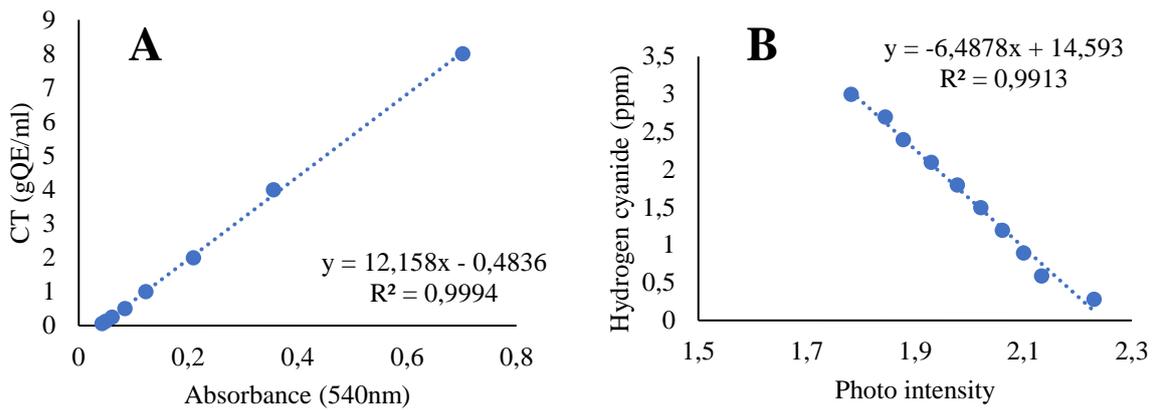


Figure 4A: The reference standard curve used to calculate CT production. **B:** The reference standard curve used to calculate HCN production.

2.2.5 Statistical analysis

All data were tested for normality using the Shapiro-Wilks test and normal probability plots of residuals. Generalised Linear and Mixed models were used to assess variation in secondary metabolite production of different plant species as a function of season and location. A p-value smaller than 0,05 was regarded as significant. STATISTICA version 13.3 (Tibco Software, USA) was used for all statistical analyses.

2.3 Results

2.3.1 Condensed tannins (CT)

2.3.1.1 *Variation among study sites*

Seasonal CT production in the selected plant species collected seasonally from the three study sites are summarised in Table 2. Condensed tannin produced by *V. karroo* differed significantly between the three study sites ($F_{2,26} = 7.944$, $p = 0.002$). At the Doornrivier and Klipkraal sites, CT in *V. karroo* leaves differed significantly during winter (July 2017) ($p = 0.040$) as well as during autumn (April 2018) ($p = 0.006$). Condensed tannin concentrations in *V. karroo* leaves differed significantly among Doornrivier and Touwsberg during winter (July 2017) ($p = 0.036$). However, CT production did not differ among sites (Doornrivier & Touwsberg) in two of the other shared species *S. afra* var. *afra* ($F_{1,34} = 1.926$, $p = 0.174$), and *Euclea undulata* ($F_{1,28} = 0.021$, $p = 0.885$).

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Table 2: Mean CT concentrations (gQE/kgWM \pm SE) of all plant species sampled at Doornrivier, Touwsberg and Klipkraal during winter (July 2017), spring (September 2017) and autumn (April 2018), as well as p-values. Dissimilar characters indicate significant differences for similar plant among between study sites, p - values indicate differences within study sites.

Location	Plant Species	Family	Condensed tannin concentration (gQE/kgWM \pm SE)			p-value
			Winter (July2017)	Spring (September 2017)	Autumn (April2018)	
Doornrivier	<i>V. karroo</i>	Fabaceae	247,19 \pm 40,72 ^a	unavailable	199,35 \pm 32,50 ^a	p = 0.380
	<i>S. afra</i> var. <i>afra</i>	Caesalpiaceae	87,58 \pm 22,97 ^c	72,28 \pm 12,02 ^c	65,10 \pm 9,78 ^c	p = 0.657
	<i>R. longispina</i>	Anacardiaceae	29,98 \pm 28,28	90,9 \pm 16,94	83,45 \pm 13,37	p = 0.102
	<i>P. capensis</i>	Sapindaceae	23,51 \pm 23,51	23,42 \pm 10,08	45,56 \pm 5,92	p = 0.364
	<i>E. undulata</i>	Ebenaceae	57,38 \pm 29,19	68,07 \pm 12,00 ^d	84,95 \pm 12,76 ^d	p = 0.573
Touwsberg	<i>V. karroo</i>	Fabaceae	153,21 \pm 23,27 ^b	unavailable	124,21 \pm 11,61 ^b	p = 0.254
	<i>S. afra</i> var. <i>afra</i>	Fabaceae	46,63 \pm 21,56 ^c	50,54 \pm 16,90 ^c	70,99 \pm 10,45	p = 0.536
	<i>R. pallens</i>	Anacardiaceae	32,02 \pm 32,02	126,22 \pm 8,38	128,78 \pm 33,22	p = 0.036
	<i>G. integrifolia</i>	Celastraceae	238,66 \pm 31,65	118,17 \pm 8,05	176,19 \pm 19,41	p = 0.013
	<i>E. undulata</i>	Ebenaceae	69,57 \pm 24,66 ^d	96,9 \pm 25,11 ^d	50,85 \pm 15,31	p = 0.311
Klipkraal	<i>V. karroo</i>	Fabaceae	139,2 \pm 27,50 ^b	unavailable	96,49 \pm 16,84 ^b	p = 0.150
	<i>R. lancea</i>	Anacardiaceae	131,38 \pm 24,98	171,64 \pm 27,45	116,53 \pm 10,24	p = 0.226
	<i>R. burchellii</i>	Anacardiaceae	19,27 \pm 9,40	51,33 \pm 12,10	18,03 \pm 9,75	p = 0.079

2.3.1.2 Seasonal variation within study sites (see Table 2)

Doornrivier: - The plant species sampled on Doornrivier varied significantly in overall seasonal CT production ($F_{4,69} = 24.144$, $p < 0.01$). The following plant species CT production did not vary significantly among seasons: *V. karroo* ($F_{1,10} = 0.843$, $p = 0.380$), *S. afra* var. *afra* ($F_{2,19} = 0.429$, $p = 0.657$), *Pappea capensis* ($F_{2,8} = 1.150$, $p = 0.364$) and *E. undulata* ($F_{2,16} = 0.576$, $p = 0.573$). CT production in *Rhus longispina* did not vary significantly among seasons ($F_{2,16} = 2.639$, $p = 0.102$). However, there was a significant pairwise difference in CT production in *R. longispina* at Doornrivier between winter (July 2017) and spring (September 2017) ($p = 0.044$).

Touwsberg: - On Touwsberg there was a significant variation ($F_{4,56} = 16.043$, $p < 0.001$) in overall CT production were recorded among plant species. The following plant species did not differ significantly in seasonal CT production: *V. karroo* ($F_{1,6} = 1.591$, $p = 0.254$), *S. afra* var. *afra* ($F_{2,15} = 0.596$, $p = 0.563$) and *E. undulata* ($F_{2,12} = 1.291$, $p = 0.311$). However, CT production in *Gloveria integrifolia* leaves varied significantly among seasons when considered collectively ($F_{2,11} = 6.629$, $p = 0.013$); however, *post hoc* pairwise comparisons indicated a significant difference in CT production between winter (July 2017) and spring (September 2017) ($p = 0.004$). Condensed tannin production varied significantly at seasonal scale in *Rhus pallens* ($F_{2,12} = 4.389$, $p = 0.037$). In particular, the *post hoc* tests showed winter (July 2017) and spring (September 2017) differed significantly ($p = 0.021$) as well as samples taken during the winter (July 2017) and in autumn (April 2018) ($p = 0.023$).

Klipkraal: - On Klipkraal overall CT production among species varied significantly ($F_{2,37} = 24.793$, $p < 0.001$). Condensed tannin production in *V. karroo* leaves did not differ significantly in seasonal CT production ($F_{1,10} = 1.75$, $p = 0.15$). The same was true for *R. lancea* ($F_{2,15} = 1.644$, $p = 0.226$). Condensed tannin production in *Rhus burchellii* leaves did not differ significantly in overall seasonal CT production ($F_{2,12} = 3.147$, $p = 0.079$), however there was a significant difference in CT production between spring (September 2017) and autumn (April 2018) ($p = 0.04$).

2.3.1.3 New plant growth on selected plant species

Condensed tannin production in new growth (shoots & leaves) of *V. karroo* during summer (December 2017) varied significantly among study sites ($F_{2,15} = 11.514$, $p < 0.01$). *Post hoc* tests revealed significant differences in CT production between Touwsberg and Doornrivier (p

< 0.01) as well as Doornrivier and Klipkraal ($p < 0.01$), but no significant differences among Touwsberg and Klipkraal ($p = 0.831$).

On Klipkraal, CT production in new-growth leaves of *V. karroo* varied significantly among seasons (winter [July 2017] vs. summer [December 2017]; $p = 0.019$; summer [December 2017] vs. autumn [April 2018]; $p < 0.01$) (Figure 5). Condensed tannin produced by new-growth leaves varied significantly on Doornrivier between seasons (winter [July 2017] vs. summer [December 2017]; $p < 0.01$; summer [December 2017] vs. autumn [April 2018]; $p = 0.012$) (Figure 5). Similarly, on Touwsberg CT in new-growth leaves of *V. karroo* varied significantly among winter (July 2017) and summer (December 2017); $p = 0.03$) as well as summer (December 2017) and autumn (April 2017); $p = 0.014$) (Figure 5).

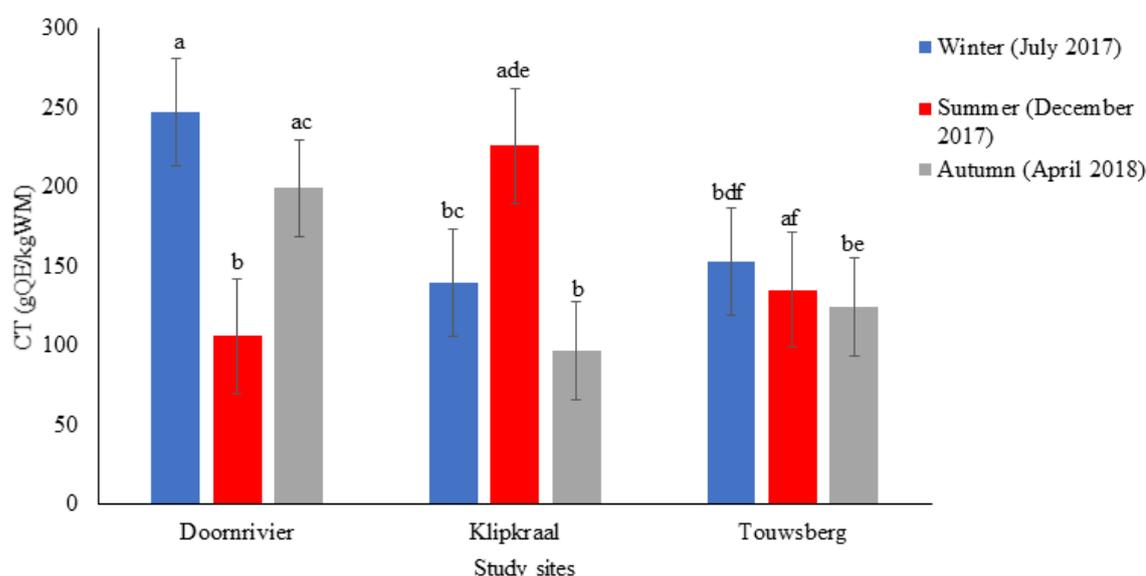


Figure 5: Mean (\pm SE) CT concentrations (gQE/kgWM) in new-growth leaves (December 2017) of *V. karroo* compared to CT concentrations of “older” mature leaf samples taken in winter (July 2017) and autumn (April 2018) from the different study sites. Dissimilar characters indicate significant differences.

