

Host specificity of the hemiparasitic mistletoe, *Agelanthus natalitius*

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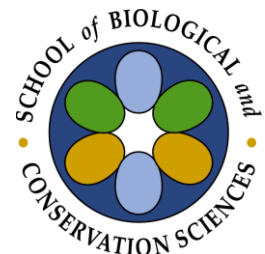
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SUMMARY

Mistletoes are a group of hemiparasitic plants that grow on a wide variety of host trees and differ in their degree of host specificity, ranging from specialists to generalists. Mistletoes can also be locally host specific where host preference varies geographically, i.e. at a given location a mistletoe species may infect only part of its overall host set. The mistletoe *Agelanthus natalitius* parasitises at least 11 tree genera distributed throughout South Africa. However, there is geographic variation in infection patterns over the parasite's range, suggesting that *A. natalitius* may be locally host specific. We quantified the degree of host specificity and tested the mechanisms that direct host specialisation in two distinct mistletoe populations at Highover and Mtontwane (about 110 km apart) in KwaZulu-Natal, South Africa.

We investigated the distribution, abundance and community composition of woody species that host the mistletoe. We also assessed the effect of light on germination and early survival of the mistletoes in a greenhouse experiment. We conducted field reciprocal transplant experiments at both sites to investigate the compatibility of these mistletoes with their hosts *Acacia karroo* and *A. caffra* during early development. We then analysed the nutrient and water contents of the mistletoe-host pairs to investigate the role of nutrient and water status in directing host specificity in mistletoes. We further studied avian dispersal in the field and in captivity to investigate optimal dispersal distance and germination success, and evaluated their role in determining mistletoe host specificity.

At both study sites, five host species were recorded as being parasitised by the mistletoe *A. natalitius*. *A. karroo* and *A. caffra* appear to be the two most common host species in the region; both grow abundantly at the study sites and were recorded with high infection by *A. natalitius*. However, *A. karroo* is the most abundant host species and the mistletoe showed a high degree of host specificity on *A. karroo*. Infection by mistletoes was positively correlated with tree size, and was highly aggregated, both individually and locally. Field observations and greenhouse shade experiments showed that light can influence mistletoe distribution. Germination of mistletoe seeds

was independent of host species and site. However, hypocotyls (the structures that develop into haustoria) grew longer when placed on their source host species within their locality. Additionally, they showed preference for the most abundant host species, *A. karroo*.

Water and nutrient status of the host species *A. karroo* and *A. caffra* had no significant effect. Thus, host nutrient and water content may not account for host specificity in this mistletoe species. Mistletoes accumulated more nutrients and maintained more negative Ψ than their host trees. We also investigated the mistletoes' use of passive nutrient uptake (from host xylem) and active nutrient uptake (from host phloem) by using the N:Ca ratio as an index of nutrient access. Mistletoes growing on *A. caffra* had a ratio > 1 , i.e. the mistletoe actively accessed nutrients from the phloem of host trees. However, mistletoes on *A. karroo* had a N:Ca ratio < 1 , which implies that they passively accessed nutrients from the xylem. The difference in mechanism of nutrient acquisition on different host species may reflect the level of compatibility between mistletoe and host.

Several bird species were frequently observed to feed on mistletoes, many of which were used in our captivity studies. Although birds did not consume mistletoe fruits in captivity as they do in the field, they were effective in removing the pulp cover of mistletoe fruits and exposing seeds in germinable condition. In captivity, the Red-winged Starling ingested whole fruits and regurgitated seeds, deliberately wiping their bills on twigs to remove the sticky seeds. As a result, germination success of mistletoes processed by Red-winged Starlings was higher than any other bird species tested in captivity.

Overall, there appears to be host specificity in morphologically identical mistletoes. Understanding the mechanisms that result in host race evolution are potentially important to the process of speciation in hemiparasitic mistletoes. We need to take into account genotypic matching in conserving these different forms of mistletoes and their host *Acacia* genotypes. Further research into the mechanisms of host specificity and patterns of genotypic matching is warranted.

PREFACE

The work described in the thesis was carried out at the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa, from January 2008 to November 2009, under the supervision of Prof. David Ward and Dr. Megan E. Griffiths.

This thesis, submitted for the degree of Master of Science in the Faculty of Science and Agriculture, University of KwaZulu-Natal, Pietermaritzburg, represents original work by the author and has not been submitted in any form to another university. Where use was made of the work of others, it has been duly acknowledged in the text.

Chapter 2 to 5 in this thesis were written in paper format following the format of the journal they were/will be submitted to.



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December 2009

I certify that the above statement is correct



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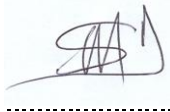
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DECLARATION 1 - PLAGIARISM

I, Desale Yosief Okubamichael, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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DECLARATION 2 - PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis.

Publication 1

DY Okubamichael, MZ Rasheed, ME Griffiths and D Ward. **Host abundance can direct local host specificity in the hemiparasitic mistletoe, *Agelanthus natalitius* (Loranthaceae)**

Author contributions:

DYO conceived the paper with MZR, MEG and DW. DYO collected and analysed the data, and wrote the paper. MZR did the shade experiment on mistletoe germination in the greenhouse. MEG and DW contributed valuable comments to the manuscript.

Publication 2

DY Okubamichael, ME Griffiths and D Ward. **Source host species recognition and preference in the hemiparasitic mistletoe, *Agelanthus natalitius* (Loranthaceae)**

Author contributions:

DYO conceived the paper with MEG and DW. DYO collected and analysed the data, and wrote the paper. MEG and DW contributed valuable comments to the manuscript.

Publication 3

DY Okubamichael, ME Griffiths and D Ward. **Nutrient and water relationships of the hemiparasitic mistletoe, *Agelanthus natalitius* (Loranthaceae), and its host species, *Acacia karroo* and *A. caffra***

Author contributions:

DYO conceived the paper with MEG and DW. DYO collected and analysed the data, and wrote the paper. MEG and DW contributed valuable comments to the manuscript.

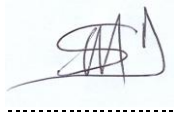
Publication 4

DY Okubamichael, MZ Rasheed, ME Griffiths and D Ward. **Host specificity and bird dispersal in the hemiparasitic mistletoe, *Agelanthus natalitius* (Loranthaceae)**

Author contributions:

DYO conceived the paper with MZR, MEG and DW. DYO and MZR collected and analysed the data, and DYO wrote the paper. MEG and DW contributed valuable comments to the manuscript.

Signed:

A handwritten signature in black ink, appearing to be 'DYO', is written on a light blue rectangular background. Below the signature is a dotted line.

Desale Yosief Okubamichael

December 2009

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First and above all I would like to thank God for seeing me through it all.

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Chapter 1

Literature review: An overview of mistletoes

Introduction

Parasitic plants access at least some of their nutrients from other living plants (Kuijt 1969, Visser 1981). Through evolution, the root systems of parasitic plants have been modified to invade the host's tissue so that they can access nutrients and water (Kuijt 1969, Vidal-Russell and Nickrent 2008). They physically attach at least at one point either to the aboveground (aerial/stem/shoot parasites) or belowground (root parasites) parts of the host plants (Polhill and Wiens 1998, Tomilov et al. 2005). Parasitic plants tap the vascular tissue of host plant via a specialised vascular attachment called a haustorium, a structure unique to parasitic plants that distinguishes them from epiphytic or mycoheterotrophic plants that either use host plants for physical support or associate via mycorrhizal intermediates (Kuijt 1969).

Parasitic plants can be categorised based on the absence or presence of chlorophyll and their degree of dependence on host trees. Hemiparasites have chlorophyll and photosynthesise but obtain nutrients and water from their host plants (Kuijt 1969, Nickrent and Musselman 2004).

Holoparasites lack chlorophyll, do not photosynthesise, and completely rely on their host plants for nutrients and carbon (Nickrent and Musselman 2004). Hemiparasites and holoparasites are obligate parasites that require host plants to complete their life cycle. However, some parasitic plants can grow to maturity in the absence of host plants. Such facultative parasites can parasitise a broad spectrum of hosts (Kuijt 1969). It is important to note that obligate parasite haustorium formation is a permanent modification of the primary root tip, unlike facultative parasites that still grow lateral roots, and consequently can grow without a host (Tomilov et al. 2005).

Like other parasitic plants, mistletoes tap the xylem or occasionally the phloem of the branches of host plants via the haustorium to access water, water-conducted mineral nutrients and,

to some extent, carbohydrates and organic solutes (Reid et al. 1995, Sargent 1995, Ladley and Kelly 1996). Mistletoes photosynthesise and hence they are hemiparasites, but they can also gain a significant amount of carbon and organic solutes from their hosts (Hull and Leonard 1964a,b, Stewart and Press 1990, Bowie and Ward 2004, Wang et al. 2008). However, not all mistletoes are aerial and stem parasites (Nickrent et al. 2004, Mathiasen et al. 2008). A few species of mistletoe are endophytic (e.g., *Viscum minimum* and *Tristerix aphyllus*) and live entirely within their hosts except when they come out to produce flowers and fruits (Thody 1951, Amico et al. 2007). Several mistletoe species belonging to the families of Loranthaceae and Santalaceae are also root parasites (Amico et al. 2007, Mathiasen et al. 2008).

Mistletoes can also grow on other mistletoes and this mistletoe-mistletoe parasitism manifests itself in four different forms. **A.** Some mistletoe species occasionally grow on other mistletoe species, but usually grow on host trees. This is referred to as incidental epiparasitism or hyperparasitism (Kuijt 1969, Mathiasen et al. 2008). **B.** Some mistletoe species can auto-parasitise individuals of their own species (Nickrent and Musselman 2004). **C.** Some mistletoe species can grow on host trees but commonly grow on other species of mistletoe with some indication of coupling or co-adaptation to their host mistletoes, referred as facultative epiparasitism. **D.** Some mistletoe species have also been observed (rarely) to form an obligate tripartite association, confined to and closely adapted to their host mistletoes, which in turn are closely adapted to a host. This is known as obligate epiparasitism (Nickrent and Musselman 2004, Mathiasen et al. 2008).

Biogeography, diversity and evolution of mistletoes

Mistletoes are a diverse group of plants found in the order Santalales, grouped in five families: Eremolepidaceae, Loranthaceae, Misodendraceae, Santalaceae and Viscaceae (Kuijt 1969, Watson 2001, Vidal-Russell and Nickrent 2008). Mistletoes comprise over 1400 species, dominated by species in the Loranthaceae (about 940 species in 73 genera), followed by Viscaceae (about 540 species in seven genera) and the rest from the other families (Downy 1998). Loranthaceae is a

Gondwanan lineage that originated in the Southern Hemisphere and dispersed, apparently early, between fragments of Gondwana; thereafter there was dispersal from Africa into Europe, and later they dispersed to the Americas (Wilson and Calvin 2006). Mistletoes' habitat encompasses boreal climates, temperate, tropical, and arid zones, with the group absent only from extremely dry or cold regions (Norton and Carpenter 1998, Norton and de Lange 1999, Watson 2001). The greatest mistletoe diversity is found in forests and woodlands (Kuijt 1969). However, mistletoes can also be selective; they prefer conifers in boreal forests (Hawksworth and Wiens 1996), succulent euphorbs (Euphorbiaceae) in African deserts, and cacti (Cactaceae) in the South American desert (Martínez del Rio et al. 1996, Polhill and Wiens 1998). Mistletoes parasitise a wide range of host species and they can also be locally limited in the subset of host species. The local adaptation of mistletoes to different host species in various parts of the range is known as host specificity (Clay et al. 1985, Norton and Carpenter 1998, Norton and de Lange 1999). Mistletoe host specificity will be explored in greater depth later in this chapter and it is the main theme of this research.

It is commonly accepted that stem-parasitic sandalwood order (Santalales) evolved from root parasites (Kuijt 1969), but there is a debate about how many times the mistletoe habit has been evolved (Vidal-Russell and Nickrent 2008). Based on recent studies, the five mistletoe families are not sister families and aerial parasitism has evolved independently five times (Mathiasen et al 2008, Vidal-Russell and Nickrent 2008). Therefore mistletoes are a polyphyletic functional group that encompasses all hemiparasitic species within the Santalales (Amico et al. 2007, Mathiasen et al. 2008, Vidal-Russell and Nickrent 2008). Vidal-Russell and Nickrent (2008) estimated that the first mistletoe developed in the Misodendraceae 80 million years ago (Mya), subsequently in Viscaceae (72 Mya), Eremolepidaceae (53 Mya), Santalaceae (46 Mya) and finally in Loranthaceae (28 Mya). Even though Loranthaceae evolved more recently, the high species diversity in this family shows the rapid adaptive radiation and speciation that occurred in the family which probably coincided with the appearance of savanna biomes during the Oligocene (34 Mya) (Vidal-Russell and Nickrent 2008).

Hemiparasitic mistletoes evolved from autotrophic plant life forms principally to acquire water and nutrients in dry areas (Ehleringer et al. 1985). This is based on the fact that mistletoes exhibit high species diversity in arid and semiarid regions where water and nutrients are limited (Ehleringer et al. 1985). It is obvious that mistletoes evolved from non-parasitic autotrophs (Kuijt 1969), but it is not clear whether the haustorium is endogenous or exogenous in origin (Matvienko et al. 2001, Tomilov et al. 2005, Calvin and Wilson 2006). The most accepted theory is that the accumulation of selected mutations modified the primary or lateral roots within the plants (Atsatt 1973). Alternatively, the haustorium may have developed from a pathogenically induced neoplasm (Atsatt 1973, 1988), i.e. externally-acquired genes, perhaps from a haustorial-producing fungus or bacteria, modified the plants. Haustorium development is morphologically and functionally very diverse, which could have phylogenetic implications and needs further investigation (Calvin and Wilson 1998, Wilson and Calvin 2006, Vidal-Russell and Nickrent 2008) (Fig. 1).

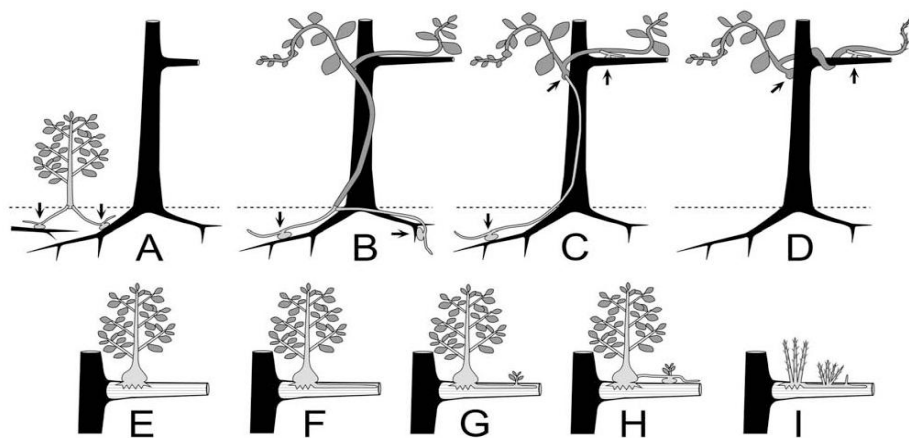


Fig. 1. Hypothetical parasitic modes in the order Santalales (after Vidal-Russell and Nickrent 2008). This assumes that there is a progression from A to I. Arrows represent haustorial connection points. This assumes that there is a phylogenetic switch from a root parasite (A) through to an aerial parasite with haustorial connections to host stems (C). Thereafter, a range of different haustorial connections evolves. With permission of D.L. Nickrent.

Seed dispersal mechanisms of mistletoes

Seed dispersal in mistletoes involves direct and indirect mechanisms (Restrepo et al. 2002). A few mistletoe species disperse directly by wind (Vidal-Russell and Nickrent 2007) or explosive fruits (Hinds et al. 1963, Vidal-Russell and Nickrent 2007). However, most mistletoe species disperse indirectly, which can be either bird- or mammal-mediated. In this case, an interaction of the vector, host and mistletoe are necessary prerequisites for seed dispersal. The specific requirements by mistletoes for dispersal vectors and host plants with suitable twig sizes makes them much more dependent on vector mediated dispersal than other plant species (Amico and Aizen 2000, Watson 2001, Restrepo et al. 2002).

Bird dispersal is the most common mode of dispersal in mistletoes (Watson 2001). This involves a high degree of co-evolution between the mistletoe and the birds that are specialised to consume the berries of particular mistletoe species and disperse them (Reid 1991, Reid et al. 1995, Watson 2001). Additionally, birds are involved in the pollination biology of some mistletoe species, which further contributes to the tight co-evolution of birds with mistletoes (Davidar 1983, 1987, Reid 1986, Ladley and Kelly 1996, Watson 2001). However, the pollination biology of mistletoes has received markedly less attention than fruit dispersal (Watson 2001). This topic is beyond the scope of the present study and will not be covered in the literature review.

The co-evolution of mistletoes with their avian vectors has influenced many nutritional, morphological and phenological attributes of fruit traits (Reid 1990, Martínez del Rio et al. 1995a, López de Buen and Ornelas 1999, 2001). Mistletoe fruits offer a substantial reward to frugivorous birds that consume them and subsequently disperse the seeds of the mistletoe (Polhill and Wiens 1998). The fruits are large, high in sugar content and brightly coloured (white, yellow, red, or purple) in order to attract bird dispersers (Godschalk 1983, 1985). The mistletoe fruit phenology effectively manipulates and maintains disperser birds by being available to them in the winter months when few other food sources are available in the ecosystem (Ladley and Kelly 1996, Polhill and Wiens 1998, Watson 2001). In addition, there is prolonged discontinuous ripening of the fruits

within individual mistletoes and asynchrony in peak fruiting time among mistletoes in a population (Davidar 1983, Hawksworth and Wiens 1996, Watson 2001).