In contrast, the CT produced in new growth (leaves) of *S. afra* var. *afra* collected from the Doornrivier and Touwsberg reserves in the summer (December 2017) did not vary significantly ($F_{1,12} = 2.719$, $p = 0.125$). Furthermore, *S. afra* var. *afra* new-growth samples collected on Doornrivier did not show significant seasonal variation when comparing summer (December 2017) samples to new-growth samples taken in winter (July 2017) ($p = 0.634$), spring (September 2017) ($p = 0.536$) and autumn (April 2018) ($p = 0.175$) (Figure 6). Similarly, new-

growth leaf samples of *S. afra* var. *afra* collected on Touwsberg, during summer (December 2017) and compared to samples collected during other seasons did not differ significantly from samples collected in winter (July) (Figure 6).

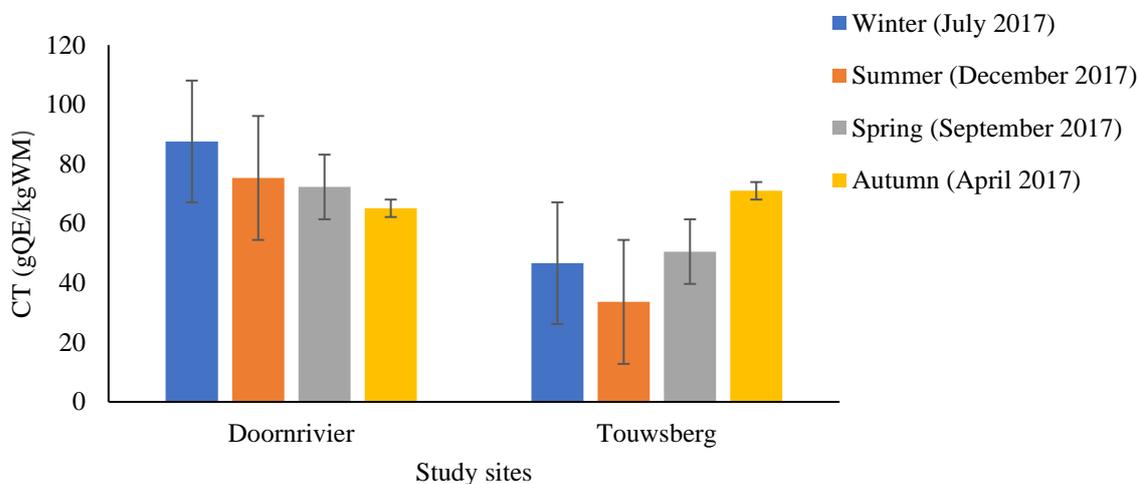


Figure 6: Mean (\pm SE) CT concentrations (gQE/kgWM) in new-growth leaves (December 2017) of *S. afra* var. *afra* compared to CT concentrations of “older” mature leaf samples taken in winter (July 2017), spring (September 2017) and autumn (April 2018) from the different study sites.

2.3.2 Hydrogen cyanide (HCN)

The methods used to determine HCN production was validated by testing *Vachellia sieberiana* var. *woodii* using the procedure described in section 2.2.4. All *V. sieberiana* var. *woodii* samples tested positive. However, no HCN was detected in the plant species (see list in Table 1) sampled at Doornrivier, Touwsberg and Klipkraal throughout the seasonal sampling events.

2.4 Discussion

Seasonal changes in production of defence chemicals and palatability affects forage selection by herbivores, impacting temporal patterns of herbivory (Boeckler et al., 2011; Zweifel-Schielly et al., 2012). However, fenced off areas (game farms/reserves) do not allow herbivores (native and extralimital) to move through the landscape in search of more palatable plant species (Frustenburg & Van Hoven 1994). The introduction of extralimital herbivores such as giraffe may have a detrimental impact on the ecosystem structure. For example, Bond & Loffel (2001) reported selective mortalities in several *Acacia* (*Vachellia*) species, as well as local extinctions caused by giraffe browsing. Giraffes are almost exclusively browsers and require large amounts of plant foliage to sustain their metabolic and reproductive requirements. These requirements may lead to an increase in secondary metabolite production, especially in semi-arid environments with limited palatable plant species, as plant species may become over-browsed. Therefore, the introduction of giraffes may affect food selectivity and availability to native herbivores, especially browsers, as they cannot move through the landscape to seek more palatable plant species.

The chemical composition of plant foliage vary seasonally, as young plant tissue is generally rich in nutrients such as nitrogen and starch, and low in secondary defence compounds that reduce palatability (Augustine & McNaughton, 1998; Wan et al., 2014). However, in semi-arid environments, new plant growth comes at an energy cost, and needs to be chemically defended (Frustenburg & Van Hoven, 1994). The secondary metabolite defence response in young leaves to giraffe browsing in the Karoo is still unknown, and may differ from other regions, as these plants evolved in the absence of such browsing (trees browsed at higher heights and greater browsing intensity).

Secondary metabolite production by plants may vary due to plant phenology and environmental stressors such as drought and browsing. Therefore, the production of HCN and CT was assessed among species, localities and seasons in selected locations in the Karoo. From the results of the study no HCN was detected in any primary plant species. However, high concentrations of CT were recorded, which varied seasonally in and among study sites.

2.4.1 Condensed tannins variation among study sites

Touwsberg can be considered as a control site for Doornrivier, as no giraffe mortalities have yet occurred on the property. During winter (July 2017) and autumn (April 2018) CT production by *V. karroo* was the highest on Doornrivier compared to Touwsberg and Klipkraal. However, other plant species occurring on both properties, such as *E. undulata* and *S. afra* var. *afra*, did not differ significantly from each other. Condensed tannin concentrations exceeding 100g/kg are considered high (Mangan, 1988; Min et al., 1998), while several studies have indicated that CT concentrations below 50g/kg may be beneficial to herbivores (Min et al., 1998; Idamokoro et al., 2016). The greater kudu (*Tragelaphus imberbis*) has been observed to avoid plant species with high tannin content as their diet consists largely of woody plants species that produce CT as an herbivore repellent (Cooper & Owen-Smith, 1985). Van Hoven (1991) found that 20-25% of the total kudu population have died due to fencing of private land, following a severe drought, showing CT production may increase during drought when limited browse is available, affecting herbivores, especially browsers, to the extent of severe population mortalities. Athanasiadou et al. (2001) showed in an experimental setup, sheep that were fed food containing 162-220gQE/kg stopped eating by day 30 and needed to be removed from the experiment. In the present study many of the plant species collected from all three study sites exceeded the 100gQE/kg threshold during the sampling events. To place the CT concentrations recorded in the present study, in context, plant species such as *V. karroo*, *R. lancea*, *G. interifolia* and *R. pallens* that giraffes primarily browse on in the Karoo (Skidmore, 2012; Gird, 2012; Gordon et al., 2016), were found to be substantially higher in CT concentrations than other plant species giraffe browse on in other locations (Table 3). Frustenburg & Van Hoven (1994) stated, when fencing off small land units consisting of homogenous vegetation and allowing the animal population to increase above the fluctuating ecological carrying capacity (as influenced by drought) of the habitat, the animals will be forced to utilise the same plants more frequently. Their results indicated that CT production increased by 13-78% within 2-7min of physical disturbance and recovery to normal equilibrium was accomplished after 40-66h (Frustenburg & Van Hoven, 1994). Thus, if plants are browsed before normal equilibrium of CT is reached, browsers may suffer from inadequate protein uptake, reduced digestion and tannin toxification when their habitat is put under severe overstocking rates or environmental stress such as drought. The browsing of plants before the recovery of normal tannin equilibrium may cause the plants to increase CT production even

more, with the equilibrium then being set at a higher level (Frustenburg & Van Hoven, 1994). This may cause CT to become a fixed defence mechanism by plants, leading to a sustained abnormally high production of CT within the plants.

Table 3: Condensed tannin concentrations (g/kg) of different plant species from various studies and study sites (SA- South Africa; ZI- Zimbabwe; IL- Israel; KNP- Kruger National Park)

Plant species	Authors	Location	Condensed tannin (g/kg)
<i>V. karroo</i>	Present study	Doornrivier, SA	184,1 ^a
<i>V. karroo</i>	Present study	Touwsberg, SA	135,0 ^a
<i>V. karroo</i>	Present study	Klipkraal, SA	153,9 ^a
<i>V. karroo</i>	Mapiye et al. (2009c, 2010)	SA	80,7 ^b
<i>V. karroo</i>	Sebata (2016)	Bulawayo, Zi	90,7 ^a
<i>V. karroo</i>	Marume et al. (2012a)	South Africa	21 ^c
<i>V. raddiana</i>	Rohner & Ward (1997)	Yotvata, IL	82,27 ^d
<i>V. tortilis</i>	Rohner & Ward (1997)	Yotvata, IL	48,92 ^d
<i>V. tortilis</i>	Frustenburg & van Hoven (1994)	KNP, SA	13,2 ^c
<i>V. gerrardii</i>	Sebata (2016)	Bulawayo, Zi	40,1 ^a
<i>V. nilotica</i>	Sebata (2016)	Bulawayo, Zi	60,9 ^a
<i>V. nilotica</i>	Frustenburg & van Hoven (1994)	KNP, SA	161,9 ^b
<i>V. rehmanniana</i>	Sebata (2016)	Bulawayo, Zi	60,87 ^a
<i>V. erioloba</i>	Viljoen (2013)	Kgalagadi, SA	33,1 ^e
<i>V. haematoxylon</i>	Viljoen (2013)	Kgalagadi, SA	29,09 ^e
<i>V. robusta</i>	Frustenburg & van Hoven (1994)	KNP, SA	13 ^c
<i>V. nigrescens</i>	Frustenburg & van Hoven (1994)	KNP, SA	33,3 ^c
<i>V. welwitschii</i>	Frustenburg & van Hoven (1994)	KNP, SA	23,6 ^c
<i>V. exuvialis</i>	Frustenburg & van Hoven (1994)	KNP, SA	66,4 ^c
<i>V. xanthophloea</i>	Frustenburg & van Hoven (1994)	KNP, SA	68,5 ^c
<i>V. sieberiana</i>	Zinn & Kirkman (2009)	Ottos Bluff district, SA	34,26 ^a

^a Quebracho reference standard

^b Cyanidin reference standard

^c Respective purified tannin standards

^d Catechin reference standard

^e Sorghum tannin standard

2.4.2 Seasonal variation within study sites

Gordon et al. (2016) focused on how seasonality affected giraffes' diet in the Little Karoo, South Africa, and found *V. karroo* to be the most important dietary plant species throughout the summer but offered limited browse during the winter months. During winter *E. undulata* and *S. afra* var. *afra* were browsed significantly more than other plant species (Gordon et al., 2016). Similar results were showed by Parker & Bernard (2005), who reported that *V. karroo* and *R. longispina* were the most important dietary plant species across all seasons at all three their study sites in the Eastern Cape. Similarly, during winter, when *V. karroo* provided limited browse, the consumption of *R. longispina* and *S. afra* var. *afra* increased significantly (Parker & Bernard 2005).

On Doornrivier, *R. longispina* had a significant increase in CT production from winter to spring and summer. For the other plant species sampled, no significant variation in CT was recorded. The relatively high CT concentrations throughout the year may be the result of overstocking, increasing the browsing pressure on the plants, resulting in CT to be a fixed defence mechanism/deterrent or creating a resistance to over-browsing by the plant species (Rohner & Ward, 1997; Zinn et al., 2007). However, the sustained increase in CT production in most dietary plants sampled on Doornrivier needs further investigation. One possible explanation may be the reported association/correlation between CT and water availability. Horner (1990) has shown CT to correlate positively (or curvilinearly) with water stress. In the present study Doornrivier received less rainfall than Touwsberg, during 2017 and 2018 when compared to the same period in 2017, up until April 2018. The influence of water stress on CT production is addressed in Chapter 4.

On Touwsberg, *R. pallens* showed a significant increase in CT production from winter (July 2017) to spring (September 2017). However, except for *G. integrifolia* that showed decreased CT production from winter (July 2017) to summer (December 2017), no significant differences were found in CT production for other plants.

On Klipkraal, *R. burchellii* differed significantly in seasonal CT production, with the highest value recorded during spring (September 2017) when *V. karroo* browsing (leaves) was still unavailable. An increase in CT production during winter (July 2017) and spring (September 2017) was recorded on Klipkraal mainly due to an increase in CT production by both *R. lancea* and *R. burchellii*. The difference in seasonal CT production on Klipkraal may be caused by

the lower plant species diversity (with regards to plants browsed by giraffe), thus forcing giraffes and other game species to increase utilization of the same plant species when *V. karroo* offers limited browsing (Sauer et al., 1977).