The sticky viscin that coats mistletoe seeds is another attribute that facilitates the attachment of seeds to host branches, otherwise they would fall down easily to the ground (Reid 1991, Reid et al. 1995, Aukema 2003). The viscin contains a mucilaginous pectic material known for its water-holding capacity and capable of undergoing repeated drying and rehydration events (Paquet et al. 1986). Moreover, viscin-coated seeds often stick to the bill or the abdomen or other body parts of birds. This usually creates difficulties for birds to expel the attached seeds, forcing them to wipe or rub against a twig to dislodge the seed (Reid 1991). This facilitates safe site dispersal because this behaviour often takes place on suitable host branches (Roxburgh 2007).

Aside from birds, there are rare examples of marsupials, squirrels and other mammals that disperse mistletoe seeds in South and North America (Hawksworth and Wiens 1996, Mathiasen 1996, Amico and Aizen 2000). In many instances, seeds that stick to animals' bodies may rub off onto a branch of a potential host (Mathiasen 1996). Gut passage through one marsupial, *Dromiciops australis*, is required for seed dispersal of the mistletoe *Tristerix corymbosus* in the Lake district of southern Argentina (Amico and Aizen 2000). Amico and Aizen (2000) found that most seeds removed from the exocarp by hand failed to germinate and were unable to infect host plants, while over 90% of the seeds collected from marsupial faeces germinated and most of these seeds developed a holdfast. It is also suggested that marsupial dispersal of mistletoe seeds might represent a very primitive mutualism. As the Loranthaceae fossils of Palaeocene microbiotheriids (Little Mountain Monkeys) were found at different localities along the tropical Andes and the marsupial-mistletoe mutualism described here may have been widespread in the past (Amico and Aizen 2000).

Fruit handling in relation to germination success and dispersal distance

Birds apply different modes of handling and processing of fleshy mistletoe fruits; they may peel off the outer exocarp of fruits and ingest either the seed or the exocarp. They may even take only a portion of the exocarp (Kuijt 1969, Reid 1991), swallow mistletoe fruits and reflux seeds to the foregut (regurgitation) or swallow whole fruits and pass them through the gut (defaecation) (Kuijt 1969, Watson 2001, Roxburgh 2007). Each of the observed handling mechanisms has different implications for dispersal distance and the germination success of mistletoe seeds (Ladley and Kelly 1996, Roxburgh 2007). Pecking involves eating fruit pulp without ingesting the whole fruit (Roxburgh 2007). The bird grips the fruit in its bill, squeezes the sticky viscin-coated seed out to the side and then wipes the bill clean on a branch or substrate. Most of the time, the pulp is eaten and the seeds are wiped near the parent plant resulting in the dispersal of seeds on the same host as the parent (Overton 1994, Green et al. 2009). Similarly, regurgitation involves a short retention time in the gut which likely decreases the dispersal distance and increases the viability of seeds by avoiding the damaging effects of enzyme activity which mainly happens in the small intestine (Roxburgh 2007). Birds that regurgitate seeds wipe their bills against a branch, which often results in the seed adhering to a suitable site that is at a short distance from the parent plant (Roxburgh 2007).

Defaecated seeds usually fall randomly below the birds' perches (Roxburgh 2007), but systematic defaecation and subsequent cloaca wiping to remove viscin-covered mistletoe seeds has been recorded in Mistletoebirds (*Dicaeum hirundinaceum*) (Reid 1987). This systematic mechanism observed in Mistletoebirds likely results in the deposition of seeds on a suitable twig (Reid 1987). Similarly, Yellow-vented Bulbuls (*Pycnonotus xanthopygos*) feeding on the mistletoe *Plicosepalus acaciae* shake off the seeds from the cloaca which most likely increases the probability of reaching a safe site (Green et al. 2009). Defaecated seeds are often in groups piled on top of each other, which creates difficulties for each of them to come in contact with the substrate. Such piled-up seeds experience density-dependent mortality and their chance of success is lower

than regurgitated seeds that all have direct contact with a branch and are more widely spaced (Roxburgh 2007).

Many studies have investigated the effect of gut passage on subsequent mistletoe seed germination and compared this to manual removal of the mistletoe fruit exocarp (Lamont 1982, Yan 1993a, Ladley and Kelly 1996). Most studies conclude that removal of fruit from the seed is sufficient to initiate germination. However, Green et al. (2009) found that germination success of mistletoe seeds defaecated by Yellow-vented Bulbuls was higher (51.5%) than the germination success (35%) of hand-cleaned seeds. They concluded that passage through the bulbul gut increases the germination rate in *Plicosepalus acaciae* (Green et al. 2009).

Differential seed-dispersal of mistletoe fruits by birds

Tall, large and previously infected trees are usually observed with more mistletoes (Reid and Stafford Smith 2000, Aukema and Martínez del Rio 2002a,b,c, Roxburgh and Nicolson 2007). Overton (1994) hypothesised that increased infection accumulation is an effect of age through time, i.e. larger trees have more time to acquire an infection. Although this hypothesis explains most cases of mistletoe distribution pattern over time, it is rather simplistic, assuming no differences in host quality and no differential dispersal as the tree grows (Roxburgh and Nicolson 2007). The model is adequate for explaining mistletoe prevalence (percent of host trees in a given area that are infected) but not for explaining the infection intensity (number of mistletoes in a single tree). Many subsequent studies have demonstrated a weak relationship between host size and infection intensity (Donohue 1995, Aukema and Martínez del Rio 2002a,b, Roxburgh and Nicolson 2007).

There are many additional factors such as differential dispersal by birds, variation in host characteristics and differential establishment success of mistletoes on different hosts, which will all influence the distribution patterns of mistletoes. Differential seed-dispersal of mistletoes by birds has been extensively researched and these studies have determined that birds are responsible for disseminating fruits on tall rather than short trees (Reid and Stafford Smith 2000, Aukema and

Martínez del Rio 2002a,b,c, Roxburgh and Nicolson 2005). Roxburgh and Nicolson (2005) observed that birds differentially perch on and disperse seeds of the mistletoe *Phragmanthera dschallensis* to tall host trees of *Acacia sieberiana*. A similar removal and re-infection experiment on the desert mistletoe *Phoradendron californicum*, which is dispersed by Phainopeplas (*Phainopepla nitens*), showed that birds preferentially perch both on already parasitised trees and tall trees (Aukema and Martínez del Rio 2002c). Similarly, Reid and Stafford Smith (2000) showed that larger trees on which mistletoes had been experimentally removed were disproportionately re-infected with mistletoes. Martínez del Rio et al. (1996) also found that larger trees received more mistletoe seeds. In addition, Green et al. (2009) noted that bulbuls perched in infected trees for a substantial amount of time, thus enabling a high probability of infected trees being re-infected. Therefore, multiple infections often arise, resulting in the aggregation of mistletoes on individual hosts (López de Buen and Ornelas 2001, Aukema and Martínez del Rio 2002a,b,c, Aukema 2004, Carlo and Aukema 2005).

Birds prefer tall trees for perching because they provide more nesting sites; particularly old, tall trees provide more holes for cavity nesters (Roxburgh and Nicolson 2007). Large trees produce flowers and fruits that may themselves act as food, and attract more insects which can serve as supplementary protein sources for birds. Tall trees are also more likely to have mistletoes, which are the most available fruit for consumption by birds in winter, thus birds are attracted to and differentially infect tall trees (Aukema and Martínez del Rio et al. 2002b,c).

Trees close to an infected tree or close to a nesting site receive seeds at a high rate (Aukema and Martínez del Rio 2002b). For example, nest trees of Mistletoebirds attracted a disproportionately large number of mistletoe seeds (Reid and Stafford Smith 2000). Similarly, Yellow-fronted Tinker Barbets (*Pogoniulus chrysoconus*) carry mistletoe fruits to their nestlings and discard the seeds on the nest tree or on neighbouring trees (Roxburgh and Nicolson 2007). Differential dispersal is also possibly linked to host sex (Carlo and Aukema 2005). For the mistletoe *Phoradendron hexastichum*, a bird-dispersed mistletoe that infects the dioecious tree

Cecropia schreberiana, female host trees have twice as many mistletoes as male host trees because both the parasite and female host trees share frugivores (Carlo and Aukema 2005). In addition, mistletoe frugivores visit uninfected fruiting female host trees twice as frequently as male host trees, even though there are no other morphological or physiological differences between the sexes of this species (Carlo and Aukema 2005).

Recent experimental evidence reported that germination and establishment success of mistletoes did not differ significantly between tall and short trees, but success of established mistletoes was significantly higher on tall trees than short trees (Roxburgh and Nicolson 2005, 2007). When trees grow they have access to more nutrients and water due to deeper and more widespread roots (Norton et al. 1997, Bowie and Ward 2004, Roxburgh and Nicolson 2007). Mistletoes that grow in tall trees also have greater access to sunlight (Lamont and Perry 1977, Lamont 1982, Polhill and Wiens 1998), hence maximising photosynthesis and avoiding the shading effect that negatively affects mistletoe survival (Norton et al. 1997). Mistletoes on short trees can be vulnerable to fire and browsing which negatively affects their survival (Hawksworth and Wiens 1996, Kelly et al. 1997, Roxburgh and Nicolson 2007). Overall, tall trees are more likely to be higher quality hosts than short trees.

Local-aggregation of mistletoes

Birds are also responsible for local aggregation because they spend most of their time perching in highly infected areas (Aukema 2003, 2004, Ward and Paton 2007, Green et al. 2009). This local aggregation is strongly facilitated by gut passage rate (GPR), which in turn depends on the bird's size, physiology, morphology, behaviour and specialisation. For example, seed shadows increase with body mass of dispersers (Charalambidou et al. 2003). Most mistletoe-dispersing birds have a short gut that enhances the fast passage of the fruits (see e.g., Green et al. 2009). Mistletoe seeds are large and constrain flight by increasing the weight of the bird, so fast release makes it easier for

birds to fly and increases gut capacity to take and process more fruits (Roxburgh 2007). This enhances direct dispersal to the same host tree as the parent mistletoe or to neighbouring hosts.

An aviary study by Murphy et al. (1993) showed that avian dispersers with specialised guts provide higher quality seed dispersal than unspecialised frugivores feeding on *Amyema quandang* mistletoe fruits. Mistletoebirds (*Dicaeum hirundinacum*) have a specialised gut with a relatively short alimentary tract, allowing rapid passage of a large number of berries (Richardson and Wooller 1988), unlike Spiny-cheeked Honeyeaters (*Acanthagenys refogularis*). Phainopeplas (*Phainopepla nitens*) also have a short gut with the same function as in Mistletoebirds (Walsberg 1975).

Some studies have investigated the movement of birds and gut passage rate to predict the potential dispersal distance. Green et al. (2009) showed that Yellow-vented Bulbuls spent a large portion (66-93%) of total time in the *Acacia* trees that serve as hosts to the mistletoe *Plicosepalas acaciae*, allowing for direct dispersal of these seeds. Mistletoe dispersal is common within the ephemeral river (wadis), but not among wadis in the host *Acacia* trees in the Negev Desert of Israel. This is directly related to the flight behaviour of bulbuls, which seldom move among wadis. In addition, the combined result of transit time and movement pattern showed that 73% of seeds were deposited within 100 m of parent plants. In a reciprocal transplant study, Rödl and Ward (2002) showed that *P. acaciae* mistletoes recognise their own parent site and will not germinate on foreign hosts of the same species in different wadis. Under this scenario, even if bulbuls carry the seeds outside their discrete wadi, the seeds are not likely to germinate in the new site. Ward and Paton (2007) have also showed that seed shadow (seed dispersed around a parent plant) of the mistletoe *Amyema miquelii* is strongly leptokurtic, i.e. seeds being deposited a short distance from the parent plant. Similarly, seed rain (seeds dispersed within the population) was aggregated to areas with high mistletoe infection, which was directly related to bird movements (Ward and Paton 2007).

Effect of mistletoes on individual host trees and at the community level

There is little economic damage reported by mistletoes except in the dwarf mistletoes growing on gymnosperms used for timber in North America (Hawksworth and Wiens 1996). Other reports in Australia indicated that heavy infestation of eucalyptus hosts by *Amyema miquelii* and *A. pendulum* reduces the growth of host trees and ultimately can result in death (Yan and Reid 1995). There are unquantified reports that parasitism by mistletoes can directly affect the host, with damage ranging from minor swellings of the branches of host trees to death, depending on the mistletoe species, severity of infection, and health of the host (Martínez del Rio et al. 1996, Tennakoon and Pate 1996, Norton and Reid 1997). Infected trees can be weakened by mistletoe parasitism and become susceptible to insects and fungal attack, which indirectly leads to increased mortality rates (Kuijt 1969).

Mistletoes have lower water use efficiency than their hosts so they can cause considerable water stress to host trees in arid and semiarid environments. Death of *Ziziphus spina-christi* trees with high infection of the mistletoe *P. acaciae* has been observed in Israel (Ward et al. 2006). These authors did not find evidence of water stress in highly infected host trees. However they found that high infection positively correlated with the number of dry branches on *Z. spina-christi* trees. Moreover, fewer fruits were produced on highly infected trees, indicating that heavy infection negatively affected the reproductive ability of host trees (Ward et al. 2006).

Mistletoes cannot be considered as merely destructive pests; rather they can serve as sensitive indicators of overall community integrity and ecosystem health through their network of interactions with other organisms (March and Watson 2007, Mathiasen et al. 2008). Many recent studies have indicated that mistletoes serve as keystone species in many forest ecosystems (Watson 2001, Press and Phoenix 2005) and boost productivity of the soil by the contribution of leaf litter with high nutrient content (March and Watson 2007). Mistletoes provide food for herbivores and nesting sites for birds (Bennetts et al. 1996, Hawksworth and Wiens 1996, Watson 2001). Mistletoes are high in nutritional quality; they have abundant nectar and often nutrient-rich

foliage available year round and an almost complete absence of structural defence makes them very easy to be consumed (Owen and Norton 1995, Ladley et al. 1997, Watson 2001, Mathiasen et al. 2008). Recent studies have indicated that there is a high diversity of animal species that feed on mistletoes (Mathiasen et al. 2008). Mistletoe foliage has been documented in the diet of Brushtail Possums (Ogle 1997, Powell and Norton 1994, Sessions and Kelly 2001, Sessions et al. 2001), Eland (*Taurotragus oryx*), Greater Kudu (*Tragelaphus strepsiceros*) and other large mammals (Roxburgh and Nicolson 2007).

Water and nutrient acquisition of mistletoes

It is believed that mistletoes predominantly tap the xylem of the host tree passively (Hull and Leonard 1964a,b, Leonard and Hull 1965, Reid et al. 1995). However, some actively access nutrients from the phloem of the host tree (holoparasites) and others use both pathways (Ehleringer et al. 1985, Marshall et al. 1994). Water is readily available in the xylem of host plants and mistletoes tap host trees via the haustorium. In addition, mistletoes obtain mineral nutrients (K, N, P, Na, Mg, Cu, Zn, Mn, Fe and Ca) from the host xylem. Compounds such as amino and organic acids, low molecular mass carbohydrates and plant growth substances can also be present in host xylem (Raven 1983, Panvini and Eickmeier 1993, Reid et al. 1995, Press and Whittaker 1993).

The most accepted mechanism of mistletoe nutrient access is described in the passive uptake theory, which suggests that water and nutrient ions passively accumulate from the host's xylem stream to the mistletoe (see e.g., Dean et al. 1994). Mistletoes have a high transpiration rate that allows them to maintain the flow of xylem by opening their stomata (Glatzel 1983, Ehleringer et al. 1985). Thus, most of the time they maintain more negative water potentials (Ψ) than their hosts and as a result they have low water use efficiency (WUE) (Davidson and Pate 1992, Bowie and Ward 2004). By doing so, they accumulate osmotically active solutes in their tissues and the lack of any phloem connections for re-translocation of these elements out of mistletoe tissues enables them to maintain a higher nutrient concentration than their host trees (Ehleringer et al. 1985, Marshall et al.

1994, Glatzel and Gells 2009). Therefore, according to the passive uptake theory, nutrients are acquired only from the xylem, and nutrient and water procurement is directly and inexorably coupled (Schulze and Ehleringer 1984, Bowie and Ward 2004).

It is believed that mistletoes have developed passive uptake to acquire sufficient nitrogen from a very dilute nitrogen source in the host xylem solution (Schulze et al. 1984, Schulze and Ehleringer 1984, Ehleringer et al 1986). The water potential of mistletoes that grow on nutrient-rich host trees, especially on those that fix nitrogen, is close to their host trees Ψ (Ehleringer et al. 1985). Mistletoes can adjust their transpiration rate in response to the nutrient status of their host trees and regulate their WUE in response to the nitrogen supply (Schulze and Ehleringer 1984, Schulze et al. 1984, Ehleringer et al. 1985). In light of this, when mistletoes grow on nitrogen-fixing hosts where the supply of nitrogen is high, the WUE is greater and closer to that of their host than on non nitrogen-fixing hosts (Schulze and Ehleringer 1984, Ehleringer et al. 1985, Schulze et al. 1991).

Mistletoes can also actively take up some carbon nutrients from their hosts as dissolved compounds from the phloem (Pate et al. 1991, Panvini and Eickmeier 1993, Bowie and Ward 2004). For example, based on a stable isotope study, Wang et al. (2008) estimated that mistletoes (*Tapinanthus oleifolius*) take between 35% - 75% carbon and nitrogen from their host. According to the active uptake theory, nutrient and water acquisition are not tightly coupled (Panvini and Eickmeier 1993, Bowie and Ward 2004). Thus, WUE may not directly relate nutrient uptake to water status of the host (Schulze and Ehleringer 1984, Panvini and Eickmeier 1993, Bowie and Ward 2004). Regardless of the pathways involved in nutrient uptake, the concentration range of solutes in mistletoes is consistently higher than in the host (Schulze and Ehleringer 1984, Ehleringer and Schulze 1985, Bowie and Ward 2004, Glatzel and Gells 2009).

The nitrogen:calcium ratio in mistletoe tissues has been used as an index for the phloem mobility of a nutrient (Panvini and Eickmeier 1993, Bowie and Ward 2004). Calcium is phloem-immobile, while other nutrients such as nitrogen are highly phloem-mobile (Lambers et al. 1998, Bowie and Ward 2004). If the N:Ca ratio is > 1 , then host phloem contribution exists in mistletoes

and mistletoes accumulate nutrients in excess of what would have been delivered by the host xylem (Panvini and Eickmeier 1993, Bowie and Ward 2004). However, the mechanism of water and mineral movement from host to mistletoe xylem is not fully understood and consistently explained. This requires further research, and can be addressed, in part, by anatomical studies of the connections between mistletoes and their host's xylem.

Host specificity

Mistletoes parasitise a considerable number of host species and they range from generalists to specialists with wide variation in the degree of specificity, both locally and throughout their range (Monteiro et al. 1992, Downey 1998, Norton and Carpenter 1998, Norton and de Lange 1999). Generalists use various host species, belonging to different families, with no apparent preference. For example, *Viscum album album* parasitises over 450 host species (Barney et al. 1998), and *Amyema miquelli* hosts 17 plant families (Downey 1998). Contrastingly, some mistletoes exhibit a very high degree of host specificity predominating on a few host species (e.g., dwarf mistletoes *Arceuthobium minutissimum* which parasitises only the pine species, *Pinus wallichiana* (Chaudhry and Badshah 2007). Above all, a wide-ranging generalist parasite can be locally limited in the subset of host species it uses, exhibiting local adaptations to specific host species (Clay et al. 1985, Norton and Carpenter 1998, Norton and de Lange 1999, Zuber and Widmer 2000).

The host specificity of parasites can occur as a result of adaptive phenotypic plasticity. In many different types of organisms, a number of changes in phenotypic traits across a continuous environment results in an enormous complexity of interrelationships expressed as phenotypic plasticity (Roff 1992, Lynch and Walsh 1998, Joshi et al. 2001). This can be either adaptive or non-adaptive plasticity. This phenotypic plasticity can be understood by means of reaction norms (see Fig. 2). Reaction norms are lines that have a slope component to reflect the performance of genotypes across environmental changes. If the genotypic responses to environmental changes are non-parallel, it indicates that there is a genotype-by-environment interaction (GxE) (Fig. 2). In the

case of mistletoes, a reciprocal transplant experiment can be done, moving some mistletoe individuals to other host plants of the same host species in the same site and transferring others to different host plants in the same site and/or different species. Such an experiment can be analysed by two factor analysis of variance (ANOVA), where one factor is the source population (the hosts where the plant originally occurred), the second factor is the current population (the current hosts). Most interesting is the interaction effect between these factors, which if significant indicates a GxE interaction. Such a significant interaction indicates that there are crossing reaction norms. This plasticity increases the fitness or performance of individuals and is adaptive for particular local conditions. If the plasticity is indicated by parallel reaction norms, it can be simply a passive consequence of resource limitation (Dudley and Schmitt 1995, Japhet et al. 2009). Adaptive plasticity as a result of GxE interaction elicited by the local host species in the local habitat may be favoured through natural selection. Thus, local specialisation may evolve. In particular, mistletoes have the ability to infect host species that are morphologically and genetically different (Rödl and Ward 2002, Clay et al. 1985). However, it is not apparent whether there are genetically based differences that determine the ability of mistletoes to infect and grow on particular hosts. Such differences could ultimately lead to the evolution of host races (Glazner et al. 1988).

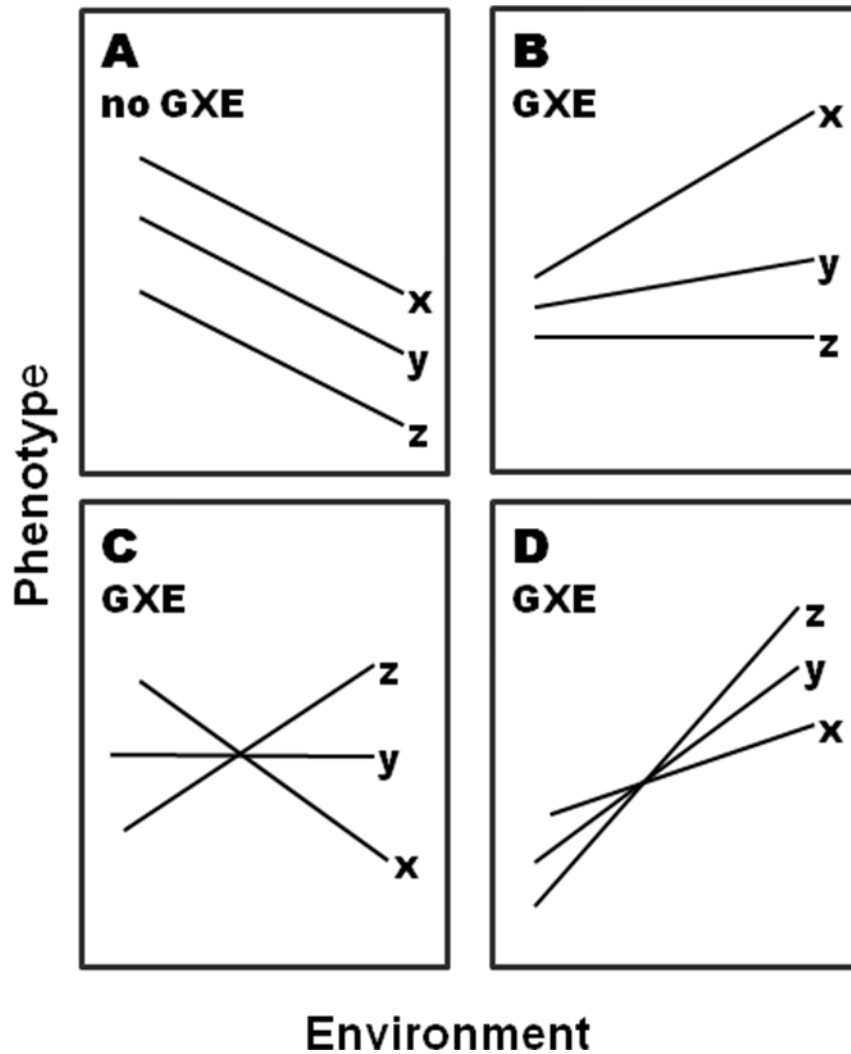


Fig. 2. Reaction norms for three genotypes (x, y and z) in response to two environments. If all reaction norms are parallel to one another, there is no genotype (G) by environment (E) interaction although there is an environment effect (A). If the reaction norms are non-parallel, there is a GxE interaction in which adaptive phenotypic plasticity exists (B, C and D) (after Lynch and Walsh 1998).

The avian dispersers, hosts and environment, along with traits of the mistletoe species, determine the local host specificity of mistletoes. Birds differentially disperse seeds based on their perching behaviour, movement, morphology, gut passage rate, and recognition of mistletoes and host trees. Movement of birds is non-random and this enhances local host specificity in mistletoes.

Differential dispersal by birds is often responsible for host-specificity (Wiens and Tölken 1979, Lamont 1982, Midgley and Joubert 1991).

Similarly, many host plant features affect the life cycle of mistletoes. The attributes of the host include abundance, quality in terms of nitrogen content, and compatibility, which can be manifested at any stage of development of seed deposition, germination, establishment and survival success. Potential hosts can be different in terms of abundance in a given area and this can affect host specificity locally, i.e. mistletoes are abundant on abundant hosts. The relative benefits of specialising on frequently encountered or abundant hosts may outweigh the benefits of interacting with less encountered potential hosts (Norton and Carpenter 1998, Norton and de Lange 1999). Host species nitrogen is a potentially limiting factor for the growth of mistletoes (Dean et al. 1994). Thus, mistletoes may selectively parasitise high nitrogen host species. Based on the Dean et al. (1994) study in South Africa, mistletoe species richness is positively correlated with the average nitrogen level of the plant community in major vegetation types. Average nitrogen levels of host genera and the numbers of mistletoe species that parasitise them are significantly positively correlated. Species of the genus *Acacia* host the largest number of mistletoe species (24 species) because they fix nitrogen, followed by *Combretum* (14 species), *Maytenus* (13 species) and *Rhus* (12 species) (Dean et al. 1984).

Host trees can exhibit different compatibility in terms of infection susceptibility or resistance (Hoffman et al. 1986, López de Buen and Ornelas 2002, Arruda et al. 2006). Host species can evolve means of avoiding mistletoe attack by the acquisition of mechanical and chemical defence mechanisms that can act at any stage of parasite development processes, including adhesion, germination, establishment and differential survival (Monteiro et al. 1992, Martínez del Rio et al. 1995b, Yan and Reid 1995, Medel 2000). Trees without suitable branches are less likely to host mistletoes. Seeds on smooth branches can more easily be washed away by rain and wind than seeds that drop on rough branches (Arruda et al. 2006). Twig diameter is also an important factor for the establishment of mistletoes because seedlings are unable to penetrate twigs with thick

bark (Sargent 1995, Yan and Reid 1995, Ladley and Kelly 1996, Norton and Ladley 1998, Arruda et al. 2006). Yan and Reid (1995) found that maximum survivorship occurred on intermediate-sized branches (7-20 mm in diameter) and the lowest establishment was recorded on small (5-6 mm) or large (>20 mm) branches. Similarly, Sargent (1995) found that seedling establishment was most frequent on twig with 10-14 mm diameters. Apparently, death is common on the smaller and bigger branches due to the frequent death of small twigs, leading to death of the seedlings on them, whereas germinated seeds often died *in situ* on large branches because they are unable to penetrate the thick bark (Sargent 1995). Infections of *Eucalyptus fasciculosa* by the mistletoe *Amyema miquelii* have been observed on the less foliated individuals than more densely covered individuals because dense crowns limit bird perching and light penetration (Ward 2005). Spines also deter bird perching in columnar cacti and this provides defence against mistletoe infection. For example, mistletoe dispersers such as Chilean Mockingbirds (*Mimus thenca*), avoid perching on hosts with extremely long spines (Martínez del Rio et al. 1995b, Medel et al. 2004). Moreover, radicle establishment is deterred as germinated seeds remain hanging on the spine, and cannot reach the host to form a haustorium and penetrate the host trees.

Initial infection may stimulate the host's resistance that prevents further morphological, biochemical and/or physiological development of the mistletoes (Hoffman et al. 1986, Yan 1993b, Reid et al. 1995, Norton and Carpenter 1998). Several potential hosts of *Amyema preissii* and *Lysiana exocarpi* can block haustorial penetration of the bark or xylem through mechanical resistance by means of change in the host tissue surrounding the haustorium (i.e. development of wound periderm) (Yan 1990, 1993b). Hoffman et al. (1986) artificially inoculated several host species with *Tristerix tetrandrus* (Loranthaceae) and found that one host, *Kageneckia oblonga*, rarely bears more than one infection and suggested that no other parasite established after initial infection because it became resistant to further infection.

On the other hand, chemical releases of host trees stimulate the germination and facilitate infection by initiating haustoria formation in many root parasitic plants (Chang and Lynn 1986,

Albecht et al 1999, Tomilov et al. 2004). Root parasitic plants have the ability to recognise the chemical releases of the host trees but, this is unknown in mistletoes (Yoder 1997, 1999, Rödl and Ward 2002, Runyon et al. 2006). Specifically how mistletoes become host specific for a particular host species is unclear. Several studies concluded that germination of mistletoes is either site-insensitive or less influenced by substrate if favourable environment conditions are present by only examining the percentage germination success of mistletoe seeds (Yan 1993a, Roxburgh and Nicolson 2005). These studies were simplified and ignored the detailed process involved in seed germination like morphological and biochemical process involved in hypocotyl, radicle, holdfast, and haustorium formation (e.g., Yan 1993a reported germination of *Amyema preissii* and *Lysiana exocarpi* to be site insensitive).

A recent study on mistletoes by Rödl and Ward (2002) found that holdfast formation and establishment are dependent on the host origin in some mistletoe species. There was also evidence presented by Clay et al. (1985) that the development of haustorial disks was significantly greater when the experimental and source host trees were the same species than when experimental and source host species were different. Nonetheless, there were no significant differences in seed germination between the two groups when source and experimental hosts were the same species and when they were different species (Clay et al. 1985). It is not clear how mistletoes recognise their host at any stage of the development process.

Research gaps, problem statement and justification

Many authors have attempted to investigate the mechanisms that contribute to host specificity in mistletoes (Thompson and Mahall 1983, Norton and Carpenter 1998, Norton and de Lange 1999, Rödl and Ward 2002). Host specificity is an elusive concept, and the mechanisms behind it remain one of the most challenging areas of mistletoe biology. Therefore, understanding the mechanisms of host specificity has the potential to resolve many scientific arguments of evolutionary concepts (Glazner et al. 1988, Norton and Carpenter 1998). Based on this justification, I will explore and test

experimentally the mechanisms of host specificity in the hemiparasitic mistletoe,

Agelanthus natalitius.

Agelanthus natalitius

Taxonomic note: Several names have been assigned to *Agelanthus natalitius* subsp. *natalitius* (Loranthaceae), including *Loranthus natalitius* Meisn.; *Loranthus natalitius* (Meisn.) Sprague; *Acranthemum natalitius* (Meisn.) Tiegh.; *Loranthus moorei* Sprague ; *Tapinanthus moorei* (Sprague) Danser; *Tapinanthus natalitius* (Meisn.) Danser; *Tapinanthus natalitius* (Meisn.) Danser subsp. *natalitius*; and recently *Agelanthus natalitius* (Meisn.) Polhill & Wiens subsp. *natalitius* (Polhill and Wiens 1998).

A. natalitius is distributed from KwaZulu-Natal to the Northern Cape in South Africa and widely distributed in southern Africa (Wiens and Tölken 1979, Visser 1981, Germishuizen and Meyer 2003). The flowering phenology of *A. natalitius* starts in September, with peaks in November followed by fruit production that can occur as late as July (Visser 1981). *A. natalitius* parasitises many plant genera, such as *Acacia*, *Carya*, *Citrus*, *Combretum*, *Dichrostachys*, *Dombeya*, *Grewia*, *Pterocarpus*, *Punica*, *Sclerocarya* and *Terminalia* (Visser 1981). Interestingly, host species of *A. natalitius* can be spatially isolated on a wide range of potential hosts, i.e. a number of isolated populations of potential hosts which are discontinuously distributed. This suggests that *A. natalitius* may be locally specialised on particular host species. For example, in the Umkomaas Valley of southern KwaZulu-Natal, *A. natalitius* parasitises mainly *Acacia karroo*, while in Witwatersrand it is found on *A. caffra*, even in the presence of *A. karroo* (Ward, pers. obs.). Recent studies have indicated tight genotypic coupling between mistletoe genotypes and their host *Acacia* populations (Rödl and Ward 2002).

Structure of the thesis

This thesis has been written as separate papers for publication. This necessarily results in some overlap between papers, particularly with regard to the Methods sections. The aims of this study are to quantify the degree of host specificity in different populations of the mistletoe species *A. natalitius*, and to determine the mechanism causing local specialisation. Chapter 2 presents the survey results in terms of distribution, abundance of host trees and the mistletoes at the study sites. This chapter provides extensive discussion on host abundance in relation to infection prevalence and infection intensity. Characteristics of the host trees that affect infection by mistletoes, such as tree height and diameter at breast height, are discussed. Chapter 3 determines the variation in growth patterns of *A. natalitius* seeds from two populations and two host species to test host compatibility in germination success and investigates the GxE interaction effect by means of a reciprocal transplant experiment. I investigated the germination success and examine the development patterns among the potential hosts and the preference mechanisms to determine possible early host recognition of the mistletoes to the host species.

Chapter 4 addresses the water status and nutrient dynamics of the host and the mistletoe. These are discussed in view of nutrient acquisition via passive or active pathways from the host's xylem or phloem, to highlight the physiological processes evolved in the parasite-host interactions. I investigate the local variation of water and nutrient dynamics among potential host trees at particular field sites that potentially determine the establishment success of mistletoes and drive the host specialisation. In addition, the chapter discusses the use of the nitrogen:calcium (N:Ca) ratio as an indication of phloem access by the mistletoes.