On all three study sites, *Rhus spp.* increased in CT production during winter months, which may be the result of dietary switching and therefore increased utilization by browsers. Similarly, Parker et al. (2003) found *Rhus spp.* to increase significantly in dietary importance, as the utilisation of the species increased during autumn and winter months for giraffe populations in the Eastern Cape.

2.4.3 New plant growth on selected species

Condensed tannins (CT) production increased significantly in young *V. karroo* sprouts and leaves in summer (December 2017) on both Touwsberg and Klipkraal compared to “older/mature” leaf samples taken during winter (July 2017) and autumn (April 2018). Several studies have shown giraffe to prefer new and growing *V. karroo* shoots when they are available, suggesting that they serve as a good food source due to their increased succulence or water content, and higher protein content (Hall-Martin & Basson, 1975; Sauer, 1983; Cooper et al., 1988). Furthermore, several studies have shown *V. karroo* to be the preferred browse compared to any other available plant species by giraffes in the Karoo, as well as in the Eastern Cape (Parker et al., 2003; Parker & Bernard, 2005; Gordon et al., 2016). From observations during the present study, *V. karroo* shoots were severely browsed during late spring (September 2017), limiting the availability of material. Sufficient shoot and leaf samples could only be taken during summer (December 2017) when trees produced excess of shoots. Thus, the increased CT production being higher on both Touwsberg and Klipkraal compared to Doornrivier may be caused by an investment in chemical defence or browsing resistance by *V. karroo* in an effort to protect new plant growth in summer (December 2017). The decrease in CT production by new *V. karroo* shoots on Doornrivier during the same time, may be the result of a decrease in browsing pressure, compared to the other two farms. From July 2017 the Doornrivier farm management started reducing the game on the property, in an attempt to reduce the browsing and game mortalities, possibly linked to malnutrition. The total large game head remaining at Doornrivier by April 2018, was reduced to almost half of the game population in January 2017.

New growth (leaves) of *S. afra* var. *afra* did not differ from “older”, mature leaves collected during other sampling events on both Doornrivier and Touwsberg. *Schotia afra* var. *afra* is the second most utilised plant species in the Little Karoo (Skidmore, 2012; Gordon et al., 2016).

This tree species has been suggested to provide browse throughout the year, therefore the continuous browsing, but less intense, may result in a sustained, but less variable CT production, as a fixed (resistance) defence strategy against herbivory. However, further studies are needed to understand the chemical defence biology of *S. afra* var. *afra*, to determine if any other, yet undetermined chemicals are produced by young leaves that may affect leaf selection and/or digestibility.

2.4.4 Hydrogen cyanide (HCN)

Fabaceae is a plant family known to contain species that produce HCN, however, *V. karoo* belonging to Fabaceae, did not produce any measurable HCN throughout the sampling period. In fact, HCN could not be detected in any of the plant species evaluated, this result consequently meant the relationship between HCN and CT in the selected Karoo plants could not be explored. In July 2018 two giraffe mortalities occurred on the Doornrivier property, and samples of the trees they browsed were taken immediately after their death. No measurable HCN was detected in the plant samples, confirming the results of previous sampling events that HCN poisoning by the selected plant species in this study is highly unlikely. The possibility of false negatives in the analysis can be largely out-ruled as *Vachellia sieberiana* var. *woodii* (a tree known to produce HCN) tested positive for HCN production at sample sizes as small as 2mg of fresh plant material. The certified HCN reference standard was used at concentration range varying from 1.9mg/l to 998mg/l, making the test sensitive enough to detect small amounts of HCN.

2.5 Conclusions

In the present study, it was confirmed that HCN remained below detection in plant species giraffe primarily may browse in the Karoo. However, high concentrations of CT were recorded throughout seasons and among study sites, compared to CT production reported in other studies including all relevant/important (giraffe diet) plant species in the Karoo. However, unlike sheep and various other herbivores, giraffe have the ability to consume feed relatively high in CT, as they secrete tannin-binding salivary proteins (proline) which counter the digestibility-reducing effect of ingested CT (Robbins et al., 1987; Shimada, 2006). However, Furstenburg & Van Hoven (1994) reported giraffe to avoid leaves high in CT. Therefore, further investigation is required. The high CT concentrations will pose a direct health threat to herbivores without this capability, therefore adaptive management should be used in semi-arid environments, like the Karoo, to ensure the well-being and survival of other game species. The severe drought experienced during the seasons of 2017 and 2018, made these farms even more vulnerable to game loss. Furthermore, the overstocking may result in plants investing too much energy in defence mechanisms, ultimately causing increased plant mortality, and therefore limited browsing together with increased browse switching during drought situations.

Although the present study could not confirm that HCN may be a defence mechanism that lead to mortalities, following periods of drought and limited availability of preferred browse, more studies are needed regarding the range of potential lethal secondary metabolites (phenolic glycosides, Rhodes et al., 2018) that lesser known plant species produce at specific locations or seasonally when giraffes are forced to include such species in their diet.

Chapter 3

Condensed tannins and hydrogen cyanide concentrations of secondary plant species giraffe browse in the Karoo

3.1 Introduction

Plants have evolved various mechanical and chemical defence mechanisms to protect themselves from browsing, particularly in resource limited environments (Robbins et al., 1987; Augustine & McNaughton, 1998; Aganga & Tshwenyane, 2003). Chemical defence mechanisms include the production of secondary metabolites such as condensed tannins (CT) and cyanogenic glycosides (enabling the release of hydrogen cyanide, HCN) (Cooper & Owen-Smith, 1985; Poulton, 1990; Furstenburg & Van Hoven, 1994; McKenzie et al., 2009). Condensed tannins may defend plants against herbivory by reducing cell wall, protein, and sodium digestion and retention (Freeland et al., 1985; Furstenburg & Van Hoven, 1994; Rohner & Ward, 1997). Various studies have reported young new-growth plant tissue to be rich in nutrients such as nitrogen and starch, and low in secondary defence compounds such as CT (Augustine & McNaughton, 1998; Wan et al., 2014). However, Furstenburg & Van Hoven (1994) showed young new-growth plant leaves contained higher CT concentrations.

Cyanogenic glycosides have been recognised in over 3000 plant species from all vascular plant taxa, distributed throughout 130 different plant families (Conn, 1981; Poulton, 1990; Gleadow et al., 2009). The mechanism of acute cyanide poisoning is well known, it occurs through the inactivation of mitochondrial cytochrome c and disruption of aerobic respiration, which results in potentially fatal cellular hypoxia and cytotoxic anoxia (Ballantyne, 1987; Villalba et al., 2002; McKenzie et al., 2009)

Cyanogenic potential of plants is affected by species variety, weather, soil fertility and state of plant growth (Poulton, 1990; Robson, 2007). Various studies have shown the cyanogenic potential of plants to increase during drought conditions, and even more so after drought breaks, when stressed, stunted plants begin to grow (McKenzie et al., 2007; Robson, 2007). Temperature can also affect HCN levels, as shown by, Hayden & Parker (2002) who reported HCN production to decrease during cold weather conditions, as enzyme activity decreases with a decrease in temperature. In the Karoo most of the woody plant species are palatable, but slow growing due to limited resources (Cowling & Roux, 1987; Esler et al., 2010). In drought scenarios or when a fenced-off area is over-stocked, or even more so when these factors are

combined, palatable plant species may be over-browsed, in extreme cases causing plant mortalities (Bond & Loffel, 2001; McKenzie et al., 2009). The loss or reduction of palatable plant species allows unpalatable (exhibiting heavy chemical and/or mechanical defences) plant species to increase in abundance in the veld (McNaughton 1983a; McInnes et al., 1992; Augustine & McNaughton, 1998). Augustine & McNaughton (1998) reported changes in herbivore density affected foraging selectivity (due to changes in frequency and amount of tissue loss). As herbivore density increases, selectivity is expected to decline due to reduced availability of preferred forage. With a decrease in plant availability of selectivity, the consumption of potentially poisonous plants by herbivores may increase. Forced resource switching may cause herbivores to rely more heavily on detoxification pathways to deal with increase intake of toxins.

Under normal circumstances, herbivores may be capable of detoxifying secondary metabolites as only small amounts are ingested (Fowler, 1983; Foley et al., 1999; Villalba et al., 2002). Furthermore, during periods of drought herbivores may/are known to experience a reduction in gastrointestinal microflora, which may further decrease the capacity to detoxify secondary compounds, such as HCN and CT (Longhurst et al., 1968; Fowler, 1983). Added to the resource switching phenomenon is also the potential that increased toxin production could occur as a response to initial increased grazing of general unpalatable plants.

The Karoo is a semi-arid environment (Esler et al., 2010), which experienced a drought during 2015-2018 (Personal communication: Freddie la Grange). The results presented in Chapter 2 of the present study, showed the most important plant species in the giraffes' diet in the Karoo to have high CT production compared to plant species in the giraffes' native habitats. The results of Chapter 2 suggested that plants giraffes primarily browse in the Karoo were over-utilised, which caused plants to produce large quantities of CT, reducing the plant palatability.

Two giraffe mortalities occurred in 2016, as well as in 2018 on the Doornriver farm, Ladismith, Western Cape, and post mortem examinations suggested acute poisoning (possibly HCN) to be the cause of death. No data are however available concerning HCN production and concentrations in preferred (primary) and non-preferred giraffe forage on Doornrivier, or other game farms in the Karoo where giraffes occur.

From the reviewed literature, the possibility of HCN poisoning cannot be out ruled and needs to be investigated, in particular, at times when palatable primary dietary plant species are excessively browsed to such an extent that they provide limited nutritional value. In response,

herbivores may seek alternative food sources (secondary plants) to sustain their reproductive and metabolic requirements (Sauer et al., 1982; Jacobs, 2008; Gordon et al., 2016). Various studies have reported a shift in giraffe browsing preference to evergreens during the winter months as deciduous species from the genus *Vachellia* (*Vachellia karroo*) becomes unavailable (Parker & Bernard, 2005; Jacobs, 2008; Gordon et al., 2016). Impact studies done before the introduction of giraffe in the Karoo identified palatable shrubs occurring below 1.5m as available browse to giraffe and provide reports of giraffe browsing plants below this foraging height (Theron, 2005; Gird, 2012; Martin, 2012). This behaviour suggest that giraffe need to seek additional food in order to sustain their metabolic and reproductive requirements (Theron, 2005). The need to meet their daily dietary requirements may cause giraffe in the Karoo to forage on plants they are unfamiliar with, such as annuals and bulbs, that emerge during spring (September). Because the Karoo is such a resource stressed environment, various plants rely on chemical and mechanical defence mechanisms (Van Wyk et al., 2002) to deter herbivores. However, these plants evolved in the absence of such browsing (increased browsing pressure) (Parker & Bernard, 2005) and how they respond to giraffe browsing with regards to chemical defences in young new-growth plant tissue are still unknown. Moreover, whether these chemical defence response by Karoo plants pose a potential health risk to giraffe still needs to be investigated.

Therefore, this study aims to assess the possibility of CT and HCN poisoning in the Karoo, especially during spring (September) as woody secondary plants species grow new-leaf shoots and various annuals and bulbs emerge and flower. The objectives of this study were to assess CT and HCN concentrations in a selection of evergreen plant species giraffe may browse in the Karoo. Furthermore, to assess the possibility of HCN production by annuals, bulbs and various shrub species giraffe may potentially browse in the Karoo.

3.2 Materials and Methods

3.2.1 Study sites

The study was conducted on three study sites namely Doornrivier, Touwsberg and Klipkraal. Refer to section 2.2.1 for a detailed description of the study sites.

3.2.2 Study species

Potential secondary dietary plant species (Table 1) were selected from published dietary reports (Parker & Bernard, 2003, 2005; Theron, 2005; Skidmore, 2014; Gordon, 2016) and impact assessments (Gird, 2012; Martin, 2012), and confirmed to be present and available to giraffe in the Karoo study areas (Detailed plant list in Appendix A, Section 2.1).