Chapter 5 determines the potential for avian dispersal of mistletoes in two distinct populations of *A. natalitius*. I examine seed dispersal in mistletoes focusing on the co-evolution of mistletoes and their bird dispersers. I explain fruit processing, regurgitation and defaecation by birds in relation to germination success and dispersal distance of mistletoes. This chapter provides a comprehensive discussion of the mechanisms that determine the distribution patterns of

mistletoes such as aggregation on individual host trees and in areas with abundant mistletoes. Gut passage rate of mistletoe seeds is important to determine the dispersal distance and germination success which both affect host specificity.

Finally, in chapter 6, I summarise the overall findings with regard to host specificity and make explicit predictions to guide future research.

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Chapter 2

Host abundance can direct local host specificity in the hemiparasitic mistletoe, *Agelanthus natalitius* (Loranthaceae)

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We investigated the abundance and community composition of woody species that host the hemiparasitic mistletoe, *Agelanthus natalitius*. We carried out field surveys at two sites (110 km apart) in South Africa. The host species community composition, host species frequency, and number of mistletoes per tree were recorded in each site. We measured host tree height and diameter at breast height (DBH), diameter of the infected twig and distribution of mistletoes within the canopy to test for their effects in the infection patterns of *A. natalitius*. We assessed shade effects on mistletoe germination by inoculating *A. natalitius* seeds on host substrates (sections of branches) under three shade levels (20, 40 and 80 % shade). We evaluated the abundance of each host species in relation to infection prevalence (number of trees with infection by ≥ 1 mistletoe) and infection intensity (number of mistletoes per tree). The results showed that *Acacia karroo* was the most abundant host species in both study sites. Mistletoe abundance (number of mistletoes per host species) and infection intensity were higher on *A. karroo* than *A. caffra* in both sites. Moreover, we

observed a greater number of infected trees than expected by chance on *A. karroo*. Infected trees were taller and bigger than the uninfected host trees for both host species in both sites. Prevalence and intensity of infection showed a significant positive relationship with tree host height and DBH. Mistletoes were more frequent on small twigs (0.01-3 cm) in the lower canopy. *A. natalitius* seed germination was significantly higher in treatments with lower shade levels (20 and 40 % shade) than at 80% shade, demonstrating that shade limits the development of *A. natalitius*. Overall our findings suggest that the mistletoe *A. natalitius* is most compatible with the most frequently encountered host species.

Introduction

Mistletoes comprise a diverse group of hemiparasitic flowering plants that have specialised to access nutrients and water from the branches of host trees via a haustorium (Kuijt 1969; Stewart and Press 1990; Watson 2001). Hemiparasitic mistletoes perform photosynthesis but also derive some carbon compounds from the host trees (Marshall et al. 1994; Wang et al. 2007). Mistletoes are usually dependent on frugivorous birds for seed dispersal, in which the mistletoe-bird relationship can be highly specialised (Godschalk 1985; Reid 1991; Aukema and Martínez del Rio 2002*a*; López de Buen and Ornelas 2001). Birds benefit by consuming the fruits and, in turn, disperse the seeds by removing the pericarp and depositing the sticky viscum-covered seeds on twigs of host trees (Godschalk 1985; Aukema and Martínez del Rio 2002*a*). Therefore, birds play an important role in directing the dispersal of mistletoe seeds to host species (Aukema and Martínez del Rio 2002*a, b*; Roxburgh and Nicolson 2007; Green et al. 2009).

Like many other groups of parasites, mistletoe species vary widely in their degree of host specificity, ranging from extreme specialists that parasitise a single species to generalists that use many different host species with no apparent preference for any of them (Norton and Carpenter 1998; Norton and de Lange 1999). Moreover, a mistletoe species can infect a different set of host species in different areas. This may lead to the development of races within a single mistletoe species that specialise on a subset of host species along a geographic gradient (Clay et al. 1985; Overton 1994; Rödl and Ward 2002). It is clear that host species compatibility directs host specificity because host species may differ in their susceptibility to infection by mistletoes (Hoffmann et al. 1986; Yan 1993*a, b*). Moreover, hosts can differ in nutrient availability, water status and size, which can affect the infection pattern of the host species (Bannister et al. 1999; Bowie and Ward 2004). Some mistletoe species become specialised on the most frequently encountered host, which allows the mistletoe to use the most abundant resource in a site (Norton and Carpenter 1998; Norton and de Lange 1999). This may be further enhanced by host recognition in the mistletoes in the form of a gene-by-environment interaction that ensures host specificity

(Rödl and Ward 2002). The study by Rödl and Ward (2002) of mistletoes in Israel shows that there is tight genotypic coupling between mistletoe genotypes and their host *Acacia* populations. Genetic differences in the ability to infect and grow on different hosts may lead to the evolution of host races and subsequently to sympatric speciation (Roff 1992). Consequently, detailed examination of host race evolution, specifically in mistletoes, can provide insight into its potential as a pathway for speciation.

The mistletoe *Agelanthus natalitius* is widely distributed throughout southern Africa, from KwaZulu-Natal to the Northern Cape (Visser 1981; Polhill and Wiens 1998). *A. natalitius* parasitises at least eleven tree genera, including *Acacia*, *Carya*, *Citrus*, *Combretum*, *Dichrostachys*, *Dombeya*, *Grewia*, *Pterocarpus*, *Punica*, *Sclerocarya* and *Terminalia* (Visser 1981). However, geographic variation in the infection patterns over the parasite's range suggests that *A. natalitius* may be locally specialised on particular host species. Many studies have not quantified and investigated local abundance of mistletoes and host species in relation to the mechanisms that cause local host specificity. The aims of this survey are to quantify the degree of host specificity in two populations of the mistletoe species *A. natalitius*, and to evaluate the factors that determine the local distribution and local specialisation of *A. natalitius*.

Methods

Study sites and species

We conducted a survey on the tree communities and the population distribution of the mistletoe *A. natalitius* in two sites in KwaZulu-Natal, South Africa, from April to September 2008. The study sites were located at Highover (29° 54'S, 30° 05'E) and at Mtontwane (28° 80'S, 29° 93'E), about 110 km from one another. Mean annual precipitation at Highover is 763 mm and at Mtontwane is 769 mm. Temperatures vary from 1 °C in winter to 37 °C in summer in both sites. The vegetation of Mtontwane is characterised by *Acacia caffra*, *A. karroo*, *A. tortilis* and *A. nilotica* woodlands and thickets. The vegetation of Highover is also characterised by *A. karroo*,

A. caffra and *A. ataxacantha* woodlands and thickets, except that the vegetation is thicker and the terrain steeper than at Mtontwane.

Field survey

Host availability was assessed by quantifying the host tree community composition in the two study sites. We surveyed a total of 64 plots (20 m x 50 m), in areas where there were high aggregations of trees infected by the mistletoe *A. natalitius*. Plots were excluded if they were intersected by roads, rivers, steep slopes, or thickly vegetated areas that were difficult to access. We identified all potential host tree species in each plot, and verified species identifications by comparing digital photographs and voucher specimens with the herbarium collection at the University of KwaZulu-Natal in Pietermaritzburg. To determine the frequency of *A. natalitius* infection on different host species, we identified and counted all *A. natalitius* individuals in each tree. We then quantified host abundance, *A. natalitius* infection prevalence (the number of trees of a species with infection by one or more mistletoes), *A. natalitius* abundance or mistletoe load (total number of mistletoes in each host species), and *A. natalitius* infection intensity (number of mistletoes per tree) in different host species (classification following Martínez del Río et al. 1996).

Height of each tree was measured with a measuring pole. If the tree was inclined or growing on a slope, trigonometric calculations were applied. Circumferences were measured approximately 1.5 m above the base of the stem and circumference was averaged for multi-stemmed trees. Trees below 2 m in height and < 10 cm in circumference were excluded, because these were never parasitised by a mistletoe. The measured circumferences were used to calculate diameter at breast height (DBH) for each tree. We also measured the circumference of all twigs with mistletoes, from which the twig diameter was later calculated. The diameter of twigs with mistletoes was subsequently categorised into three classes: 0.01 – 3.00 cm, 3.01 – 7.00 cm and > 7.01 cm. In addition, the position of the mistletoe in the canopy was recorded as being in the lower, middle or upper third within the host canopy.

Effect of shade

We conducted a greenhouse experiment to assess the effect of light on germination and early survival of the mistletoes. Shade houses were built using 20, 40 and 80 % shade levels in the greenhouse, with three replicates at each shade level. We used a 3 cm-long section of branches that ranged in diameter from 38 mm to 56 mm from the two main host species (*A. karroo* and *A. caffra*) from both sites (Highover and Mtontwane). For each shade level, we used 40 branch sections; 10 branch sections for each combination of host species and site. For each section of a branch, we inoculated four *A. natalitius* seeds. Two seeds obtained from mistletoes on *A. karroo*, one from Highover and one from Mtontwane and the other two seeds obtained from mistletoes on *A. caffra* (one from Highover and one from Mtontwane). This design enables the seeds to have a source and non-source substrate in terms of host species and sites. The length of the hypocotyl (which gives rise to the haustorium, that attaches to the host) was recorded weekly and survival of the mistletoe seedling was determined at the end of five weeks.

Statistical analysis

All data were analysed using SPSS 15.0 for Windows (χ^2 tests and ANOVA) and GenStat version 11 (binomial logistic regression and generalised linear models). Host species and mistletoe abundance, prevalence of infection, size classes of parasitised twigs and canopy distribution of the mistletoes were analysed for differences in frequency using χ^2 tests. We used ANOVA to analyse the differences in mean height and DBH of the two host species with and without infection by *A. natalitius*. Binomial logistic regression was applied to examine the relationship the probability of prevalence of infection (0, 1) of host trees and height and DBH of host species on. We also analysed the probability of intensity of infection in relation to the tree height and trunk diameter using generalised linear models (GLIM) (GenStat version 11). To determine the effect of shade on growth of *A. natalitius*, we performed Scheffe multiple comparisons tests among the three shade levels.

Results

Field survey

At the two study sites, five host species were recorded as being parasitised by *A. natalitius*, namely *A. caffra*, *A. karroo*, *A. tortilis*, *A. nilotica*, and *Leucaena leucocephala* (all Fabaceae; scientific names after Van Wyk and Van Wyk 1997). *A. tortilis*, *A. nilotica*, and *L. leucocephala* were excluded from further analyses because these species were either rare in the study sites or had few infected individuals. *A. tortilis* and *A. nilotica* were completely absent in Highover, except for one tree (*A. nilotica*) recorded in the survey plots. Similarly, only two individuals of *L. leucocephala* were recorded in a single plot at Highover, both of which were infected. At Mtontwane, only a few individuals of *A. nilotica* (n = 3) and *A. tortilis* (n = 9) were infected, each supporting a single *A. natalitius* individual. Thus, all statistical analyses were applied to the two most common host species, *A. karroo* and *A. caffra*, which grow abundantly at both sites and were recorded with high infection rates of *A. natalitius*.

A. karroo was significantly more abundant than *A. caffra* in both sites (fig. 1) ($\chi^2_{1, 1464} = 428.46$, $p < 0.001$). Prevalence of infection (number of trees with infection by ≥ 1 mistletoe) was higher for *A. caffra* than *A. karroo* at both sites (fig. 2). However, assuming an equal probability of infection for both species, infected trees of *A. karroo* were more frequent than expected by chance ($\chi^2_{1, 366} = 73.49$, $p < 0.001$). The mistletoe load (number of mistletoes per host species) was higher for *A. karroo* in both sites ($\chi^2_{1, 529-673} = 116.75-293.80$, $p < 0.001$, n = 1202 total, with 529 mistletoes on *A. karroo* at Highover and 673 at Mtontwane) (fig. 2). There was no significant difference in tree height and trunk DBH of *A. karroo* and *A. caffra* trees in either site (height, $F_{1, 1464} = 0.76$, $p = 0.39$; DBH, $F_{1, 1464} = 2.16$, $p = 0.142$). However, the mean height and trunk DBH of infected trees were significantly greater than for uninfected trees for both species in both sites (height, range of $F_{1, 157-622} = 12.688-46.038$, $p < 0.001$; DBH, range of $F_{1, 157-622} = 5.15-61.51$, $p < 0.05$) (fig. 3).

The relationships of prevalence (i.e. carrying at least a single mistletoe infection) and infection intensity (number of mistletoes per tree) to tree height and trunk DBH were tested with binary logistic regression and generalised linear models (GLIM), respectively. The logistic regression analysis indicated that both height and trunk DBH had a significant positive effect on the probability of infection (slopes for prevalence and height ranged from 0.38-0.85, range in Wald = 12.07-40.25, $p < 0.001$, range in N = 157-622). A similar result was obtained for trunk DBH, although there was a lower slope (slopes for prevalence and DBH ranged from 0.050-0.096, range in Wald = 4.70-40.62, $p < 0.001$, range in N = 157-622). Prevalence of grouped host trees were positively correlated with height (1 m class width) and with DBH (10 cm class width) (results after prevalence was arcsine square root transformed, height, range in $r = 0.90 - 0.95$, range in $F = 17.33-39.76$, $p < 0.05$; and DBH, range in $r = 0.90 - 0.97$, range in $F = 12.07 - 50.04$, $p < 0.05$). However, the DBH class of *A. caffra* in Highover was not significantly positively correlated with prevalence ($r = 0.50$, $F = 1.00$, $p = 0.39$) (fig. 4).

The relationship between intensity of infection and tree height and trunk DBH was further analyzed with GLIM, as the frequency distribution of parasitism among the two host species followed a negative binomial distribution (see Krebs 1989) (variance/mean = 6.30/0.79 and $k = 0.16$, $N = 1464$, χ^2 for goodness of fit = 9.17, $df = 3$, $p = 0.027$). This analysis demonstrated that the distribution of *A. natalitius* among host trees was strongly aggregated, meaning that most potential hosts were not infected, while a few individual host trees supported most of the parasites and only a few were highly infected (e.g. we observed a single host with 56 mistletoes in our study). The GLIM analysis showed that the number of mistletoes per host tree (infection intensity) had a positive significant relationship with tree height (range in slopes = 0.30-0.70, $p < 0.001$, range in N = 157-622). A similar result was obtained for trunk DBH, although with a lower slope (range in slopes = 0.024-0.032, $p < 0.001$, range in N = 157- 622). Overall, infection intensity and the probability of infection increased with tree height and trunk DBH. *A. natalitius* grew primarily on small twigs with a circumference of 0.01-3.00 cm (81 %), followed by 3.01-7.00 cm (14 %), and

least on twigs > 7.00 cm (5 %) ($\chi^2_{3, 1168} = 1931.36, p < 0.001$). The distribution of *A. natalitius* within the canopy of the host species was mainly in the lower canopy (61 %), followed by the upper canopy (31 %), and with the fewest in the middle canopy (9 %) ($\chi^2_{1, 1066} = 238.17, p < 0.001$).

Growth under different shade levels

Mistletoe seeds germinated immediately (we detected no differences in germination rate among treatments), but mistletoe seeds did show response in their hypocotyl growth. The hypocotyl length of *A. natalitius* seeds grown under different shade levels was significantly different across independent variables; source, shade level, source x shade level and source x current substrate x shade level (table 1). A Scheffe multiple comparisons test indicated no significant difference between the hypocotyl length of the seeds grown under 20 % and 40 % shade levels ($p = 0.59$), but both were significantly greater than those of seeds grown under 80 % shade level ($p < 0.001$) (fig. 5). In addition, our results showed that the hypocotyl length of *A. natalitius* was greater when placed onto their original host substrate than on different substrates (fig. 5).

Discussion

Host abundance

A. natalitius parasitises several host species and its local distribution can be patchy, depending on diverse factors such as host abundance, host compatibility, bird seed dispersal and host nutrient and water content. Based on the results of our study, *A. karroo* can be considered as the most suitable and compatible host species for *A. natalitius*. The prevalence of mistletoes (the number of infected host trees within a species) was greater on *A. caffra* in both sites, but most *A. caffra* individuals had only a single infection. The parasitic load and the expected number of infected trees (expected prevalence considering only infected trees and assuming equal probability of infection) were greater on *A. karroo*. This result demonstrated that prevalence alone does not allow one to evaluate host specificity. For example, *L. leucocephala* was represented by only two

trees in the study sites and both were infected. If prevalence was considered, it would be 100 %, which fails to explain host specificity.

The results from our study were consistent with those of other studies demonstrating that host specificity can be directed by host abundance because abundant host species are most frequently encountered, and are more reliable through time and space than less abundant host species (Norton and de Lange 1999; López de Buen and Ornelas 2002). Thus, interacting with abundant host species is advantageous compared to rare species. Host species selection can favour the most abundant host trees (Norton and Carpenter 1998; Zuber 2000). A field reciprocal transplant germination experiment also showed that mistletoe seeds grew better on *A. karroo* than *A. caffra* regardless of their source host species, demonstrating a preference of *A. natalitius* for the most abundant host species, *A. karroo* (D.Y.O. et al.; in prep.).

Host tree traits

A. karroo and *A. caffra* were not significantly different in size (height and DBH) in both sites, so it can be assumed that any differences in mistletoe infection cannot be attributed to size differences in the host species. If we compare other traits that influence infection patterns, *A. caffra* has either small spines or is almost spineless, which can affect the post-dispersal development process of the seed (e.g. Hoffmann et al. 1986; Yan 1993a, b; Martínez del Rio et al. 1995a, b). Seeds that are dispersed to spineless branches that have rougher fissured-bark on *A. caffra* can easily contact the substrate and establish more easily (see other similar studies on traits of host traits in relation to mistletoe infection; Sargent 1995; Arruda et al. 2006). Based on the ability of mistletoe seeds to attach to *A. caffra*, it might be expected that this host species would be more frequently infected by mistletoes than *A. karroo*. However, greater infection intensity was observed on *A. karroo* trees than on *A. caffra* trees.

Tree height and trunk diameter of infected and uninfected trees

In spite of a lack of size difference between host species, the infected trees were taller and had a greater trunk diameter than uninfected host trees for both host species in both sites. This result may be a consequence of the behaviour of dispersers, as birds differentially perch on tall trees and may deposit mistletoe seeds in the process. Moreover, if trees are tall, they are probably older and have had more time to become infected by mistletoes. Thus, tall and big trees are frequently observed with a greater number of mistletoe infections than short and smaller trees (Donohue 1995; Aukema 2004; Roxburgh and Nicolson 2007; Kartoolinejad et al. 2007). In addition, tall trees are usually more branched and provide more twigs with a suitable diameter than short trees because mistletoes can only penetrate and establish on small twigs (Sargent 1995). Mistletoes deposited on tall trees also have greater success because tall trees are less likely to be shaded, thereby providing adequate light for mistletoes (Lamont 1982; Ward and Paton 2007; Katoolinejad et al. 2007). In addition, tall trees may supply more nutrients and water to the mistletoes due to deeper and broader root systems (Ward et al. 2006). Tall trees also protect mistletoes from browsing by large herbivores (Roxburgh and Nicolson 2007). The giraffe, a common herbivore of mistletoes, is abundant in Mtontwane. Mistletoes are often selected by herbivores over their host trees because the mistletoes are higher in nutrient content and have few physical and chemical defense mechanisms. Thus, herbivores can limit mistletoes (Midgley and Joubert 1991). Associated with this, *A. natalitius* growing on *A. karroo* may be better protected against foragers because *A. karroo* has longer spines than *A. caffra* and, thus, is better defended against herbivores (see also Martínez del Rio et al. 1995a). Although there has not been much study on the effects of fire on mistletoe survival, large trees have higher survival following fire and may maintain mistletoes better than small trees in habitats subjected to fire.

The risk of infection of host trees by *A. natalitius* positively correlates with tree height and DBH. As trees get older they generally become taller and bigger, thereby increasing the probability

of infection because birds prefer to perch on tall trees (Overton 1994; Donohue 1995; Aukema and Martínez del Rio 2002b). Overton (1994) explained the frequency of mistletoe infection as an accumulation function of infection with time as the tree gets older. However, many studies showed a weak correlation between height of tree and infection intensity which did not fit Overton's (1994) model (Donohue 1995; Aukema 2004; Roxburgh and Nicolson 2007). As in previous studies, linear regression analysis in this study produced a weak correlation. We suggest that the analysis may be inappropriate because the data from these other studies followed a negative binomial distribution and hence testing for a linear correlation without an appropriate statistic may make the comparison invalid.

An alternative (GLIM) analysis explained the observed patterns better because the frequency distribution of the number of mistletoes per tree (infection intensity) is a good fit to the negative binomial distribution, i.e. most species are free of mistletoes and a few individuals have high infection. Infection intensity (number of mistletoes per tree) was positively related to tree height (range in slopes = 0.38-0.85, $p < 0.001$) as well as trunk diameter (range in slopes = 0.050-0.096, $p < 0.001$). This pattern occurs because previous infection increases the likelihood of further infection and apparently causes a clumped or aggregated distribution which can be considered as being due to the limited dispersal of mistletoe seeds, resulting in a high rate of autoinfection directed by birds (Overton 1994; Aukema 2004; Ward and Paton 2007). Birds significantly increase the repeated infection of the host tree by voiding the seeds through regurgitation or defaecation usually on the same host tree (D.Y.O. et al.; pers. obs.). As a result, re-infection of already parasitised trees leads to a strong aggregation of mistletoes on hosts. Infection of neighbouring trees is also higher and this reinforces aggregation in mistletoes (Green et al. 2009).

Twig size and shade effect

A. natalitius predominantly infects smaller twigs of the host species in the study sites. This is similar to other mistletoes because they are unable to penetrate the thicker bark of larger diameter host stems (Sargent 1995). Mistletoes were mainly distributed in the lower canopy, followed by the upper canopy within host trees. As birds disperse mistletoe seeds through regurgitation and defaecation, the lower canopy receives more seeds than the upper canopy. Moreover, mistletoes were abundant in trees that were found in open areas and were almost absent in thick forest. Consistent with the field observations, mistletoe seed germination and early survival in our greenhouse study were significantly higher at lower shade levels. It has also been reported in other studies that mistletoes are often abundant in savannas where the canopy cover is minimal (e.g. Dean et al. 1994). Additional studies also explained that a positive correlation between the high prevalence of mistletoes and host tree height is related to a better survival of mistletoes at high light intensity (Aukema and Martínez del Rio 2002b; Roxburgh and Nicolson 2007), which is consistent with our findings. This implies that shade can limit the survival of mistletoes. In addition, our results also showed that the hypocotyl length of *A. natalitius* seeds was greater when seeds placed on the same host substrate (branch sections) as that of its host origin (or source) than on different substrates. This deserves further investigation.

Conclusions

Overall, these findings support the suggestion of Norton and Carpenter (1998) that host abundance plays an important role in determining mistletoe host specificity. Loranthaceous mistletoes tend to be generalists in heterogeneous tropical forests and tend to be host specific in temperate forests where the resources are limited. Therefore, performance of preferred hosts manifested by host abundance enhances the performance of growth and reproduction of the parasite (Norton and Carpenter 1998). The host species, *A. karroo*, is abundant in both sites, followed by *A. caffra*. In the study sites, other potential host species for *A. natalitius* are rare, so *A. natalitius* may

be selected to use the most abundant resource, *A. karroo*. We note that other studies reported that frequency of infection in mistletoes did not reflect host relative abundance (Aukema and Martínez del Rio 2002*b*; Roxburgh and Nicolson 2005).

Key factors enhancing host specialisation in mistletoes include chemical compatibility and nutrient and host water content, which deserve further study. For example, chemical interactions between the host species and the parasites can also ensure host specificity, i.e. hosts direct their infection by their chemical releases (Yoder 1999; Runyon et al. 2006). This has not been fully investigated in mistletoes (Rödl and Ward 2002) but is relatively well known in root parasites (Yoder 1999).

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Table 1

THREE WAY INTERACTION BETWEEN SOURCE, CURRENT HOST SUBSTRATE AND SHADE LEVEL (20, 40 AND 80 %). ALL FACTORS WERE SIGNIFICANTLY DIFFERENT EXCEPT THE CURRENT SUBSTRATE AND ITS INTERACTION WITH SHADE LEVEL.

Source of variation	d.f.	F	P
Source	1	162.36	< 0.001
Current substrate	1	2.91	0.089
Shade level	2	405.70	< 0.001
Source x current substrate	1	64.43	< 0.001
Source x shade level	2	39.18	< 0.001
Current substrate x shade level	2	0.98	0.377
Source x current substrate x shade level	2	16.34	< 0.001
Error	719		

Figure legends

Fig. 1 Percentage abundance of *A. natalitius* on the host species *A. karroo* (most common) and *A. caffra* at Highover and Mtontwane (frequency of each host species converted to percentage).

Fig. 2 (a) Prevalence of *A. natalitius* (number of trees parasitised/total number of trees*100) on *A. karroo* and *A. caffra* in Highover and Mtontwane. (b) Percentage of *A. natalitius* load (total number of *A. natalitius* on a particular host species/total number of mistletoes on all host species*100) in Highover and Mtontwane.

Fig. 3 Mean height \pm SE of infected and uninfected trees of *A. karroo* and *A. caffra* in Highover and Mtontwane. In Highover, infected trees were taller than uninfected trees for both host species, *A. karroo* (Hk) and *A. caffra* (Hc). Similarly, in Mtontwane, infected trees were taller than uninfected for both host species, *A. karroo* (Mk) and *A. caffra* (Mc).

Fig. 4 Prevalence was significantly positively correlated with tree height (a and b) and DBH (c and d) for both host species, *A. karroo* (solid circles) and *A. caffra* (blank circles) in both sites, Highover and Mtontwane. Prevalence of *A. caffra* was not significantly correlated with tree DBH in Highover (Highover, c).

Fig. 5 Mean \pm SE of the hypocotyl length of the seeds from *A. karroo* (mk) and *A. caffra* (mc) inoculated on different host substrates, *A. karroo* (k) and *A. caffra* (c), at three shade levels (20, 40 and 80 %) from Highover and Mtontwane. Note the consistently short hypocotyl lengths at 80% shade. In addition, our results showed that the hypocotyl length of *A. natalitius* was greater on the same host substrate as that of its host origin than on different substrates (kxmk represented by black bar and cxmc represented by white bar at 20 and 40 %).

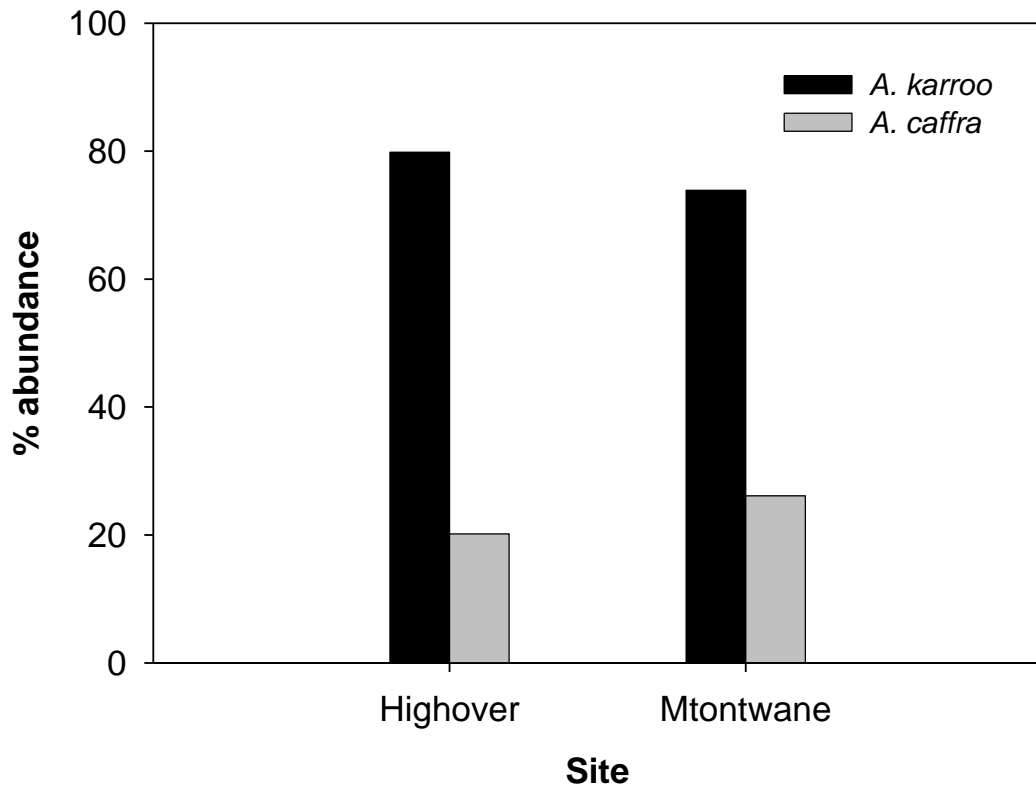


Fig. 1

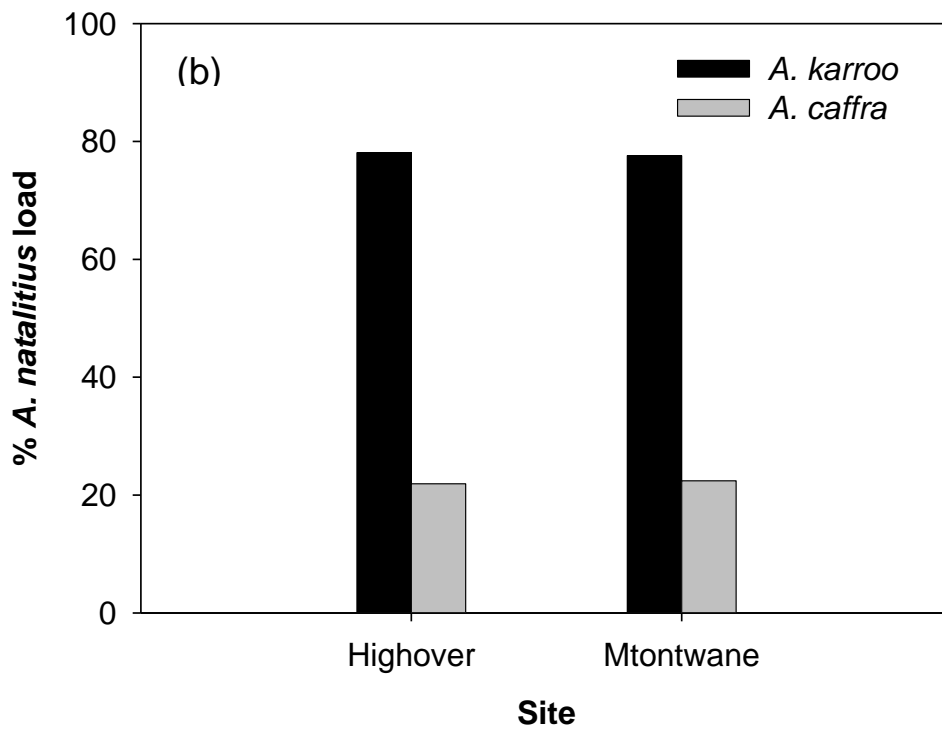
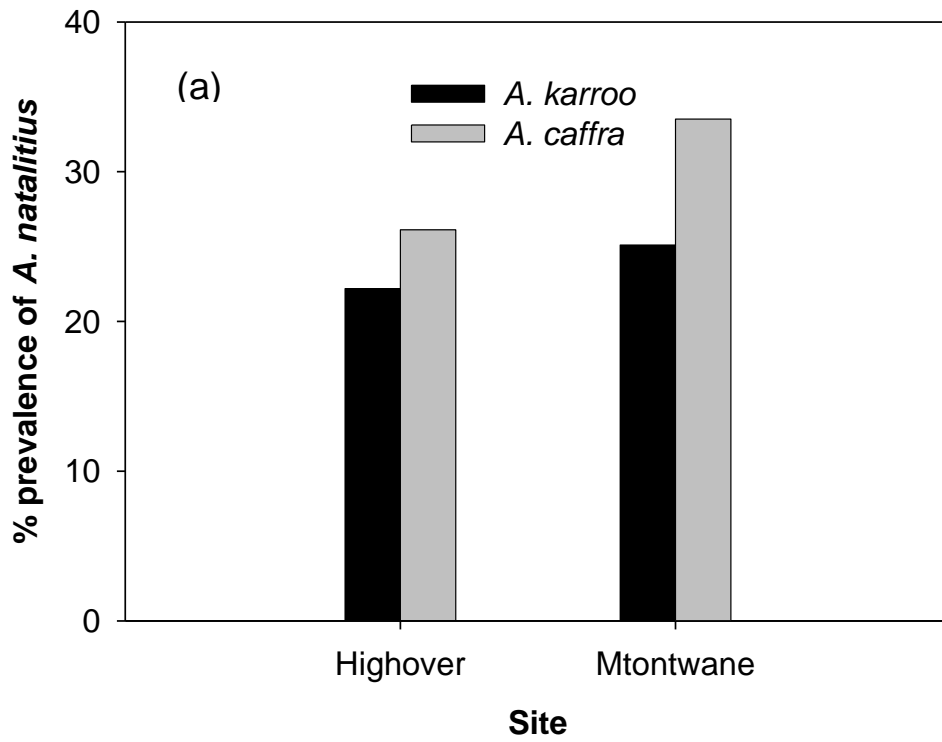