Table 1: All the plant species sampled and analysed for CT and HCN production on Doornrivier, Touwsberg and Klipkraal during spring (September 2017). ^e Evergreen, ^d deciduous, ^{s-d} semi-deciduous

Family	Plant species	Location(s)
Amaranthaceae	<i>Salsola aphylla</i> ^e	Doornrivier, Touwsberg, Klipkraal
Anacardiaceae	<i>Rhus burchellii</i> ^e	Klipkraal
	<i>Rhus lancea</i> ^e	Klipkraal
	<i>Rhus longispina</i> ^e	Doornrivier, Touwsberg
	<i>Rhus lucida</i> ^e	Touwsberg
	<i>Rhus pallens</i> ^e	Doornrivier, Touwsberg
Apocynaceae	<i>Carissa haematocarpa</i> ^e	Doornrivier, Touwsberg
Asteraceae	<i>Pteronia adenocarpa</i> ^e	Doornrivier
Bignoniaceae	<i>Rhigozum obovatum</i> ^e	Doornrivier, Touwsberg
Caesalpiniaceae	<i>Schotia afra</i> var. <i>afra</i> ^e	Doornrivier, Touwsberg
Celastraceae	<i>Gloveria integrifolia</i> ^e	Doornrivier, Touwsberg
Ebenaceae	<i>Diospyros austro-africana</i> ^e	Klipkraal
	<i>Euclea undulata</i> ^e	Doornrivier, Touwsberg
Sapindaceae	<i>Pappea capensis</i> ^{s-d}	Doornrivier, Touwsberg
Solanaceae	<i>Lycium oxycarpum</i> ^e	Doornrivier, Touwsberg, Klipkraal

3.2.3 Sample collection

Terminal leaf shoot samples were collected during September 2017 (spring) from a selection of shrubs and trees (Table 1) that giraffe may potentially browse in the Karoo (Detailed plant list in Appendix A, Section 2.1). For CT and HCN analyses, all the leaf samples were taken at the same time of day (from 05:30-07:00am and 17:30-19:00pm) to minimize variation among samples associated with circadian rhythm-linked metabolite flux. Plant samples were taken by clipping the end parts of the twigs and branches (approximately 10cm), where most of the leaves occur, from the top part of the tree (higher than 50% of the tree height), and from the lower half of the tree. Samples from the two selected heights were taken from all wind-directions around the tree (NESW), to account for chemical variation within the tree. Samples were placed in labelled plastic bags, and stored at -21°C . In the laboratory the leaves were frozen using liquid nitrogen and grinded to powder using a mortar and pestle. Thereafter the powder was stored in 50ml centrifuge tubes at -80°C until used for analysis.

Additional samples of annuals and bulbs (Table 3) giraffe may potentially browse were also taken and tested in-field for HCN production. Samples consisted out of flower heads and leaves that were placed into a 1.5ml Eppendorf tube and crushed using a pellet pestle, where after 0.5ml of MillQ water was added into the tube. A Feigl-Anger test paper was placed under the lid of the tube to test for free HCN production after 24h (Description for preparation of test papers in Appendix A, Section 1.1). If a sample tested positive, more of the plant was collected for quantification of HCN production at the laboratory.

3.2.4 Chemical analysis

All plant species listed in Table 1 was used for CT and HCN analyses. Condensed tannins (CT) were assayed using the Acid-Butanol assay described by Hagerman (2002) (Detailed description in section 2.2.4, Detailed method described in Appendix A, section 1.2.1). The release of HCN gas was tested using the Feigl-Anger test papers described by Kakes (1991) (Detailed description in section 2.2.4, Detailed method described in Appendix A, Section 1.1.1)

3.2.5 Statistical analysis

All statistical analyses were performed using STATISTICA version 13.3 (Tibco Software, USA). Condensed tannins (CT) data were tested for normality using the Shapiro-Wilks test and normal probability plots of residuals. Generalised Linear and Mixed models were used to assess variation in secondary metabolite production of different plant species as a function of location.

No statistical analysis was performed for HCN as only one plant tested positive. A p-value smaller than 0,05 was regarded as significant.

3.3 Results

3.3.1 Condensed tannins

Klipkraal had the highest average CT production (CT = 116.959gQE/kgWM), however, there were only two species that produced measurable CT on Klipkraal (Figure 2). Doornrivier had the second highest average CT production (CT = 93.353gQE/kgWM) with Touwsberg having the lowest average CT production (CT = 76.663gQE/kgWM). *Gloveria integrifolia* on Doornrivier had the highest CT production (Figure 2, CT = 191.322gQE/kgWM) but did not differ significantly from samples taken on Touwsberg (CT = 118.174gQE/kgWM, $F_{1,5} = 1.320$, $p = 0.306$). *Pappea capensis* had the lowest CT production on both Doornrivier (CT = 23.423gQE/kgWM, Figure 2) and Touwsberg (CT = 19.330 gQE/kgWM, Figure 2) but no significant difference occurred between the two study sites ($F_{1,4} = 0.069$, $p = 0.806$). Similarly, *Rhus longispina* did not differ significantly in CT production between Doornrivier and Touwsberg ($F_{1,8} = 0.099$, $p = 0.762$), as well as *Rhus pallens* ($F_{1,8} = 0.396$, $p = 0.271$) (Figure 2). *Carissa haematocarpa* did however differ significantly in CT production between Doornrivier (CT = 161.268 gQE/kgWM, Figure 2) and Touwsberg (CT = 40.745 gQE/kgWM, Figure 2, $F_{1,4} = 16.567$, $p = 0.015$). Condensed tannins production in *S. afra* var. *afra* leaves differed significantly between Doornrivier (CT = 77.115g/kgWM, Figure 2) and Touwsberg (CT = 41.416gQE/kgWM, Figure 2, $F_{1,28} = 5.394$, $p = 0.028$). Condensed tannins production in *Euclea undulata* leaves did not differ significantly between Doornrivier (CT = 68.071gQE/kgWM, Figure 2) and Touwsberg (CT = 96.904/kgWM, Figure 2, $F_{1,11} = 1.360$, $p = 0.268$). *Rhus lancea* did not differ significantly in CT production between Doornrivier (CT = 128.362gQE/kgWM, Figure 2) and Klipkraal (CT = 171.646g/kgWM, Figure 2, $F_{1,10} = 1.594$, $p = 0.244$).

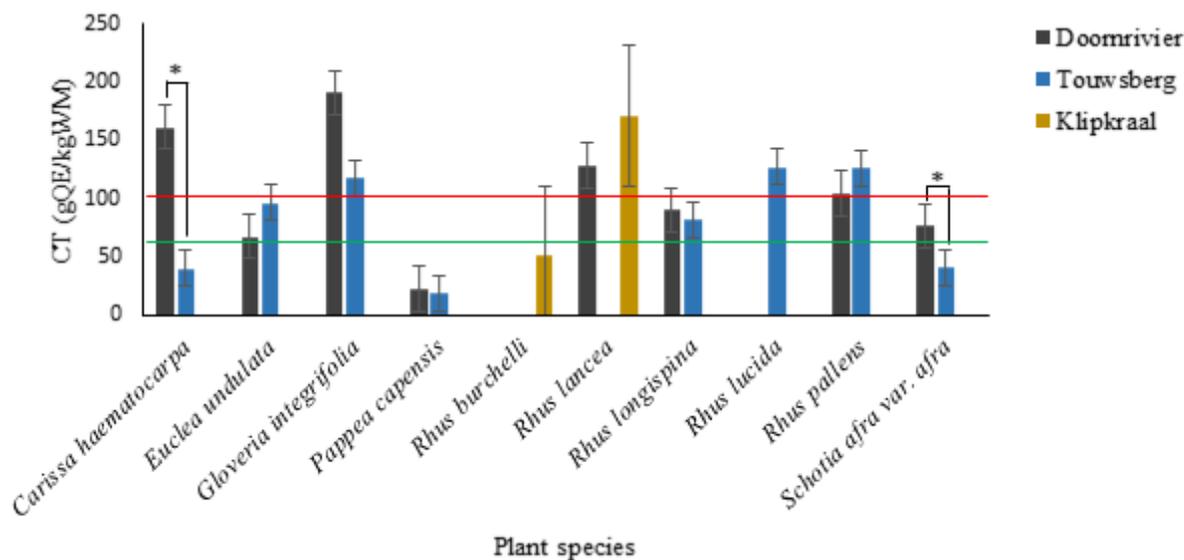


Figure 2: Condensed tannin production (gQE/kgWM \pm SE) of different plant species sampled on Doornrivier (grey), Touwsberg (blue) and Klipkraal (brown) during September 2017. The green horizontal line indicates CT concentration considered to be beneficial to herbivores (60gQE/kgWM), whereas the red horizontal line indicates CT concentration considered detrimental to herbivore health (100g/kgWM).

All three study sites contained plant species that did not produce any measurable CT (Table 2). The plant species listed in Table 2 was excluded from the statistical analysis as they contained no CT data.

Table 2: Plant species that did not produce any measurable CT at the different study sites.

Family	Plant species	Location
Amaranthaceae	<i>Salsola aphylla</i>	Doornrivier, Touwsberg, Klipkraal
Solanaceae	<i>Lycium oxycarpum</i>	Doornrivier, Touwsberg, Klipkraal
Bignoniaceae	<i>Rhigozum obovatum</i>	Doornrivier, Touwsberg
Asteraceae	<i>Pteronia adenocarpa</i>	Doornrivier
Ebenaceae	<i>Diospyros austro africana</i>	Klipkraal

3.3.2 Hydrogen cyanide

The plant species listed in Table 3 were tested for HCN production. Only *Eucalyptus cladocalyx* contained free HCN of all the plant species listed in Table 3. Additional *E. cladocalyx* samples were taken to the laboratory to quantify HCN production (Figure 3). Hydrogen cyanide production (ppm) of *E. cladocalyx* showed a positive linear relationship with the wet sample weight (mg). 1000mg of *E. cladocalyx* yielded 697.185ppm HCN (Figure 3).

Table 3: Plant species sampled in-field for hydrogen cyanide production (HCN) in the Karoo during spring (September 2017).

Family	Species	Hydrogen Cyanide (HCN)
Acanthaceae	<i>Blepharis capensis</i>	None
Aizoaceae	<i>Cylindrophyllum comptonii</i>	None
	<i>Drosanthemum crassum</i>	None
	<i>Drosanthemum giffenii</i>	None
	<i>Galenia africana</i>	None
	<i>Lampranthus dependens</i>	None
	<i>Phyllobolus splendens</i>	None
	<i>Ruschia caroli</i>	None
	<i>Ruschia pungens</i>	None
	<i>Ruschia spinosa</i>	None
Amaranthaceae	<i>Salsola aphylla</i>	None
Anacardiaceae	<i>Rhus burchellii</i>	None
	<i>Rhus lancea</i>	None
	<i>Rhus longispina</i>	None
	<i>Rhus lucida</i>	None
	<i>Rhus pallens</i>	None
Apocynaceae	<i>Carissa haematocarpa</i>	None
Asphodelaceae	<i>Bulbine frutescens</i>	None
Asteraceae	<i>Chrysocoma ciliata</i>	None
	<i>Euryops lateriflorus</i>	None
	<i>Felicia dubia</i>	None
	<i>Pentzia incana</i>	None
	<i>Pteronia adenocarpa</i>	None
	<i>Schotia afra</i> var. <i>afra</i>	None
Caesalpiniaceae	<i>Gloveria integrifolia</i>	None
Crassulaceae	<i>Crassula arborescens</i>	None
	<i>Crassula cultrata</i>	None
	<i>Crassula rupestris</i>	None
	<i>Tylecodon cacaloides</i>	None
	<i>Tylecodon paniculatus</i>	None

	<i>Tylecodon wallichii</i>	None
Ebenaceae	<i>Diospyros austro africana</i>	None
	<i>Euclea undulata</i>	None
Euphorbiaceae	<i>Euphorbia mauritanica</i>	None
Malvaceae	<i>Hermannia althaeifolia</i>	None
Meliaceae	<i>Nymania capensis</i>	None
Myrtaceae	<i>Eucalyptus cladocalyx</i>	Yes
Poaceae	<i>Cenchrus ciliaris</i>	None
	<i>Merxmullera disticha</i>	None
Polygalaceae	<i>Polygala pinifolia</i>	None
Ridaceae	<i>Homeria pallida</i>	None
Sapindaceae	<i>Dodonaea viscosa</i>	None
Solanaceae	<i>Lycium oxycarpum</i>	None
	<i>Lycium pumilum</i>	None
Zygophyllaceae	<i>Zygophyllum foetidum</i>	None