Fig. 2

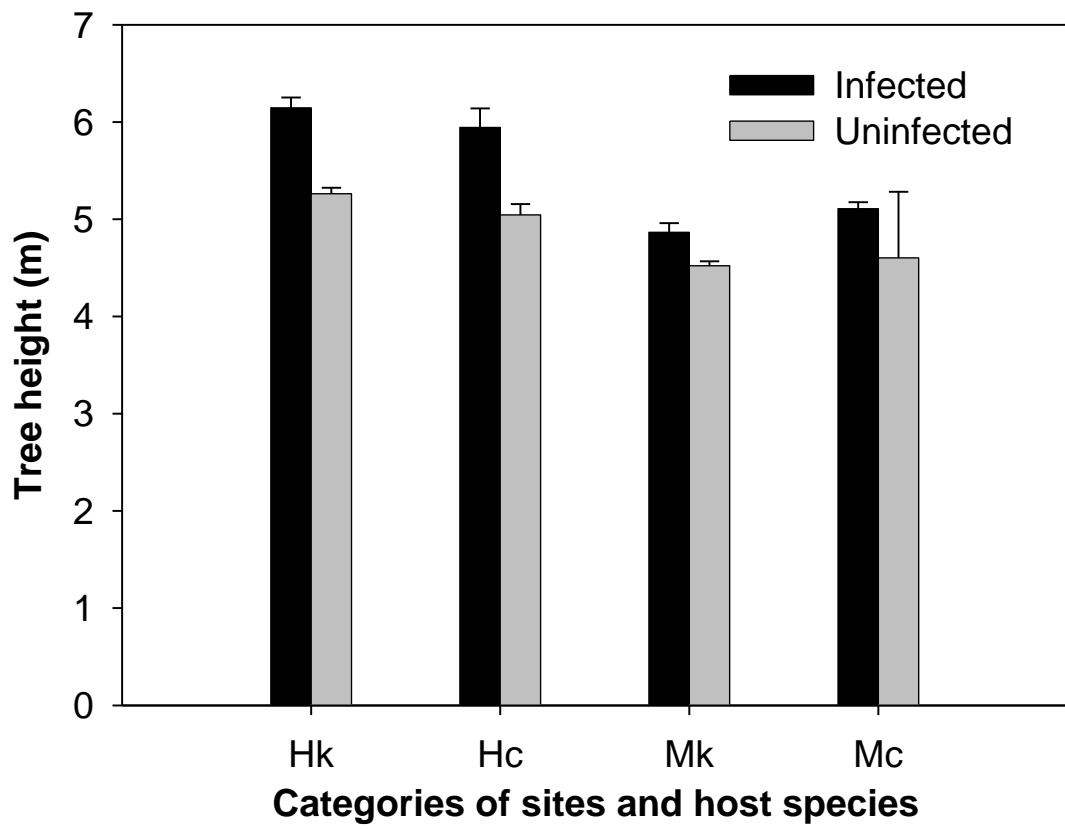


Fig 3

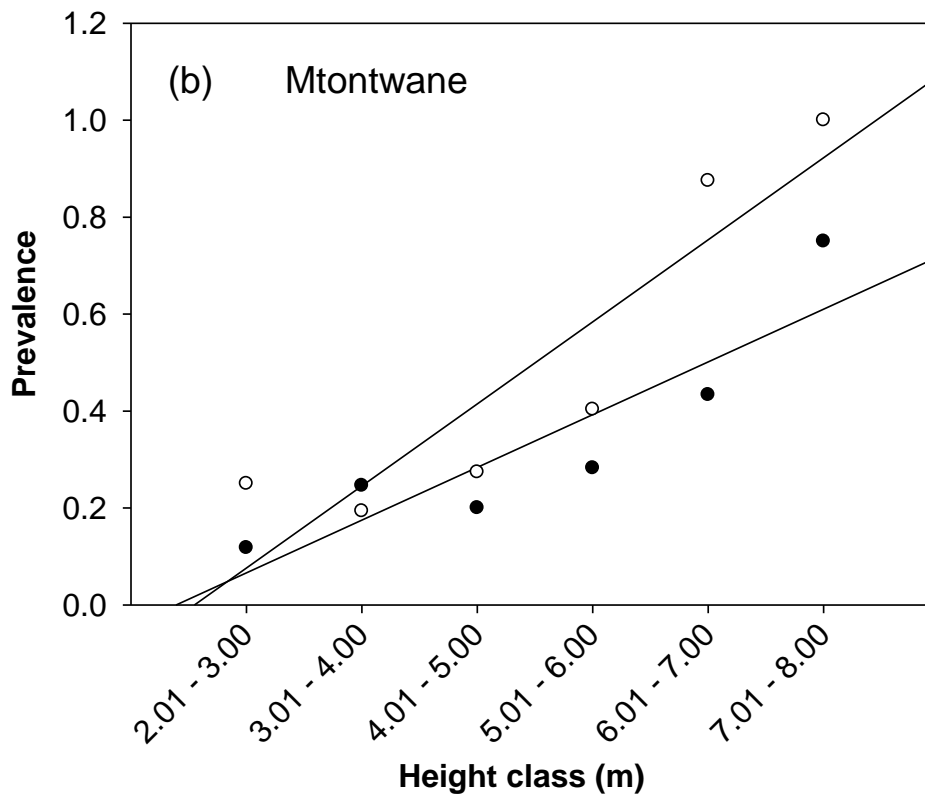
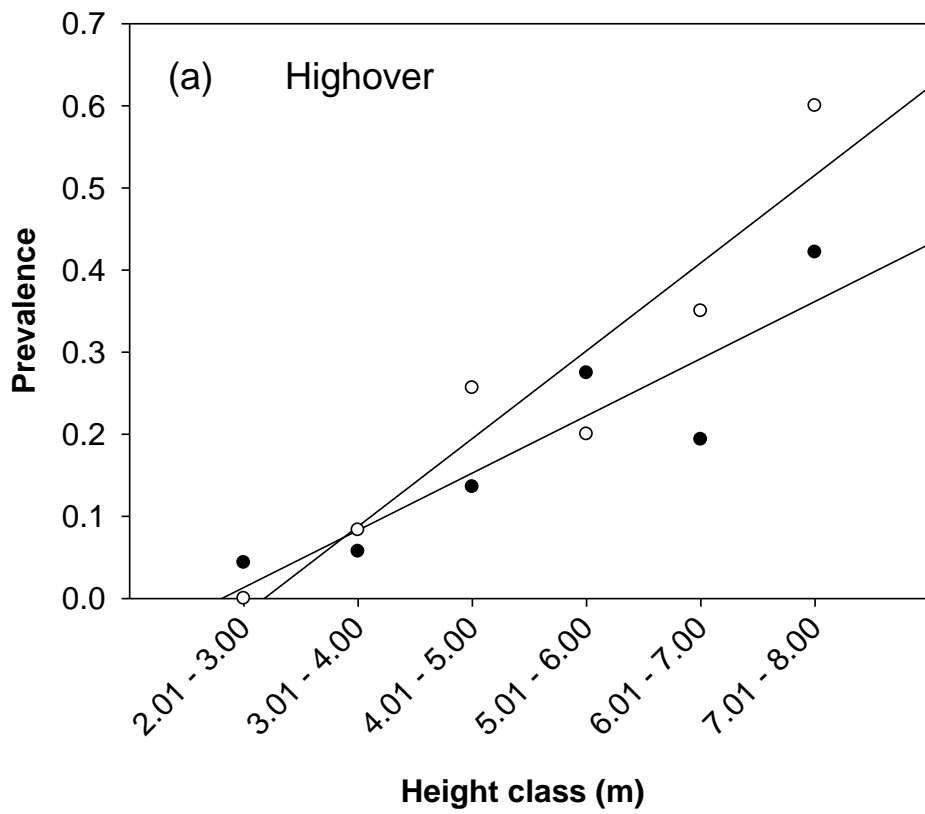


Fig. 4

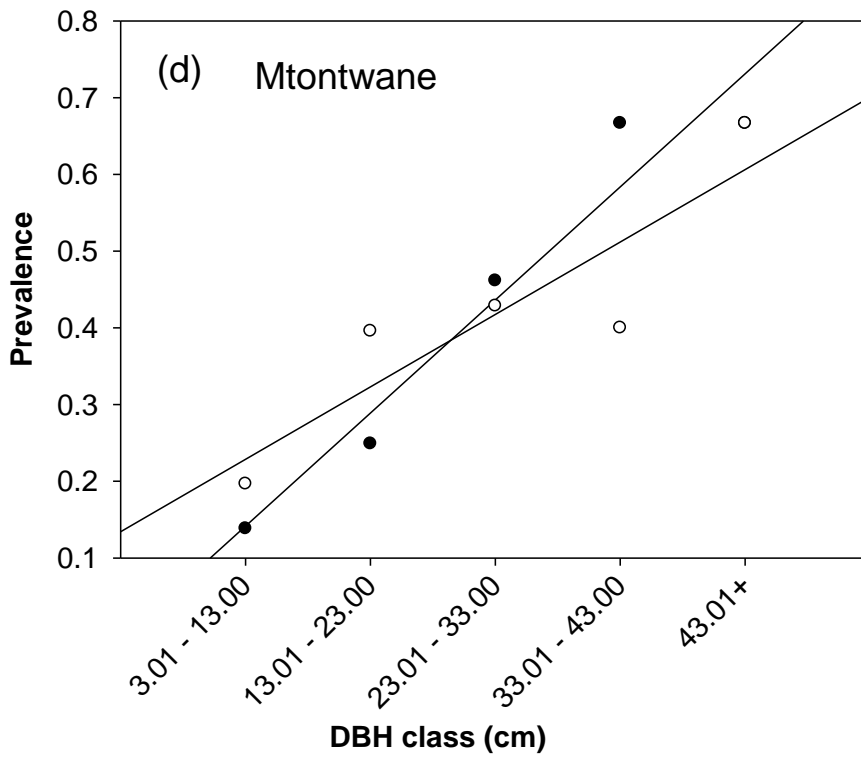
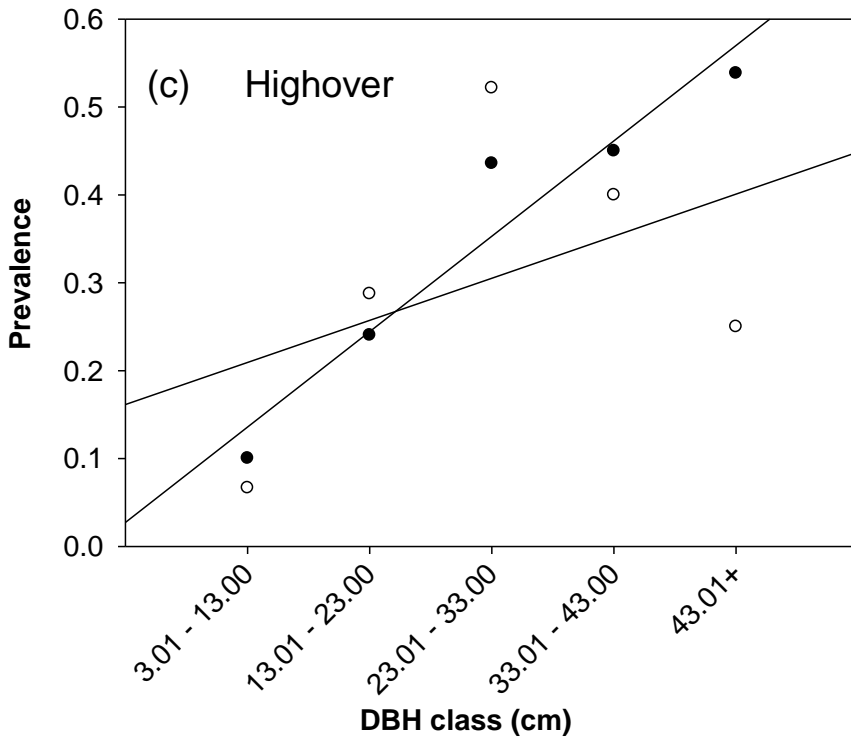


Fig. 4

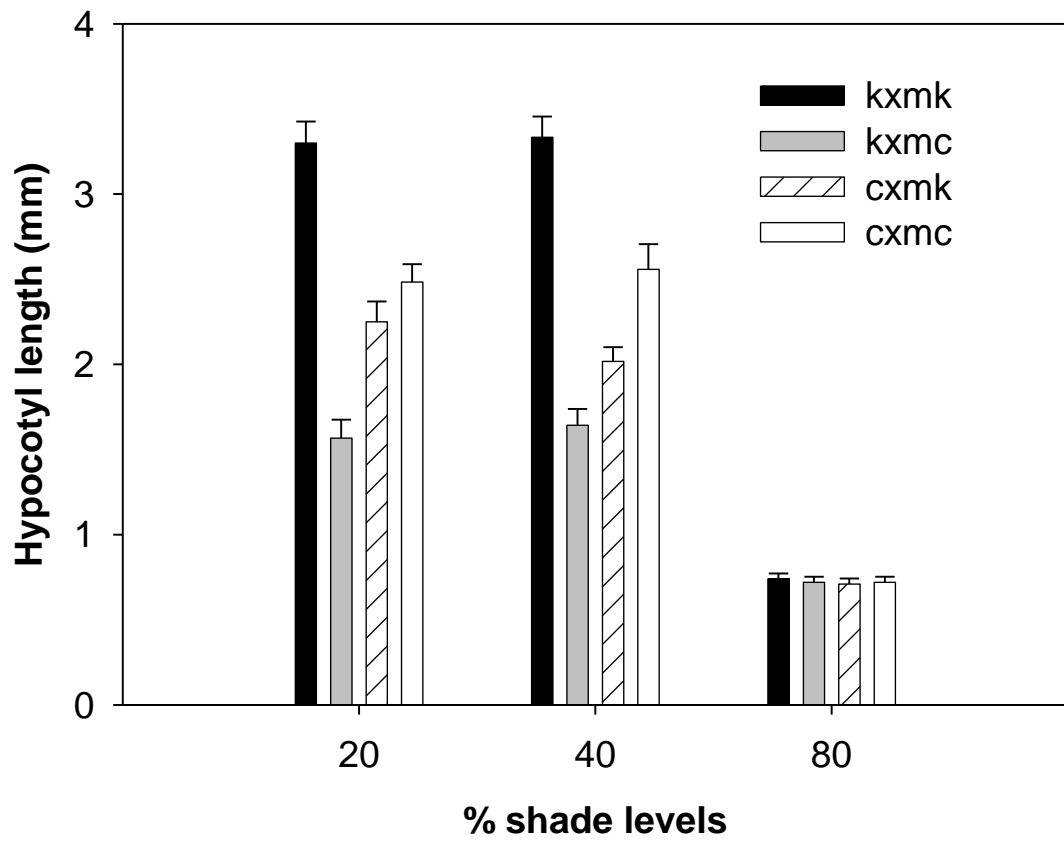


Fig. 5

Chapter 3

Source host species recognition and preference in the hemiparasitic mistletoe, *Agelanthus natalitius* (Loranthaceae)

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Running title: Host specificity of *Agelanthus natalitius*

This chapter has been written following the format of *Functional Ecology*

Summary

1. Genotype by environment (G x E) interactions influence the development of host-race differentiation, which may eventually direct local host specificity. We tested for G x E interactions in the hemiparasitic mistletoe, *Agelanthus natalitius*.
2. We conducted field reciprocal transplant experiments in two distinct populations (110 km apart) of one mistletoe species, *Agelanthus natalitius*, that commonly parasitises *Acacia karroo* and *A. caffra* in South Africa. We inoculated mistletoe seeds on individuals of source and non-source host species both within their locality and translocated between sites. We recorded germination success, hypocotyl length and the growth form of the germinated mistletoes (specifically, the direction of movement towards or away from the host substrate) to investigate their performance in different host environments.

3. Germination success was high and there was no significant difference among all combinations, indicating that germination occurs independent of substrate and site. However, the hypocotyls of the germinated seeds generally grew longer when they were placed on source host species within their locality. When seeds from mistletoes growing on *A. caffra* were transferred to *A. karroo*, they grew as well as those transferred to *A. caffra*. In contrast, seeds obtained from mistletoes growing on *A. karroo* and placed on *A. caffra* fared worst of all combinations in both sites. We found the same trends for the number of hypocotyls that successfully attached to the substrate.

4. The mistletoes performed better on their source host species and showed preferences among the available host species at an early developmental stage. In this case, *A. karroo* is the more susceptible or compatible host species. This also explains the observed infection patterns of these mistletoe populations in the field which could be the result of an adaptation of the mistletoe to the most frequently-encountered host species, *A. karroo*.

5. Hypocotyl growth in this mistletoe species showed an adaptive plasticity due a G x E interaction, the result of which is that mistletoes have strong coupling with their source host species in their own locality. This suggests that differential utilisation of hosts by mistletoes may ultimately direct host specificity.

Key-words: G x E interaction, germination, haustorium, host specificity, reaction norms, reciprocal transplant

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Introduction

Many mistletoes are hemiparasitic plants that grow on the branches of host trees and shrubs (Kuijt 1969; Schulze, Turner & Glatzel 1984; Wang *et al.* 2008). They infect a wide array of host trees and vary greatly in their degree of host specificity, ranging from specialists that parasitise a single species to generalists that use many different host species (Norton & Carpenter 1998; Norton & de Lange 1999). Mistletoes can also be locally host specific where host preference varies geographically, i.e. at a given location a mistletoe species may infect only part of its potential host set (Lamont 1982; Rödl & Ward 2002).

Behaviour of bird dispersers and various host traits (species, tree height, incidence of previous mistletoe infection) are often considered to determine the infection patterns of mistletoes (Aukema & Martínez del Rio 2002; Rödl & Ward 2002; Green, Ward & Griffiths 2009). Very few studies have indicated that host species vary in their susceptibility and resistance to infection by mistletoes (Hoffmann *et al.* 1986; Yan 1993b; Aukema & Martínez del Rio 2002; Arruda, Carvalho & Del-Claro 2006). However, bark thickness and texture of host species can affect the attachment and establishment of mistletoes (Hoffmann *et al.* 1986; Yan 1993b; Arruda *et al.* 2006); these traits vary greatly among tree species. There is speculation that some host species may develop resistance in response to previous infection that helps to protect them against further infection (Hoffmann *et al.* 1986).

It is well known that a phenotype of a trait is the expression of a genotype in a given environment (i.e. a G x E interaction) (Joshi *et al.* 2001). The genetic variation for plasticity (G x E) of organisms allows them to cope with heterogeneous environments they encounter (Dudley & Schmitt 1995; Japhet *et al.* 2009). The adaptive plasticity as a result of G x E interaction elicited by the local habitat may be favoured by natural selection. Thus, local specialisation may evolve. In particular, hemiparasitic mistletoes have the ability to infect host species that are morphologically and genetically different (e.g. Rödl & Ward 2002). However, the genetically-based differences in the ability to infect and grow on particular hosts that may lead to the evolution of host races and

subsequently to sympatric speciation are unclear (Glazner, Devlin & Ellstrand 1988). The environmental plasticity of mistletoes along a geographic gradient shows limited evidence of genetically-based variability in host preference (Glazner *et al.* 1988). Furthermore, it is unclear why differential host preference develops in mistletoe populations (Clay, Dement & Rejmanek 1985; Rödl & Ward 2002).