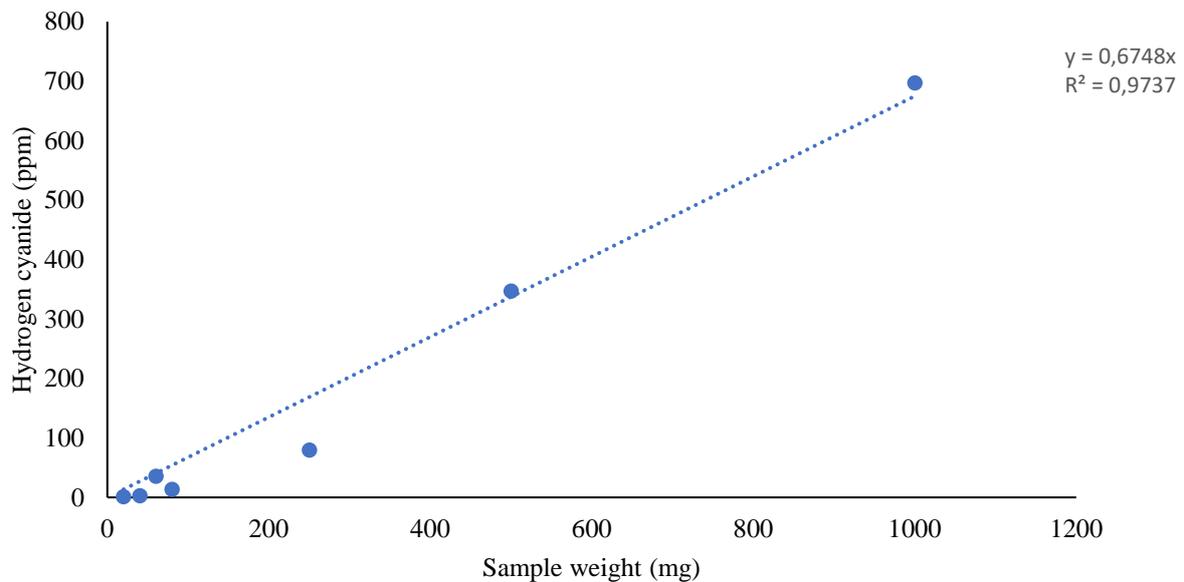


Figure 3: Free hydrogen cyanide (ppm) had a positive linear relationship with sample weights (mg) taken from *Eucalyptus cladocalyx* on Doornrivier during spring (September 2017).

3.4 Discussion

Vachellia karroo is the most preferred plant species giraffe browse in the Karoo (Skidmore, 2012; Gordon et al., 2016). However, during winter, in the Karoo, *V. karroo* is prone to leaf loss and offers limited available browse. In the present study, new *V. karroo* shoots were only available in surplus after December, therefore herbivores need to change their diet to evergreens during the winter months to sustain metabolic and reproductive requirements. The seasonal switching of plant species by giraffes are no new occurrence, various studies have shown giraffe to change their diet in their natural habitat (Limpopo) (Furstenburg & Van Hoven, 1994), as well as extralimital giraffe occurring in the Eastern Cape (Parker & Bernard, 2003, 2005) and the Free State (Theron, 2005). However, there is dramatic differences in various factors such as environmental conditions, species identity, species richness and species abundance from the above-mentioned locations and the Karoo in the Western Cape (Cowling et al., 1997). Seasonal changes, such as spring time, can influence the chemical composition of plant species, as young plant tissue starts to grow and is generally rich in nutrients such as nitrogen and starch, and low in secondary defence compounds (condensed tannins) that reduce palatability (Augustine & McNaughton, 1998; Wan et al., 2014). As new plant growth comes at an energy cost in semi-arid environments, plants may have to invest more in defence mechanisms to limit tissue loss by herbivores (Furstenburg & Van Hoven, 1994). Plants in the Karoo may therefore rely more heavily on chemical defence mechanisms (such as condensed tannin production and free hydrogen cyanide) during stages of new plant growth (spring) in the Karoo.

Therefore, new-growth plant tissue was collected from secondary plant species in the Karoo to determine whether CT production was high (exceeding 100g/kg) during spring, and whether new-growth tissue of secondary plant species, as well as various other plant species giraffe may forage contained any free HCN. From the results six out of the 15 secondary plant species produced high amounts of CT in new-growth leaves, whereas none of the 15 secondary plant species produced any detectable free HCN. From all the 35 additional plant species sampled for free HCN, only *E. cladocalyx* produced measurable free HCN.

3.4.1 Condensed tannins

In the present study *C. haematocarpa*, *G. integrifolia*, *R. lancea*, *R. lucida* and *R. pallens* produced high concentrations of CT (concentrations exceeding 100g/kg), while *E. undulata* and *R. longispina* produced moderately high concentrations of CT (exceeding 60g/kg).

Condensed tannins production may increase when the same individual plant is rebrowsed before CT concentrations can return to normal tannin equilibrium, causing the tannin equilibrium to be set at a higher level (Furstenburg & Van Hoven, 1994). Klipkraal, situated in the greater Karoo, had the highest average CT concentrations in spring when compared Doornrivier and Touwsberg in the Little Karoo. However, Klipkraal only had two focus species, both from the *Rhus* genus. Parker & Bernard (2003, 2005) as well as Theron (2005) and Jacobs (2008) have shown *Rhus spp.* to increase in importance for extralimital giraffe during the cold seasons when primary plant species are unavailable. This may explain the high CT concentrations recorded on all three study sites for all *Rhus spp.*, except for *R. burchellii* on Klipkraal. However, plant species composition and abundance are higher in the Eastern Cape (Parker & Bernard, 2003, 2005) and the Free State (Theron, 2005; Jacobs, 2008) than in the Karoo (Gordon et al., 2016). The lack/low abundance of palatable plant species in the Karoo coupled with the high Large Stock Units (LSU)/overstocking on both Klipkraal and Doornrivier may explain the high CT concentrations (Refer to section 2.1.1 for information regarding farm size and giraffe population). Furstenburg & Van Hoven (1994) reported that when fencing off small land units consisting of homogenous vegetation and allowing the animal population to increase above the fluctuating ecological carrying capacity (as influenced by drought) of the habitat, the animals will be forced to utilize the same individual dietary plants more frequently. Touwsberg had the highest species diversity as well as species abundance due to the Touwsberg Mountain (creating a micro climate) that occurs on the property. The mountain also creates more floodplains and river tributaries, which allows thick stances of *S. afra* var. *afra* and *V. karroo* (Gordon et al., 2016). The relatively higher abundance of *S. afra* var. *afra*, coupled with the higher species richness compared to Klipkraal and Doornrivier, may explain the lower average CT concentrations, as the browsing pressure on individual plants may be lower. The high (exceeding 100g/kg) CT concentrations recorded in secondary plant species may pose a greater health risk to giraffes on both Doornrivier and Klipkraal as giraffes may be forced to seek additional food sources below their preferred browsing height (2-4m).

3.4.2 Hydrogen cyanide

Eucalyptus cladocalyx is known to be the main source of HCN poisoning of livestock in the Western Cape Province, South Africa (Van Wyk et al., 2002). Basson (1987) did an experimental study where potassium cyanide powder was administered to several herbivores. The results show HCN concentrations ranging from 370ppm - 700ppm to cause clinical signs

of poisoning within 4-18 minutes (Basson, 1987; Mithöfer & Boland 2012). In the experiment done by Basson (1987) one eland (*Taurotragus oryx*) died after receiving an HCN dosage of 700ppm (Basson, 1987). From the results, *E. cladocalyx* on Doornrivier, produced high enough quantities of HCN to potentially cause acute poisoning, however *E. cladocalyx* have been present on the property since the game farm was established. When sufficient browse is available, herbivores will occasionally browse poisonous plants as the quantity of toxins are small and diluted by the more preferred plant species (Fowler, 1983; Pfister, 1999). However, in adverse environmental conditions such as the drought the Karoo experienced during 2015-2018, herbivores may be more susceptible to poisoning. Longhurst et al. (1968) stated shortage in carbohydrates or nitrogen in the diet of herbivores reduces the richness of the gut flora and allow poisoning, which would not occur when better forage is available. Therefore, the possibility exists of HCN poisoning being responsible for the giraffe mortalities on Doornrivier as giraffe may have browsed *E. cladocalyx* to maintain their metabolic and reproductive requirements. However, it is unlikely that *E. cladocalyx* was responsible for the giraffe mortalities, because the carcasses were found approximately 5km from the *E. cladocalyx* tree, a distance the animals could unlikely cover because HCN poisoning is rapid and acute (personal communication: Prof. Jan Myburgh). Furthermore, none of the plant species tested in-field contained any free detectable HCN, including the secondary plant species giraffe may potentially browse during spring. New-growth plant tissue of the selected secondary plant species therefore does not produce HCN as an herbivore deterrent in the Karoo.

3.5 Conclusions

Condensed tannins were high in new-growth plant leaves in the most important secondary dietary plant species (*Rhus spp.*). However, whether plants in the Karoo invest more in CT production in new leaves to deter herbivores, or whether high browsing pressure causes a response to produce more CT in the new-growth leaves are still unknown and needs to be further investigated. Nonetheless, the high CT concentrations in secondary plant species on Doornrivier and Klipkraal may pose health risks to the giraffe as they may be forced to browse unfamiliar plant species (potentially poisonous) in order to fulfil their reproductive and metabolic requirements. Due to the feeding behaviour of giraffe, they may increase the browsing pressure on individual plants, which may in return cause the plants tannin equilibrium to rise. Giraffe can browse trees containing high concentrations of CT, however, giraffe will still avoid plant species containing high CT concentrations due to only being able to partially degrade CT (Hagerman et al., 1993). Therefore, high CT concentrations can still reduce the availability of browse and pose constraints on metabolic and reproductive requirements of giraffe. However, other herbivores however, especially grazers, may be directly negatively affected by high CT concentrations due to their inability to degrade CT.

No free HCN was detected in any of the 46-plant species tested in-field, which included various Karoo shrubs, annuals and bulbs available during spring. The lack of any detectable free HCN indicates the probability of HCN poisoning being responsible for the giraffe mortalities that occurred on Doornrivier is highly unlikely. However, various other poisonous plants were abundant in the veld on all three study sites. Therefore, more research needs to be done to assess the possibility of plant poisoning by other secondary compounds such as cardiac glycosides in the Karoo.

Chapter 4

Condensed tannin production in Karoo tree species *Vachellia karroo* in response to simulated herbivory and watering

4.1 Introduction

Several plants have, through natural selection, developed metabolic responses to intense grazing and browsing, across both evolutionary and ecological timescales to reduce leaf loss caused by herbivory. These responses used by plants as resistance to defoliation may be costly, as trade-offs are known to exist between defence mechanisms, growth and reproduction (Coley et al., 1985; Rohner & Ward, 1997; Koricheva, 2002). Plant defence mechanisms may include chemical defence (Du Toit et al., 1990), physical responses (such as thorn production; Myers & Bazely, 1991; Growda, 1996; Sebata, 2010), varied growth strategies (growing too tall for the leaves to be eaten; Milewski et al., 1991; Pablo et al., 1993), and decreasing nutrient availability (Lundberg & Astrom, 1990).

Woody plants growing on nutrient-poor soils in general, are slow growing and may produce relatively high levels of chemical compounds such as condensed tannins (CT), making them less palatable to browsers. Fast growing plants however growing on nutrient rich soils are generally more palatable due to less chemical defences (Bryant et al., 1989; Du Toit 1995). Du Toit et al. (1990) showed that *Acacia* (*Vachellia*) species compensated in shoot regrowth in heavily browsed areas compared to lightly browsed areas. Furthermore, Du Toit et al. (1990) showed that foliage of heavily browsed *Acacia nigrescens* to be lower in CT and higher in nutrients than that of lightly browsed trees. Du Toit et al. (1990) proposed that severe pruning by browsers reduced the inter-shoot competition for nutrients, promoting rapid shoot regrowth. Carbohydrate demands associated with rapid regrowth may reduce carbon-based secondary synthesis, resulting in patches of highly palatable browse that attract further browsing, thereby creating a browsing regrowth feedback loop. Such patches may be considered analogous to grazing lawns (Pellew, 1983; Danell et al., 1985; Danell & Huss-Danell, 1985).

However, in semi-arid environments such as the Karoo biome in South Africa, plants may encounter multiple environmental stressors that potentially could alter both their chemical composition and the associated herbivore community (Garg et al., 2001; Khan et al., 2010). Environmental stresses, such as drought can directly influence physiological processes,

reducing plant growth, as well as affecting the allocation of resources and secondary metabolite profiles (Khan et al., 2010; Khan et al., 2011).

Most woody plant species in semi-arid environments are known to produce CT as an herbivore deterrent. Condensed tannins constitute of a diverse group of soluble phenolics which may decrease plant herbivory by reducing protein, cell wall, and sodium digestion and retention (Zucker, 1983; Rhoner & Ward, 1997; Sebata, 2016). Frustenburg & Van Hoven (1994) showed that the average duration of feeding per dietary plant species by giraffe declined as CT increased. Furthermore, Frustenburg & Van Hoven (1994) showed that, in fenced-off areas, herbivores utilised the same individual dietary plant species more frequently. The rebrowsing of an individual plant before the tannin equilibrium recovered to pre-disturbance concentrations potentially could result in an added effect as the CT increased even more, with the equilibrium then set at a higher level. The resulting exposure to increased CT may cause browsers to suffer from inadequate protein uptake, reduced digestion and tannin toxification, ultimately leading to death (Van Hoven, 1991; Frustenburg & Van Hoven, 1994).

However, Rohner & Ward (1997) found no consistent response in the amounts of CT produced by *Acacia (Vachellia)* tree species under intense mammalian herbivore browsing. The results showed that CT production was high in the heavily browsed sites, as well as in the control sites which had no browsing pressure. From both Chapter 2 and 3 of the present study, CT concentrations were high throughout the seasons in samples taken of *V. karroo* leaves, in the Karoo. Furthermore, secondary plant species that increased in dietary importance during winter (July) when *V. karroo* was unavailable, during this time *Rhus spp.* increased in CT concentrations. Whether these high CT concentrations measured in *V. karroo*, and the increase measured in *Rhus spp.* were caused by browsing needs to be investigated.

Stock et al. (1993) did simulated browsing studies on shrub species in the extensively utilised rangelands of the semi-arid Karoo region of southern Africa. The results showed that some woody plant species responded to simulated browsing by increasing CT production. However, Stock et al. (1993) concluded that differential responses to stimulated browsing, as shown by the enhanced production of polyphenols in slow growing evergreens versus their absence in faster growing deciduous species, is consistent with the suggestion that passive alterations in the carbon/nutrient balance of a plant determines patterns of chemical allocation to defence.

In recent years there has been an increase in farm conversions from livestock farming to game farms/reserves in the Karoo. One of the frequently introduced mega-herbivore species are

giraffes (*Giraffa camelopardalis*), which are predominantly browsers that can consume up to 48kg of fresh plant foliage daily (adult bull). Several studies have shown *V. karroo* to be the most preferred plant species in the giraffe diet in the Eastern Cape (Parker & Bernard, 2003, 2005) and in the Free State (Theron, 2005; Jacobs, 2008). This is due to the high nitrogen content of the trees, making them high in proteins and a good source of food. Similarly, dietary studies done by Skidmore (2012) and Gordon et al. (2016) showed *V. karroo* to be the most favoured plant species in the giraffes' diet in the Karoo. However, the vegetation composition differs from the Eastern Cape and Free State to the Western Cape, especially the Karoo (Cowling et al., 1997). The need therefore exists to examine the dietary quality of *V. karroo* in the Karoo, and how various environmental factors as well as intensive browsing may influence the chemical composition of *V. karroo*.

Therefore, this study aims to assess how browsing (simulated) and water availability may influence the chemical composition of *V. karroo* in the Karoo at temporal scale. The objectives of this study were to assess the effect of different intensities of simulated browsing and water availability on CT concentrations, %N, %C, C/N ratios and $^{13}\text{C}/^{12}\text{C}$ ratios in *V. karroo* leaf tissue collected over a period of 10 days.

4.2 Materials and Methods

4.2.1 Study site

The experiment was carried out on the Klipkraal study site in the Greater Karoo. This study site was selected as it contained a fenced-off area dominated by *V. karroo* and where herbivores were excluded. For a detailed description of the Klipkraal farm, refer to section 2.2.1.

4.2.2 Study species

Vachellia karroo, the primary plant species in the giraffe diet in the Karoo (Gird, 2012; Martin, 2012; Gordon et al., 2016), the Eastern Cape (Parker & Bernard 2003, 2005) and in the Free State (Theron, 2005, Jacobs, 2008) was selected as the study species. Adult trees that corresponded relatively in size and phenology were included in the investigation. As different phenology stages and growth stages may affect the chemical composition of trees as trade-offs exists between reproduction, growth and defence mechanisms (Coley et al., 1985; Rohner & Ward, 1997, Koricheva, 2002).

4.2.3 Experimental design and sample collection

The experiment consisted out of four treatment groups, each represented by three *V. karroo* trees. The trees were all about six meters from each other occurring in the dry riverbed. The experiment was performed during April 2018. The treatments included: (1) a simulated lightly browsed and not watered, (2) simulated lightly browsed and watered, (3) simulated heavily browsed and, (4) simulated heavily browsed and watered. The lightly browsed and not watered block received no treatment (LB), the lightly browsed and watered (LBW) treatment received 25l water every day, the heavily browsed (HB) treatment received simulated browsing with no water, and lastly the heavily browsed and watered treatment (HBW) was heavily browsed and received 25l water each day. The HBW and HB treatments were applied every day for 10 days, with samples taken every third day. Heavy browsing was simulated by stripping leaves from seven shoots (approximately 10cm) and waiting 10 minutes before stripping leaves off another seven shoots every day (Furstenburg & Van Hoven, 1994). The light browsing was simulated by clipping approximately 10cm off seven shoots every third day. Samples were taken before the experiment started and was there-after collected every third day to allow the trees in treatment groups LB and LBW to return to their normal tannin equilibrium levels (Furstenburg & Van Hoven, 1994). Samples were collected by clipping the end parts of the twigs and branches (approximately 10cm). The samples were placed in labelled plastic bags and stored at -21°C to prevent the breakdown of the secondary metabolites. In the laboratory the leaves

were frozen using liquid nitrogen and grinded to powder using a mortar and pestle. Grinded material was subsequently stored in 50ml centrifuge tubes at -80°C until analysed.

4.2.4 Chemical analysis

The Acid-Butanol assay described by Hagerman (2002) was used to determine CT concentrations in *V. karroo* leaves. Quebracho tannin was purified using the methods described by Hagerman (2002) and used as a reference standard. (For a detailed description see section 2.2.4, Detailed method described in Appendix A, Section 1.2.1)

4.2.5 Isotope analysis

Sample material was taken from the stored tubes and placed in an oven at 70°C until the plant material was completely dried out. The samples were again grinded into a fine powder using a mortar and pestle, where after between 0.4-0.6mg of the sample was placed into a tin cup for isotope analysis performed at iThemba LABS in Johannesburg. The isotope analysis was used to determine $\text{C}^{13}/\text{C}^{12}$ ratios, percentage carbon content (%C), percentage nitrogen content (%N) and C/N ratios of the selected *V. karroo* samples. Crude protein values were calculated by multiplying the nitrogen content (%N) by a factor of 6.25 (Boyazoglu, 1997). The enclosed tin cups containing the samples were combusted in a Flash HT Plus elemental analyser coupled to a Delta V Advantage isotope ratio mass spectrometer through a ConFloIV interface (all equipment supplied by ThermoFisher, Bremen, Germany). Carbon and nitrogen isotope values were corrected against an in-house standard (Merck Gel) and a Urea Working Standard (IVA Analysentechnik e.K., Meerbusch, Germany).

4.2.6 Statistical analysis

All statistical analyses were performed using STATISTICA version 13.3 (Tibco Software, USA). Data were assessed for normality using the Shapiro-Wilks test and normal probability plots of residuals. Homogeneity of variance was evaluated using Levene's test. Generalized Linear and Mixed Models were applied to assess variation in secondary metabolite production of *V. karroo* species as a function of treatments (Section 4.23). In addition, the relationship between water content, secondary metabolite production, and browsing intensity was assessed using Mixed Models. Pearson's r correlation coefficients were used to determine correlations between water, herbivory and plant chemical composition. A principal component analysis was performed to assess associations between secondary metabolite loads and different treatment groups, $\text{C}^{13}/\text{C}^{12}$ ratios, %N, %C and N/C ratios of *V. karroo*.

4.3 Results

4.3.1 Condensed tannin (CT) production within treatment groups

Condensed tannin production increased from day one to day 10 in all treatment groups, however this increase was not significant in the lightly browsed LB group ($p = 0.158$, Figure 1). Condensed tannins concentrations in *V. karroo* leaves did however increase in the lightly browsed and watered treatment (LBW) from day four to day 10 ($p = 0.036$). Similarly, CT concentrations increased significantly in *V. karroo* leaves in the heavily browsed but not watered treatment (HB), taken on the first and fourth day, compared to the leaf samples taken on the 10th day (day one vs. day 10; $p = 0.012$, day four vs. day 10; $p = 0.152$, Figure 1). The greatest increase in CT concentrations occurred in the heavily browsed and watered treatment (HBW) where levels increased from 43.097gQE/kgWM on day one to 165.689gQE/kgWM. Condensed tannin concentrations therefore differed significantly from day one to day seven ($p = 0.0159$), day one to day 10 ($p < 0.01$) as well as day four to day 10 ($p = 0.046$, Figure 1).

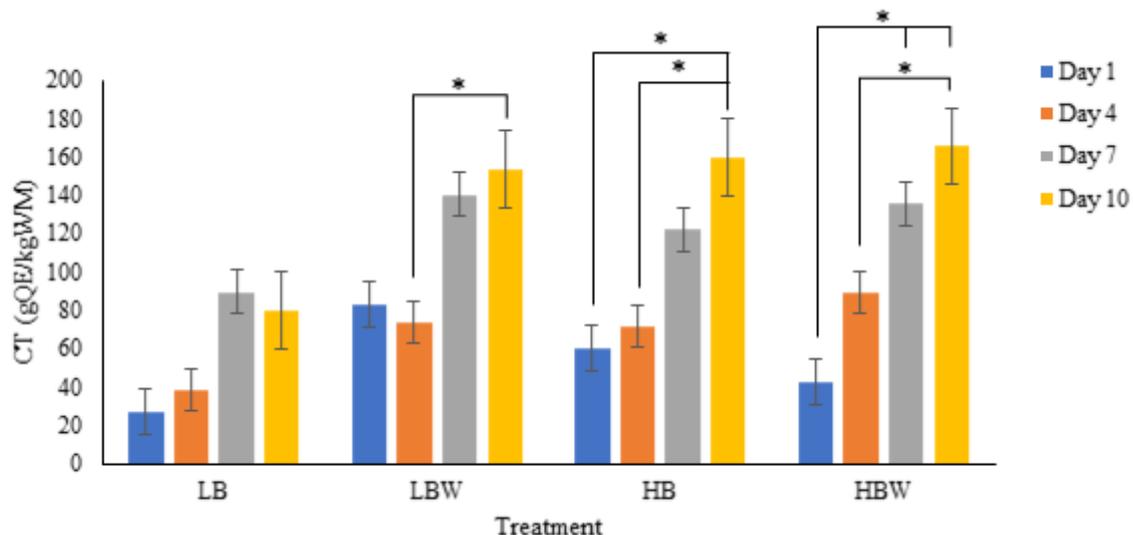


Figure 1: Condensed tannin concentrations (gQE/kgWM \pm SE) in leaves of *V. karroo* collected over a period of 10 days from different treatment groups during April 2018 (LB: lightly browsed, LBW: lightly browsed and watered, HB: heavily browsed, HBW: heavily browsed and watered).

4.3.2 Condensed tannin (CT) variation among treatment groups

Condensed tannin concentrations in *V. karroo* leaves did not vary significantly among the treatment groups when considered collectively ($F_{3,44} = 2.507$, $p = 0.071$). However, when using the CT concentrations from the 10th day of the experiment, significant pairwise differences occurred among the following treatments: Between the lightly browsed (LB) and the lightly browsed and watered treatments (LBW) ($p = 0.035$), as well as between the lightly browsed (LB) and the heavily browsed and not watered treatment (HB) ($p = 0.023$). Condensed tannin concentrations did not differ significantly between the lightly browsed and watered (LBW) and heavily browsed and watered treatments (HBW) ($p = 0.710$), as well as the heavily browsed and watered (HBW) vs. the heavily browsed and not watered treatments (HB) ($p = 0.856$).