The key initial developmental stages for mistletoes in their interactions with any host tree are germination and haustorium formation, which are the focus of the present study. Mistletoes need to germinate and attach to host plants efficiently to ensure their survival. Thereafter, a hypocotyl-radix complex is formed by the mistletoe, which then forms a bell-shaped holdfast that attaches to the host (Rödl & Ward 2002). At this contact site, a haustorium is formed through which mistletoes penetrate and access nutrients from the host vascular tissue (Rödl & Ward 2002; Roxburgh & Nicolson 2005).

Unlike mistletoes, germination and haustorium formation have been extensively investigated in root parasites due to their negative effects on crop yield (Chang & Lynn 1986; Yoder 1999; Matvienko, Torres & Yoder 2001). Root parasites use host-derived chemicals to stimulate and initiate germination (Chang & Lynn 1986; Press, Graves & Stewart 1990; Yoder 1999; Matvienko *et al.* 2001). Similarly, many *in vitro* investigations have demonstrated that applying natural and synthetic chemicals can stimulate haustorium development in root-parasitic plants (Albrecht, Yoder & Phillips 1999; Tomilov *et al.* 2004). In contrast, many aerial parasitic plants such as mistletoes and facultative parasites do not require host germination factors. Their germination is independent of the substrate as long as the exocarp (the outermost layer of fruit) is removed (Lamont 1983; Yan 1993a; Rödl & Ward 2002).

Once germinated, mistletoe haustorium formation may involve chemical cues. Rödl & Ward (2002) found that haustorium formation can be site-sensitive. In their study on the mistletoe *Plicosepalus acaciae*, seeds planted on different substrates were most successful in forming haustorial disks when placed on substrates from their own locality (see also Clay *et al.*

1985; Yan 1993b). This suggests that there is tight coupling between mistletoe genotypes and their host *Acacia raddiana* populations (Rödl & Ward 2002). It has been also demonstrated that individuals of the facultative parasite dodder, *Cuscuta pentagona*, locate their hosts through volatile chemicals released by their preferred host plants (Runyon, Mescher & De Moraes 2006). While dodder and other root parasites are capable of selecting different host species, the mechanisms involved in host location and discrimination in mistletoes are not well understood (Rödl & Ward 2002; Mathiasen *et al.* 2008).

Plasticity seems to enable mistletoes to interact to a variety conditions that subsequently directs and develops host races locally as result of G x E interactions. In light of this, we conducted a reciprocal transplant germination experiment to investigate the contribution of G x E interactions in local adaptation of the hemiparasitic mistletoes. We investigated germination and haustorium formation in the mistletoe, *Agelanthus natalitius* (Meisn.) Polhill & Wiens subsp. *natalitius*, and predicted that host recognition and preference may occur at this stage. We tested this prediction in two combinations of mistletoe-host populations and used hypocotyl growth as important traits of adaptive plasticity.

Methods

We carried out field reciprocal transplant germination experiments using *A. natalitius* seeds obtained from fruits of mistletoes parasitising *Acacia karroo* Hyne and *Acacia caffra* (Thunb.) Wild. (source host species) from two sites, Mtontwane (28° 80'S, 29° 93'E) and Highover (29° 54'S, 30° 05'E) in KwaZulu-Natal, South Africa. In this paper, we used the word *mistletoe* to refer to the mistletoe species of our study, *A. natalitius* in order to avoid confusion of the names with the host species; *A. caffra*, and *A. karroo*, especially when they appear together in the text. Mean annual precipitation at Highover is 793 mm and at Mtontwane is 769 mm. The vegetation of Mtontwane is characterised by *A. caffra*, *A. karroo*, *A. tortilis*, *A. nilotica* woodlands and thickets. The Highover

vegetation is riparian forest characterised by *A. karroo*, *A. caffra*, and *A. ataxacantha* woodlands and thickets along steep valleys of the Umkomaas River.

A. natalitius fruiting occurs from April to July (Wiens & Tölken 1979; Visser 1981; Polhill & Wiens 1998). *A. natalitius* fruits are single seeded and change in color from green to dark-red when fully ripe. Prior to fruit collection, we bagged unripe mature fruits using nylon mesh bags to protect fruits from bird consumption. To avoid pseudoreplication, we randomly selected 20 individual mistletoes in different host trees from each of the two main source host species, *A. karroo* and *A. caffra*, in both sites. When the fruits were fully ripe, we collected them by hand picking in May 2009. We stored the fruits in labeled paper bags at 4 °C for two weeks.

We selected undamaged fruits and transported them to the field sites. We manually removed the exocarp (pulp cover) and the skin covering the seeds. This is essential because the layers covering the seed can act as barriers to germination in mistletoes (Lamont 1983; Ladley & Kelly 1996). Furthermore, this enables the sticky viscin surrounding the seed to be exposed, which facilitates the temporary attachment of mistletoe seeds to host branches. We further worked the viscin by hand to increase its stickiness (for a similar method, see Sargent 1995; Ladley & Kelly 1996). We then allocated these viscin-covered seeds from the two source host species from either site to the two common host species, *A. karroo* and *A. caffra* in both sites (Fig. 1).

We used non-parasitised individual trees in our experiment to avoid any effects of previous infection and susceptibility. Trees ranged in height from 2-6 m and were all located in open areas to avoid shade effects (refer to our earlier chapter showing negative effects of shade). Global Positioning System (GPS) coordinates of each tree were recorded and the trees were marked. We monitored a total of 64 individual trees at Mtontwane and Highover (2 *Acacia* species × 2 sites × 8 replicates = 32 individual trees per site). For each host species in each site, we had two groups: one group received seeds of mistletoes obtained from *A. caffra* and the other group from *A. karroo*. Each group consisted of eight trees. For each experimental tree, we selected two healthy branches of similar size (8-12 cm circumference) and at the same position within the canopy. Each branch

received 10 seeds; five seeds from Highover and five seeds from Mtontwane. We applied a local and non-local seed paired design in order for each pair to experience identical environmental conditions including the current host species, bark surface and diameter (see Rödl & Ward 2002). The seeds were linearly orientated and placed 3 cm apart. In addition, seeds from different localities were marked with a distinctly coloured pin (Fig. 1).

We monitored the seed germination after one week, after one month and after six months in both sites. At each time period we recorded the condition of each seed as germinated (indicated by protrusion of the fresh green seed embryo), dead (where colour had changed to black and the seed had become dried and shriveled) or lost *in situ*. Where germination occurred we measured hypocotyl length from the base of the viscin layer to the distal end of the protruded embryo. We also recorded whether the hypocotyl growth was directed away from or towards the substrate. Hypocotyls that curved towards the substrate and attached to the host substrate were considered to have successfully established because the haustorium will form in this position.

The experimental design was done to manipulate the G x E interaction in response to site, source and current substrate while other abiotic factors were equivalently experienced by mistletoe seeds. The genotype responses to environmental changes are indicated by a statistical technique called reaction norms (a line that has a slope component to reflect performance of genotypes across environmental changes). If the genotype response to environmental changes of two or more reaction norms are non-parallel, it indicates genotype by environment (G x E) interaction. If this plasticity increases the fitness or performance of individuals it is adaptive for the particular local conditions. If the plasticity of two or more reaction norms is indicated by parallel reaction norms, it can be simply a passive consequence of resource limitations (Japhet, Zhou & Zhang 2009). In this experiment, we investigated the plasticity of the hypocotyl growth of the mistletoe seeds.

All data were analysed using GenStat version 11. Three sets of measurements were analysed: germination (expressed as the percentage of seeds that germinated), the hypocotyl length and growth form. χ^2 tests were used to investigate the variation in germination and growth form

between the source and non-source host species in both sites. We used ANOVA to analyse the mean hypocotyl length difference among sites, source, current substrate and their interactions.

Results

Germination of *A. natalitius* seeds started within a day in both sites and it was 100 % after one week, independent of host substrate and site. After one month, 7 % of the germinated seeds of *A. natalitius* were unsuccessful (either they died or were lost *in situ*) and there was no significant difference in germination success whether they were placed on source or non-source host species and whether they had been translocated to a different site or were germinated within their locality ($\chi^2_{1,7} = 4.77$, $p = 0.78$, $N = 64$).

After one month, hypocotyl length showed significant variance across all three predictor variables: site, source and current substrate (Table 1). There were significant interaction effects of source \times current substrate and site \times source \times current substrate. The three-way interaction was the most important because it reflects the genotype \times environment interactions. *A. natalitius* seeds placed on the same host species as the parent plant performed better within their locality in both sites. Mistletoe seeds obtained from *A. karroo* had greater success when placed on *A. karroo* than those placed on *A. caffra*, except at Highover (Fig. 2). Similarly, mistletoe seeds obtained from parent plants grown on *A. caffra* performed better on *A. caffra* than those placed on *A. karroo* in both sites.

We also analysed the hypocotyl growth form by considering the most effective attachment pattern after one month. The number of hypocotyls that curved towards and contacted the host substrate was higher when they were placed on the source host species ($\chi^2_{1,7} = 97.21$, $P < 0.01$, $N = 309$) (Fig. 3). Even when we excluded the source and site effects, by considering current host substrate only, hypocotyls attached better on *A. karroo* ($\chi^2 = 28.4$, $P < 0.01$, $N = 309$). This finding also indicates that mistletoes performed better on their preferred host species, *A. karroo*, indicating crossing or non-parallel reaction norms, consistent with a G \times E interaction.

Discussion

We studied the early developmental stages of *A. natalitius* infecting its two most common host species to examine how host compatibility and G x E interactions determine local adaptation in the hemiparasitic mistletoe, *Agelanthus natalitius*. All *A. natalitius* seeds germinated one week after inoculation, implying that germination of the mistletoe is independent of site and substrate. This is consistent with other studies that found that mistletoes are insensitive to substrate during the germination phase, provided that the pericarp is removed (Lamont 1983; Ladley & Kelly 1996; Rödl & Ward 2002; Roxburgh & Nicolson 2005). The hypocotyl length and growth form, however, showed significant differences due to G x E interactions. Mistletoes performed better on their source host species within their locality. Clay *et al.* (1985) also suggested that populations of mistletoes are genetically differentiated such that early seedling development is greatest when there is correspondence between maternal and seedling host species.

This study showed that mistletoes are able to perform better on the same host species that supported the parent plant, which implies that mistletoes and their hosts have a tight “daughter-mother” relationship. Similarly, other studies have demonstrated that the development of the haustorium is more successful when mistletoe seeds are placed on their source host species (Clay *et al.* 1985; Yan 1993b; Rödl & Ward 2002). Our study is the first to note that hypocotyl growth depends on a daughter-mother relationship of the mistletoes and host species.

In previous field studies we showed that the mistletoe *A. natalitius* preferentially grew on *A. karroo*, the most abundant host species at both sites. This was also supported by reciprocal transplant studies that demonstrated that *A. karroo* was the most compatible host species for the mistletoe, *A. natalitius*. This compatibility may be a result of a selective advantage of survival on the most frequently-occurring host species (see Norton & Carpenter 1998). In addition, many bird species disperse *A. natalitius* seeds more effectively by regurgitation of viable seeds on the same tree or a nearby tree (Okubamichael *at al.*, unpublished; see also Roxburgh 2007). This may reduce colonisation of new sites, but might improve chances of landing in a safe site, especially if

compatibility of mistletoes is positively related to host abundance. López de Buen & Ornelas (2002) also found that *Liquidambar styraciflua*, which is by far the most common host tree in the area in central Veracruz, Mexico, was the most compatible host for the mistletoe *Psittacanthus schiedeanus* (Loranthaceae) among all potential host species. Norton & de Lange (1999) also found a similar pattern in that host specificity was related to relative host abundance as a key factor determining the degree of host specialisation in the five extant New Zealand loranthaceous mistletoes (*Alepis flavida*, *Ileostylus micranthus*, *Peraxilla colensoi*, *Peraxilla tetrapetala* and *Tupeia antarctica*). In contrast, Roxburgh & Nicolson (2005) found no relationship between observed prevalence among host species and compatibility of the mistletoes, *Plicosepalus kalachariensis* in Zambia.

Many natural and synthetic chemicals have been identified that initiate haustorium formation in root parasitic plants (Matvienko, Tomilova & Nickrent 2001; Bouwmeester *et al.* 2003; Tomilov *et al.* 2004). We also suggest that the chemical interaction of the mistletoes and their host trees may happen during the post-germination development of the hemiparasitic mistletoes and this may be the basis for the G x E interactions we observed. This is because germination occurs independently of substrate and site but the post-germination processes of mistletoes (such as the hypocotyl length followed by haustorium formation) show differential development based on site, source and the substrate on which they were placed. In addition, the haustorium is a distinct and unifying structure of parasitic plants. Thus, investigating haustorium formation and adaptation may be a holistic approach to studying the infection patterns and host specificity of parasitic plants (Calvin & Wilson 2006; Vidal-Russell & Nickrent 2008). However, the mechanism by which mistletoes differentiate their source host species and their locality, and the genetic-chemical basis for mistletoes species to form host races remains unresolved.

Compatibility, selection, recognition, localisation of gene flow and spatial segregation of host species or populations all help to promote host race formation of mistletoe populations by promoting the gene pool of each putative host race (Glazner *et al.* 1988). Our findings clearly showed that differences in host utilisation of source and non-source host species have a genetic

basis, leading to differential success of individuals from one mistletoe population when grown on a different host species or in different areas. Unfortunately, measuring fitness differences in reciprocal transplant experiments in these long-lived perennial plants in an appropriate quantitative genetic study is unlikely to be possible. However, we have already performed pilot genetic studies using allozymes to examine the genetic composition of *A. natalitius* populations. Further research into the mechanisms involved in isolation and maintenance of the gene pool of mistletoe populations that infect different host species is warranted.

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Table 1. ANOVA of the reciprocal transplant experiment, showing the variation in hypocotyl growth. Most important are the interaction effects, which reflect genotype x environment interactions and showed a significant difference in the three way interactions of site, source and current substrate. Note that there was both a significant effect of source x current substrate and site x source x current substrate. Site = Highover or Mtontwane; Source = source (original) host species; Current substrate = host that the mistletoe was transferred to manually.

Source of variation	d.f.	F	P
Site	1	7.16	0.008
Source	1	39.33	<0.001
Current substrate	1	55.36	<0.001
Site x Source	1	29.03	<0.001
Site x Current substrate	1	0.89	0.345
Source x current substrate	1	7.95	0.005
Site x Source x Current substrate	1	22.61	<0.001
Error	1214		
Total	1221		

Figure legends

Fig. 1. Schematic diagram of the experimental design. Mistletoe fruits were collected from the two most common source host species, *A. karroo* (*mk*) and *A. caffra* (*mc*) from both sites, Highover and Mtontwane. The seeds obtained from the fruit were allocated and placed manually on the two host species, *A. karroo* (*k*) and *A. caffra* (*c*) at both sites. Each arrow points to the species consisting of eight individual trees; one group received from *mk* and the other group from *mc*. Each branch of the host species received 10 seeds, viz. two sets of five seeds each from each site and the same source host species (*A. karroo* or *A. caffra*). The main trait in focus, the hypocotyl of germinated mistletoe seeds, is indicated by the white arrow in the photograph.

Fig. 2. The hypocotyl length (mean \pm SE) of the germinated seed of the mistletoes of the reciprocal studies of all combinations of current substrate x source host species at both sites (Highover and Mtontwane). Abbreviations: current substrate; *k* = *A. karroo* and *c* = *A. caffra* and source host species; *mk* = mistletoe seeds obtained from mistletoes grew originally (source) on *A. karroo* and *mc* = mistletoe seeds obtained from mistletoes grew on *A. caffra*. When the source and the current substrate were the same host species, mistletoe seeds grew longer hypocotyls (see *k* x *mk* and *c* x *mc*, except in Highover). However, when mistletoe seeds were transferred to the non-source host species, they did better on *A. karroo* (*k* x *mc*) and fared worst on *A. caffra* (*c* x *mk*) in both sites.

Fig. 3. The number of hypocotyls of the germinated seeds that curved from the centre and attached to the host substrate of all combinations of current substrate x source host species in both sites (Highover and Mtontwane). Abbreviations as in Fig. 2. When the source- and the current-substrate were the same host species, they attached better on source host species-substrate, (see *k* x *mk* and *c* x *mc*, except in Highover). Similarly, mistletoe seeds obtained from the source *A. caffra*, when

placed on the current substrate *A. karroo*, also attached like those transferred to their source host species but mistletoes that were transferred to *A. caffra* fared worse.