4.3.3 Diet quality and isotopic ratios

Vachellia karroo leaves in the heavily browsed and watered treatment (HBW) contained the highest average %N and lowest average C/N ratios, however, this treatment group also had the highest average CT concentrations (Table 1). Furthermore, crude protein was the highest in the heavily browsed and watered (HBW) treatment group, and the lowest in the heavily browsed and not watered treatment (HB) group. The lightly browsed (LB) treatment had the lowest average CT concentrations, whereas the heavily browsed and watered (HBW) treatment group had the highest CT concentration (Table 1). Condensed tannin concentrations did not associate with %N, as well as %C (Table 2, Figure 2). However, CT concentrations did show a negative correlation with water use efficiency ($^{13}\text{C}/^{12}\text{C}$ ratios) (Table 2). Percentage N and %C showed a strong positive correlation (Table 2, Figure 2). The lightly browsed and watered treatment (LBW) grouped with the heavily browsed and watered treatment (HBW) with regards to chemical composition.

Table 1: The average chemical composition values measured in *V. karroo* leaf tissue taken from different treatment groups over a period of 10 days on the Klipkraal study site in the Greater Karoo, South Africa (CT = Condensed tannins, CP = Crude protein, N = nitrogen, C = carbon).

Treatment	CT			CP	
	(gQE/kgWM)	%N	%C	C/N	(g/kgWM)
Lightly browsed not watered (LB)	80,28	1,79	63,01	41,17	67.125
Lightly browsed and watered (LBW)	153,67	2,16	76,91	41,58	81
Heavily browsed not watered (HB)	159,82	0,92	39,01	49,71	34.5
Heavily browsed and watered (HBW)	165,69	3,11	92,84	34,81	116.625

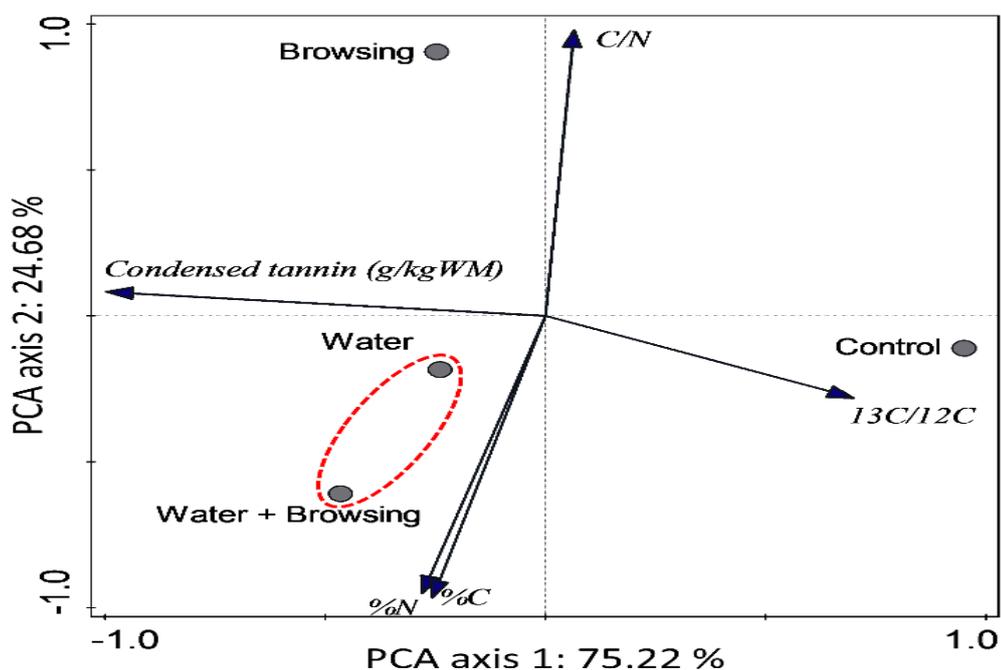


Figure 2: A Principal Component Analysis (PCA) biplot indicating the among-treatment (dis)similarities in associated with plant chemical composition in *V. karroo* leaves collected on Klipkraal. Each point represents a different treatment group, whereas the arrows represent CT production, %N, %C, C/N ratios as well as $^{13}\text{C}/^{12}\text{C}$ ratios respectively.

Table 2: Pearsons r correlation coefficients were used to assess correlations between herbivory, water, and plant chemical composition of *V. karroo* leaf tissue taken from different treatment groups over a period of 10 days on the Klipkraal study site in the Greater Karoo, South Africa.

	CT (gQE/kgWM)	%N	%C	C/N	¹³ C/ ¹² C
Condensed tannin (gQE/kgWM)	1,000				
%N	0,200	1,000			
%C	0,176	0,988	1,000		
C/N	0,016	-0,975	-0,961	1,000	
¹³ C/ ¹² C	-0,720	0,161	0,082	-0,353	1,000

4.4 Discussion

Vachellia karroo is the most important dietary plant species in the diet of extralimital giraffe populations in the Eastern Cape, Free State and Western Cape (Parker & Bernard, 2003; Theron, 2005; Jacobs, 2008; Skidmore, 2012; Gordon et al., 2016). However, the Karoo in the Western Cape differs dramatically from the Eastern Cape, as well as the Free State with regards to environmental conditions and vegetation composition such as species richness and species abundance (Acocks, 1988; Cowling et al., 2004). The results of both Chapters 2 and 3, of the present study, showed the Touwsberg farm had lower CT concentrations compared to Doornrivier. The Touwsberg farm had a higher abundance of *V. karroo* and *Schotia afra* var. *afra* and in theory provided more browse. However, the Touwsberg Mountain also occurs on the Touwsberg farm, which allows a greater water catchment area and therefore *V. karroo* may be less water stressed on Touwsberg than on Doornrivier. Du Toit et al. (1990) showed *Acacia* (*Vachellia*) tree species occurring next to waterholes were lower in CT concentrations than trees further away. How lower/higher browsing pressures and water availability, or the combination of these two factors, affects CT concentrations and the nutritional value of *V. karroo* is unknown and has not been studied experimentally.

Therefore, samples of *V. karroo* leaf tissue were collected over a period of 10 days from different treatment groups to assess how browsing and water availability may influence the chemical composition of *V. karroo*. Condensed tannin concentrations were the highest in the heavily browsed and watered treatment (HBW), however this treatment group also had the highest %N and the lowest C/N ratio. Water availability correlated negatively with CT production, while %C and %N had a strong positive correlation. Furthermore, CT production increased in both the lightly browsed (LB) and the lightly browsed and watered (LBW) treatments, showing the tannin equilibrium did not recover to the normal tannin equilibrium after three days.

4.4.1 Condensed tannins (CT)

In the present study CT concentrations were found to be high in *V. karroo* throughout the seasons (See Chapter Two). Several studies have shown CT concentrations to be high (exceeding 100g/kg) when plants are over-browsed (Cooper & Owen-Smith, 1985; Mangan, 1988; Barry & McNabb, 1998). Furstenburg & Van Hoven (1994) reported *Acacia* (*Vachellia*) *nigrescens* to increase its CT production by 22-220% after giraffe browsing occurred. The

recovery to the pre-disturbed (normal equilibrium curve) CT concentration was accomplished after 40-66h.

In the present study three of the four experimental treatment groups (LBW, HB and HBW), increased significantly in CT production from day one of the experiment to the 10th day (day 1 vs. day 10). The lightly browsed and watered treatment (LBW) trees did not return to their pre-disturbed tannin concentrations after 72h (3 days). This result indicates that trees in the Karoo may take longer to return to their normal CT equilibrium even when trees aren't heavily browsed/pruned. This is especially true in conditions where the trees have sufficient water available, as the treatment group that was similarly pruned, but did not receive water (LB) did not respond similarly with regard to CT production. Therefore, the possibility exists that the high CT concentrations in the lightly browsed and watered (LBW) as well as the (HBW) treatments may be as a response to water availability, as trees increase CT production to protect the new plant growth that will follow after receiving water. However, CT concentrations did not differ significantly between the heavily browsed (HB) and the heavily browsed and watered (HBW) treatment groups. Therefore, under conditions of over-browsing even when in drought scenarios, *V. karroo* still have the ability to produce high concentrations of CT. However, plants may not be as water stressed as anticipated as *V. karroo* trees in the Karoo occur mostly in the riverbeds, where groundwater may be accessible year-round (Halevy & Orshan, 1972). Despite the arid climatic (drought) conditions, these trees may operate well within their physiological boundaries, at least once they are established as large trees.

The results from the present study therefore show that *V. karroo* trees in the Karoo are capable of producing high concentrations of CT to deter herbivores under drought scenarios (condensed tannin concentrations exceeding 100g/kg), but even more so when trees have sufficient water availability. Therefore, trees may be less palatable even after rains occurred regardless of the browsing pressure.

4.4.2 Quality of *Vachellia karroo* as dietary source

Both the simulated lightly browsed (LB) and the heavily browsed but not watered (HB) treatment groups had the lowest nutritional value (lower %N and lower amount of crude protein). Whereas both the lightly browsed and watered (LBW) and the heavily browsed and watered (HBW) treatment groups had the highest nutritional value. The additional water allowed *V. karroo* trees to increase their photosynthesis activity, allowing more nutrient uptake from the soils, explaining the higher percentage N (Taiz & Zeiger, 2010). The increase in

photosynthesis can be seen in both the treatment groups that received water (LB and HBW) as the percentage carbon increased in both these groups. Carbon in the tree increase as a result of increased photosynthesis, as plants need to balance the uptake of CO₂ with water loss (Taiz & Zeiger, 2010). The higher percentage N in the simulated heavily browsed and watered treatment (HBW) group compared to the percentage N in the only lightly browsed and watered group (LBW), may be a result of the browsing. Du Toit et al. (1990) explained that severe pruning by herbivores reduces the inter-shoot competition for nutrients, thus increasing nutrients in the available and new plant tissue. However, the percentage N was much lower in the simulated heavily browsed treatment (HB) compared to the lightly browsed treatment (LB) group. This suggests that *V. karroo* trees are capable of increasing nutrient content, even when over-browsed, given that there is sufficient water available. However, over-browsing during times of drought such as 2015-2018 in the Karoo, will only decrease the quality of the foliage, as percentage N was the lowest and the C/N ratio the highest in the simulated heavily browsed treatment (HB) group. Therefore, the quality of *V. karroo* foliage in the Karoo changes with seasonal changes and will be influenced by water availability. However, the high CT concentrations did not associate with %N and %C showing that CT did not decrease as the nutrient content of the tree increased. Therefore, the high CT concentrations will reduce the nutrient availability to herbivores that do not have the ability to degrade CT.

4.5 Conclusions

Condensed tannins concentrations increased significantly in all treatment groups, except for the lightly browsed treatment group from the first day of the experiment to the 10th day. Condensed tannin concentrations increased significantly under light browsing simulation, which may suggest that increased CT production may be a result of increased water availability. However, during times of drought *V. karroo* are still capable of producing high CT concentrations if heavily browsed. Increased water availability increased the foliage quality even under high browsing pressure. Although CT increased significantly under high browsing pressure (HB treatment group), it may pose a greater health risk to other herbivores, as giraffe saliva contains tannin-binding salivary proteins (proline), which counter the digestibility-reducing effect of ingested CT (Robbins et al., 1987; Shimada, 2006). However, during periods of drought, such as 2015-2018 in the Karoo, the quality of *V. karroo* foliage may decrease, especially if over-browsing occurs. The reduction in available nutrients may increase the browsing pressure even more on *V. karroo*. Furthermore, all the *V. karroo* trees continuously increased their CT production even under simulated light browsing conditions (LB and LBW),

showing more than 72h are needed for trees to return to their normal tannin equilibrium. This may pose serious health risks to herbivores that do not have the ability to degrade CT, as foliage will be low in nutrients and high in CT. Therefore, giraffe will be able to survive and reproduce in the Karoo, however, during times of drought, they may have a profound effect on the chemical composition of Karoo plants that will greatly impact all other herbivores that browse the same plant species.

Chapter 5

Conclusions and Recommendations

Mortalities in extralimital giraffe populations have been suggested to be associated with secondary metabolite poisoning, for example CT and HCN. The aim of this study was to assess the variation in presence and concentrations of these compounds in favoured dietary species as well as secondary plant species utilized by giraffes when primary species are not available or in short supply.

Conclusion of Chapter 2 (primary feed): High condensed tannin concentrations were measured in primary dietary species on all three study sites. However, CT concentrations varied seasonally as evergreens increased in CT concentrations during winter. Furthermore, CT concentrations remained high in new-growth tissue of *V. karroo* which is contradicting to other studies. Whether the increase in CT concentrations were caused by an increase in browsing pressure, or the increase in water availability (rain) remain unknown. No evidence was found to prove the HCN hypothesis, as no free HCN was detected in any of the samples.

Conclusion of Chapter 3: The expanded sampling effort revealed new-growth plant tissue of secondary plant species were high in CT. Whether the high CT concentrations measured in plant species were caused by high browsing pressure on the new soft plant tissue, or as a response to rains during winter and spring remain unknown. Only one tree species *E. cladocalyx* tested positive for free HCN production; however, it was out ruled as a possible cause of the giraffe mortalities. Furthermore, no other plant species sampled contained any measurable HCN, therefore the possibility of HCN poisoning being responsible for the giraffe mortalities are highly unlikely.

Conclusion of Chapter 4: From the previous chapters an experiment was needed to determine whether water availability or browsing, or a combination of the two factors, influence CT production. Condensed tannin production increased significantly in *V. karroo* trees that received lightly simulated browsing and watered treatment (LBW), whereas trees in the lightly browsed (LB) treatment group did not increase its CT concentrations significantly. However, the lightly browsed and watered treatment (LBW) did not differ significantly from the simulated heavily browsed treatment group. Furthermore, the lightly browsed and watered, heavily browsed and watered and the heavily browsed treatment groups did not differ significantly from each other in their CT concentrations. Thus, *V. karroo* increase their CT production as a response to water availability, but also as a response to browsing. However,

the combination of water and heavy browsing does not increase CT production significantly more than under conditions of water and light browse as well as water and heavy browse.

Therefore, during times of drought giraffe browsing may increase CT production and lower the nutritional value of the trees. Giraffes are capable of degrading CT to a certain extent due to proline rich saliva. However, other herbivores that compete for the same food resources will be negatively influenced as they do not have the ability to degrade CT. The lower nitrogen content caused by over-browsing paired with high CT concentrations as a result of giraffe browsing, may pose a serious health risk to other herbivores (lack of nutritional browse).

The possibility therefore exists that high CT concentrations and lower nutritional value of *V. karroo* during drought scenarios, as well as the high CT concentrations measured seasonally and in secondary plant species, may result in giraffe browsing additional/unknown plant species to fulfil their metabolic and reproductive requirements. However, the hypothesis that HCN was potentially responsible for the giraffe mortalities in the Karoo, can be rejected based on the findings of this study, because none of the primary, secondary, annuals and bulbs produced any measurable free HCN. Future research will need to focus on other poisonous plants in the Karoo, especially plants producing cardiac glycosides, which giraffe may possible browse during adverse environmental conditions, or during times of new plant growth.

When converting a farm previously used for livestock to a game farm, various aspects should be taken into consideration. Assessing only the vegetation as an indicator of possible stocking rates of Large Stock Units (LSU) is not enough. As shown in the present study, CT concentrations can vary between farms 8km apart from each other, therefore using dietary studies done on similar plant species in the Eastern Cape as an indicator of diet quality may provide misleading information. Various factors influence the chemical composition of plant species and therefore impact studies done to assess the possibility of introducing large game into the Karoo should be site specific and must focus on more aspects that may influence dietary quality, such as chemical plant defence response to herbivory.

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Appendix A

1.1 Chemical analysis

1.1.1 *Hydrogen cyanide*

Total HCN was determined using the modified Feigl-Anger test described by Kakes (1991). Leaves collected for HCN analyses were grinded in liquid nitrogen using a mortar and pestle, 8mg of grinded leaf material was loaded per well in a 48 well microtiter plate and incubated at -20°C for at least one hour. After one hour, 200µl of distilled water was pipetted into each well. Six freshly prepared standard solutions of HCN in water was included per plate, with concentrations ranging from 998mg/L to 1.95mg/L (doubling dilution). The plates were wiped dry and a Feigl-Anger test paper was paced and secured with Perspex sheets and rubber bands over the plates. The plates were incubated at 35 °C for one hour. After incubation, the test papers were dried in a fume hood, then analysed.

1.1.2 *Test papers*

The test papers were made by mixing equal volumes of 1% w/v solutions of tetra base (4,4'-methylenebis- (*N*, *N*-dimethylaniline)) and copper (II) ethylacetoacetate in chloroform. Whatman No. 1 filter papers was cut to the size of the microtitre plate, then soaked briefly (about 15s) in the mixed solutions before being dried in a fume hood (Kakes, 1991; Seigler, 1991).

1.1.2.1 *Analysis of test papers*

The reaction is based on colour changes from white to blue-purple in the presence of HCN. Computer analysis was used to quantify the results of the colour change assay. Test paper colourations was digitised within 30 minutes of incubation. A numeric value will be obtained for the colour saturation of the spot corresponding to each sample with the NIH/ImageJ software. A standard curve was constructed for each plate by plotting the log of the mean saturation of the standard solutions against concentrations (Hayden & Parker, 2002).

1.2.1 *Condensed tannin quantification*

The acid-butanol assay was used to measure condensed tannins, using the method described by Hagerman (2002). Condensed tannins were extracted prior to the acid-butanol assay from the samples. Each sample was grinded using liquid nitrogen in a mortar and pestle. Three grams of the powder was transferred to a 50ml screw cap plastic centrifuge tube, supplemented with 20 mL of 70% acetone (acetone:water, 70:30), and sonicated for 30min at 4 °C (Hagerman, 2002). The test tube was thereafter centrifuged for 10min at 2500x g, the supernatant removed and stored at 4 °C, until used for the acid-butanol assay. It is important to ensure the analysis of the samples are performed within 24 hours after extraction. Quebracho tannin was purified and used as a standard for the acid-butanol assay. The acid-butanol assay is a colorimetric reaction and uses an acid-catalysed oxidative depolymerization of condensed tannins to yield red anthocyanidins. Although several studies have indicated problems with this colorimetric assay, due to the assay not accounting for certain insoluble and hydrolysable tannins, which may result in an underestimation of tanniferous capacity of plant tissue (Waterman & Mole, 1994; Schofield et al., 2001; Heil et al., 2002), this assay is still commonly used as the most reliable method for condensed tannin assays (Schofield et al., 2001; Scogings et al., 2013)

2.2 Study species

From published dietary reports (Parker & Bernard 2003, 2005; Theron, 2005; Skidmore, 2014; Gordon, 2016) and impact assessments (Gird, 2012) a total of 15 plant species were selected as focal species for this study. Dietary preference, plant height as well as potential sources of tannins and HCN were considered for selection.

Table 3: Observations from other literature regarding the feeding behaviour of giraffes in the Karoo have identified the following 15 plant species as the main dietary browse.

Family	Name	Present on reserve(s)	Height (m)	Flowering period	Growth type	Chemical Defence	Physical Defence	Primary/Secondary food source	Reference
Mimosiadeae	<i>Vachellia karroo</i>	Klipkraal Touwsberg Doornrivier	2-5	Spring-Summer	Deciduous	Tannin	Thorns	Primary	Coates Palgrave, M. 2002. <i>Keith Coates Palgrave Trees of southern Africa</i> , edn 3. Struik, Cape Town.
Ebenaceae	<i>Diospyros austro-africana</i>	Klipkraal	2-10	Spring-Summer	Evergreen	Unknown	None	Secondary	Coates Palgrave, M. 2002. <i>Keith Coates Palgrave Trees of southern Africa</i> , edn 3. Struik, Cape Town.
Anacardiaceae	<i>Rhus /Searsia burchellii</i>	Klipkraal	2-5	Autumn-Winter	Evergreen	Unknown	None	Primary	Van Wyk, B.-E. & Gericke, N. 2000. People's plants. Briza Publications, Pretoria.
Anacardiaceae	<i>Searsia (Rhus) lancea</i>	Klipkraal Touwsberg Doornrivier	2-7	Winter-Spring	Evergreen	Unknown	None	Primary	Van Wyk, B. & Van Wyk, P. 1997. Field Guide To Trees Of Southern Africa. 1st edition, Cape Town: Struik Publishers.
Chenopodiaceae	<i>Salsola aphylla</i>	Klipkraal Touwsberg Doornrivier	1-3	Spring	Evergreen	Unknown	None	Primary	Coates Palgrave, M. 2002. <i>Keith Coates Palgrave Trees of southern Africa</i> , edn 3. Struik, Cape Town.
Asteraceae	<i>Pteronia adenocarpa</i>	Touwsberg Doornrivier	0.4-0.6	Spring-Summer	Evergreen	Chemical	None	Secondary	The antimicrobial properties and chemical composition of leaf extracts and essential oils of indigenous <i>Pteronia</i> species

	<i>Schotia afra</i> var. <i>afra</i>	Touwsberg Doornrivier	2-5	Spring- Summer	Evergreen	Unknown	None	Primary	Van Wyk, B. & Van Wyk, P. 1997. <i>Field guide to trees of southern Africa</i> . Struik, Cape Town.
Solanaceae	<i>Lycium oxycarpum</i>	Klipkraal Touwsberg Doornrivier	1-2	Winter- Spring	Evergreen	Unknown	Thorns	Primary	Vlok, J. and Schutte-Vlok, A.L. 2010. <i>Plants of the Klein Karoo</i> . Umdaus Press, Hatfield.
Anacardiaceae	<i>Searsia (Rhus) longispina</i>	Touwsberg Doornrivier	2-4	Autumn- Spring	Evergreen	Unknown	Thorns	Secondary	Vlok, J. and Schutte-Vlok, A.L. 2010. <i>Plants of the Klein Karoo</i> . Umdaus Press, Hatfield.
Bignoniaceae	<i>Rhigozum obovatum</i>	Touwsberg Doornrivier	1-4.5	Spring	Deciduous	Unknown	Thorns	Primary	Van Wyk, B & Van Wyk, P. 1997. <i>Field guide to trees of southern Africa</i> . Struik, Cape Town.
Apocynaceae	<i>Carissa haematocarpa</i>	Touwsberg Doornrivier	1-4	Spring- Summer	Evergreen	Unknown	Thorns	Secondary	Palmer, E. & Pitman, N. 1972. <i>Trees of southern Africa</i> . Balkema, Cape Town.
Sapindaceae	<i>Pappea capensis</i>	Touwsberg	2-8	Summer- Autumn	Deciduous	Unknown	Thorns	Secondary	Migdoll, I. 1987. <i>Field guide to butterflies of southern Africa</i> . Struik, Cape Town.
Ebenaceae	<i>Euclea undulata</i>	Touwsberg Doornrivier	2-7	Summer- Autumn	Evergreen	Unknown	None	Primary	Coates Palgrave, M. 2002. <i>Keith Coates Palgrave Trees of southern Africa</i> , edn 3. Struik, Cape Town
Anacardiaceae	<i>Searsia (Rhus) lucida</i>	Touwsberg Doornrivier	2-5	Spring- Winter	Evergreen	Unknown	None	Secondary	Coates Palgrave, M. 2002. <i>Keith Coates Palgrave Trees of southern Africa</i> , edn 3. Struik, Cape Town.
Celastraceae	<i>Gloveria integrifolia</i>	Touwsberg Doornrivier	2-5	Spring	Evergreen	Unknown	Spikes	Secondary	Goldblatt, P. and Manning, J.C. 2000. <i>Cape Plants: A conspectus of the Cape Flora of South Africa</i> . Strelitzia 9. National Botanical Institute, Cape Town.
Anacardiaceae	<i>Searsia (Rhus) pallens</i>	Touwsberg	2-5	Summer- Autumn	Evergreen	Unknown	None	Primary	Van Wyk, B & Van Wyk, P. 1997. <i>Field guide to trees of southern Africa</i> . Struik, Cape Town.