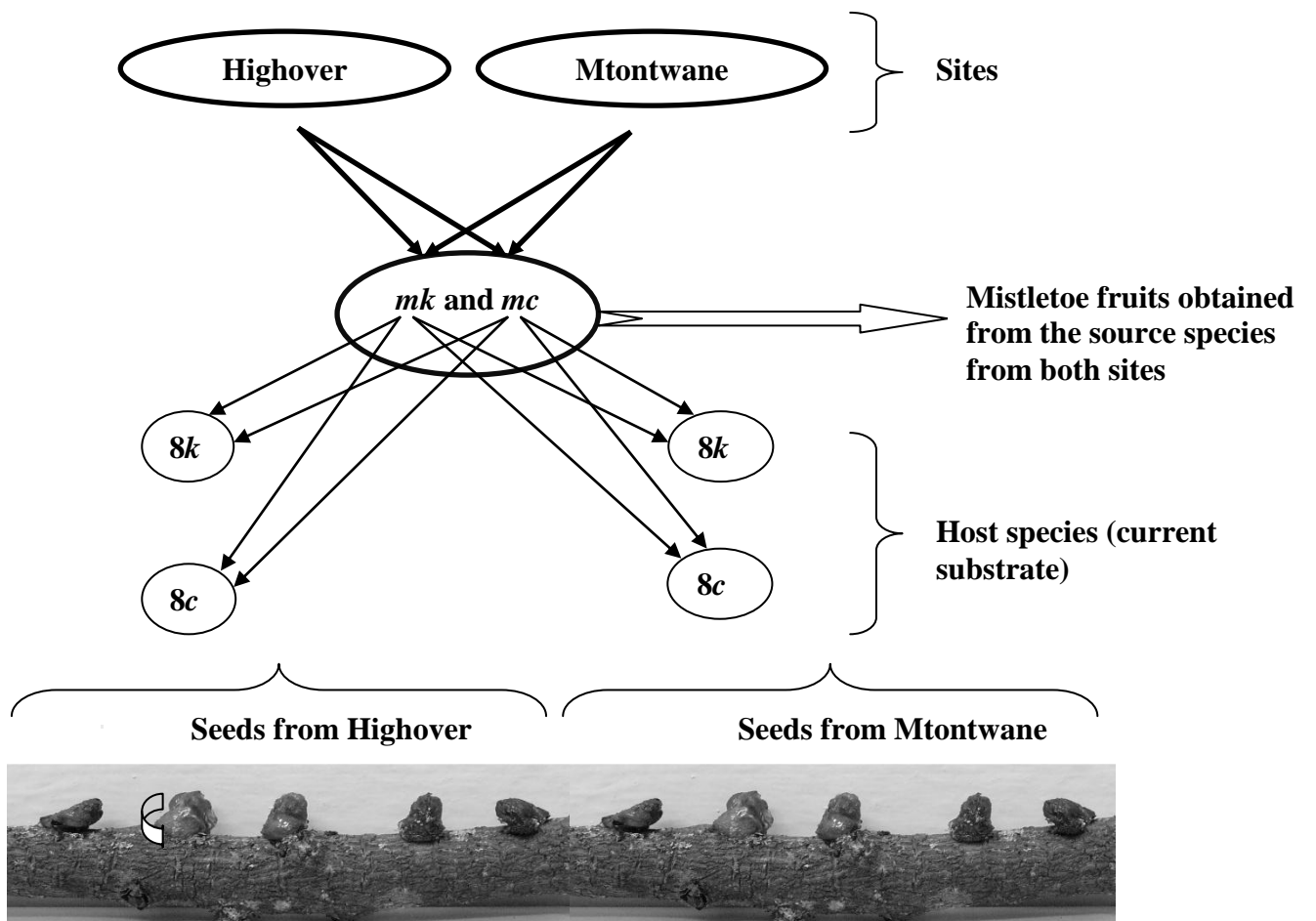


Fig. 1.

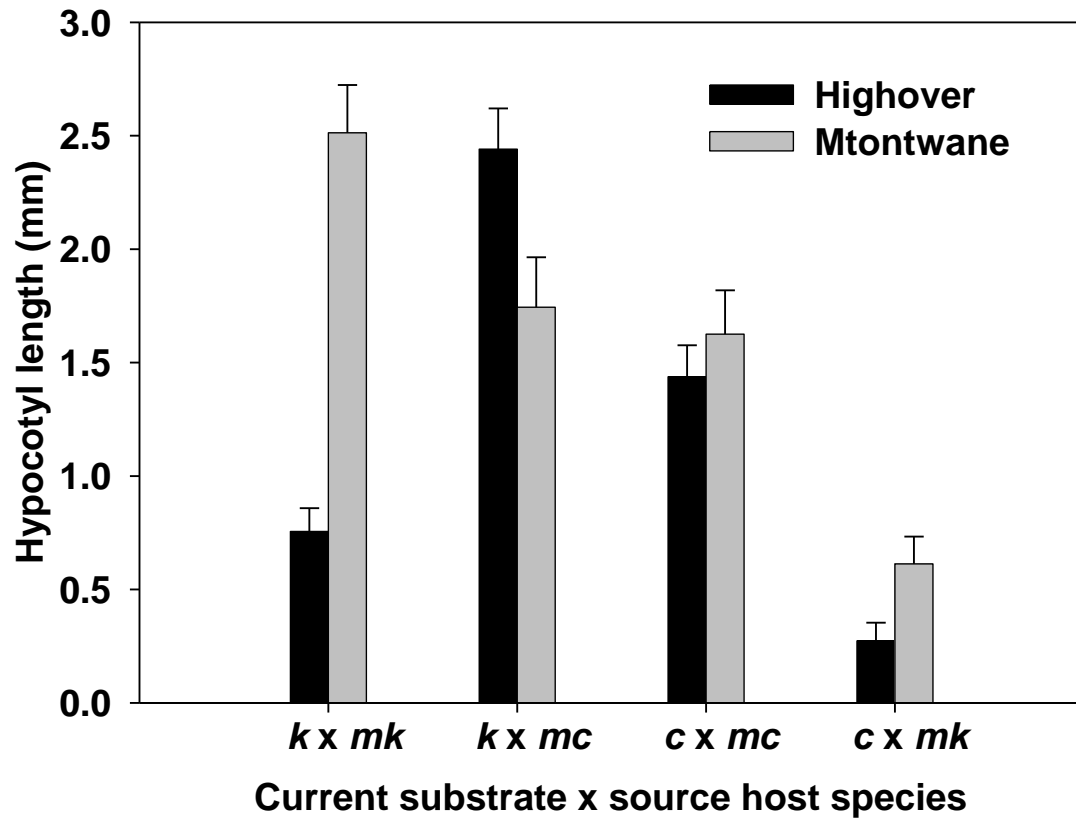


Fig. 2.

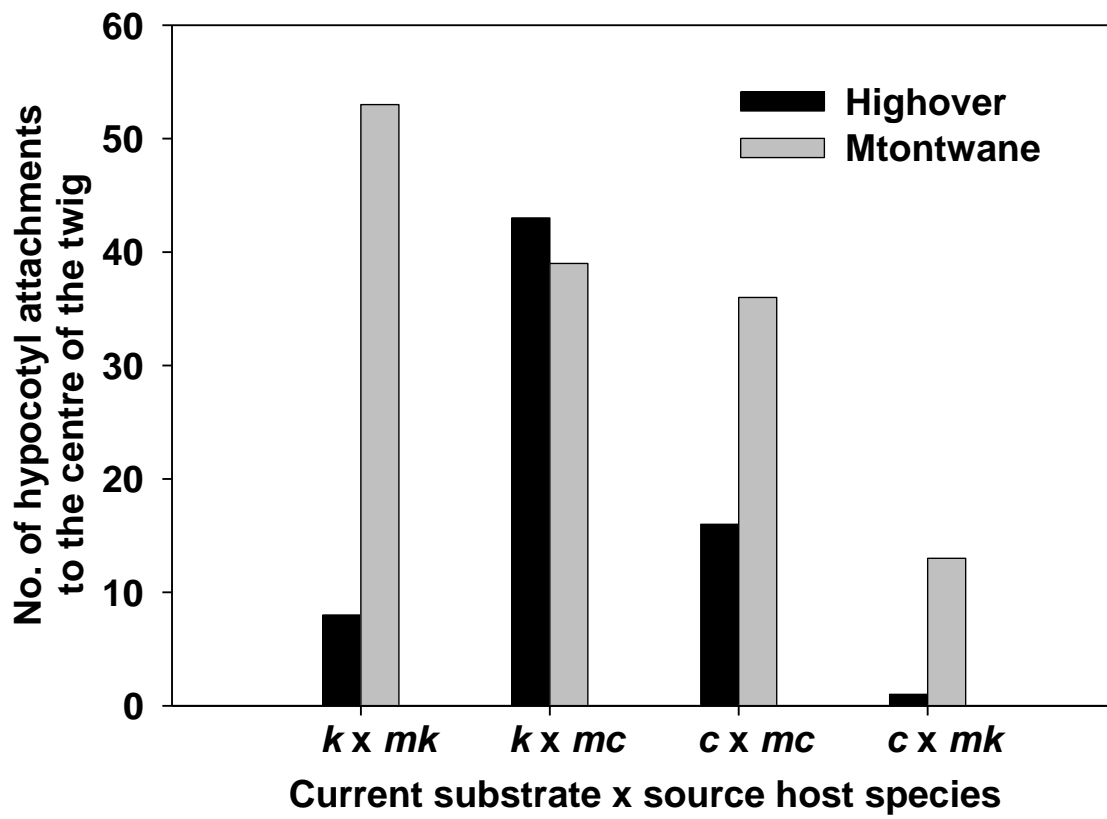


Fig. 3.

Chapter 4

Nutrient and water relationships of the hemiparasitic mistletoe, *Agelanthus natalitius* (Loranthaceae), and its host species, *Acacia karroo* and *A. caffra*

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Summary

- Mistletoe host specialisation may depend on the water and nutrient status of the host and on the mechanism used by the mistletoe for nutrient acquisition.
- We measured the nutrient and water status of two distinct populations of the hemiparasitic mistletoe, *Agelanthus natalitius*, growing on the host species *Acacia karroo* and *A. caffra* in South Africa.
- The mistletoes maintained lower water potential and accumulated higher nutrient levels than their host trees. The nitrogen:calcium ratio of mistletoes growing on *A. karroo* was < 1 , which indicates passive uptake via the xylem, while the ratio for mistletoes growing on *A. caffra* was > 1 , indicating active exploitation of the host phloem.
- Although the mistletoes preferentially grew on *A. karroo*, water status and nutrient content of *A. karroo* did not differ from *A. caffra*. Thus, nutrient and water content of the host species may not account for host specificity in this mistletoe.
- The mistletoe *A. natalitius* may use passive nutrient uptake on its most compatible host, *A. karroo*, a process which does not require energy. In the less preferred host, *A. caffra*, the mistletoe uses active nutrient uptake.

Key words: active uptake, host specificity, nitrogen:calcium ratio, passive uptake, phloem mobility, relative water content, water potential.

Introduction

Host specialisation by mistletoes may reflect differences in the water or nutrient status of their hosts. There is evidence that mistletoes are most successful on hosts that supply high levels of nitrogen (Dean *et al.*, 1994; Bowie & Ward, 2004). Mistletoe species richness on host genera is significantly positively correlated with mean host nitrogen. As a result, the *Acacia* genus hosts the most mistletoe species (24 species) in South Africa because acacias can fix nitrogen (Dean *et al.*, 1994). It has also been shown that host species that maintain high field water potentials, especially in wetter areas, are more likely to support mistletoes than those with low field water potential because mistletoes are profligate users of water (Bannister *et al.*, 1999; Miller *et al.*, 2003; Bowie & Ward, 2004).

Mistletoes are hemiparasitic and obtain nutrients passively from the xylem of the host tree, except for a few species that have been found to access nutrients actively from the phloem (Stewart & Press, 1990; Schulze *et al.*, 1991; Bowie & Ward, 2004, Wang *et al.*, 2008). Xylem-tapping hemiparasitic mistletoes often open their stomata and use a higher transpiration rate than their host trees (Scholander *et al.*, 1965; Schulze *et al.*, 1984; Strong & Bannister, 2002). This allows them to maintain a lower (more negative) water potential (Ψ) than the host trees, creating a gradient that enables the flow of xylem water and movement of nutrients passively from the host to the mistletoe by means of mass flow (Lamont, 1983; Schulze & Ehleringer, 1984; Bowie & Ward, 2004). As a result, the concentrations of mineral elements in the mistletoes are often much higher than in the host trees (Panvini & Eickmeier, 1993).

It is likely that passive uptake evolved principally as a means of water and nutrient acquisition, as xylem-tapping mistletoes exhibit high species diversity in arid and semi-arid regions of the world where water and nutrients are limited (Kuijt, 1969; Atsatt, 1973; Ehleringer *et al.*, 1985). Nitrogen is particularly important because it is the macronutrient that most limits growth in mistletoes (Dean *et al.*, 1994). Thus, passive uptake may have evolved to maximise nitrogen uptake from a very dilute host xylem solution (Ehleringer *et al.*, 1986; Press *et al.*, 1990; Dean *et al.*, 1994;

Reid *et al.*, 1995). One drawback is that mistletoes using passive uptake obtain a reduced nitrogen supply from the host xylem in nutrient-poor environments. Passive uptake can also cause considerable water stress in host trees, especially in arid environments (Bowie & Ward, 2004; Ward *et al.*, 2006; Cameron & Seel, 2007). Mistletoe transpiration rate and their nutrient content can be directly related to the nutrient status of the host (Schulze & Ehleringer, 1984; Schulze *et al.*, 1984; Ehleringer *et al.*, 1985; Wang *et al.*, 2008). For instance, when a mistletoe grows on nitrogen-fixing hosts where the supply of nitrogen is high, their water use efficiency may be greater and closer to that of their host relative to non-fixing hosts (Schulze & Ehleringer, 1984; Ehleringer *et al.*, 1985; Dean *et al.*, 1994).

Active nutrient uptake predominantly explains the contribution of the host phloem to the nutrient status of the mistletoes. In active uptake, mistletoes selectively take up specific solutes from the phloem of the hosts. Some mechanisms exist in the haustorium of the mistletoe to facilitate active nutrient accumulation, which implies that nutrient and water acquisition may not be tightly coupled (Panvini & Eickmeier, 1993; Bowie & Ward, 2004). The nitrogen:calcium (N:Ca) ratio of mistletoes has been used as an indirect method for estimating the effects of transpiration on the nutritional status of mistletoes (Lamont & Southall, 1982; Lamont, 1983; Panvini & Eickmeier, 1993; Bowie & Ward, 2004). Nitrogen is a highly phloem-mobile nutrient while calcium is phloem-immobile (Panvini & Eickmeier, 1993; Bowie & Ward, 2004). Thus, if host phloem is being actively exploited by the mistletoe, the N:Ca ratio should be > 1 in the mistletoes. Otherwise, if the ratio of N:Ca of the mistletoe is equal to or less than 1 it indicates passive uptake from the xylem of the host tree.

Mistletoes and acacias both play an important role as keystone species (Watson, 2001; Münzbergová & Ward, 2002). However, high mistletoe infection may ultimately harm the population of host acacias because mistletoes can reduce the reproductive output of their host trees (see Ward & Rohner, 1997; Wiegand *et al.*, 1999; Ward *et al.* 2006). It is important to understand the ecophysiology of mistletoes within the context of host specificity, i.e. the role that water and

nutrient status of host trees plays in determining host specificity of mistletoes. We investigated host specificity of the mistletoes by using a variety of physiological approaches. We determined the water and nutrient status of two populations of the mistletoe *A. natalitius* and its two most common host trees. We also determined whether the nutrient acquisition mechanism of mistletoes was by active or passive uptake.

Methods

Study populations

The study sites were located at Mtontwane (28° 80'S, 29° 93'E) and Highover (29° 54'S, 30° 05'E), about 110 km apart in KwaZulu-Natal, South Africa. Mean annual precipitation at Highover is 793 mm and at Mtontwane is 769 mm. Temperature varies from a minimum of 1 °C in winter to a maximum of 37 °C in summer in both sites. The vegetation of Mtontwane is characterised by *Acacia karroo*, *A. caffra*, *A. tortilis* and *A. nilotica* woodlands and thickets. The vegetation at Highover is riparian forest, characterised by *A. karroo*, *A. caffra*, and *A. ataxacantha* woodlands and thickets along the steep valleys of the Umkomaas River. The mistletoe *A. natalitius* occurs naturally in both sites and parasitises many tree species but the two most common host species in both sites were *A. karroo* and *A. caffra*. We have shown elsewhere (Okubamichael *et al.*, unpublished) that the mistletoe *A. natalitius* shows a clear preference for the host tree *A. karroo*, although it also parasitises *A. caffra*.

Nutrient status

We investigated the nutrient status of the mistletoe *A. natalitius* and its two most common host trees, *A. karroo* and *A. caffra*, in terms of total nitrogen and total calcium concentration. We specifically measured these two elements because nitrogen is highly phloem mobile while calcium is phloem immobile. This enabled us to determine whether the mistletoes access nutrients from the phloem or xylem of the host trees (see above). At each site and for each host species, we selected 15

different host trees infected with only one *A. natalitius* mistletoe and we collected a pair of leaf samples, one from the host and the other from the mistletoe parasitising the host tree (four pairs of 15 different host mistletoe-host pairs in both sites, $4 \times 15 \times 2 = 120$ leaf samples). All samples were transported to the University of KwaZulu-Natal (UKZN) and oven-dried for 48 h at 70 °C and then ground in a Wiley mill at 40 μm . Crude protein was analysed in a LECO FP2000 nitrogen analyser using the Dumas combustion method (AOAC 2000) and later converted to nitrogen concentration (mg gDWT^{-1}). Calcium was analysed by the acid digestion method and measured by an atomic absorption spectrophotometer (AOAC 2000).

Water potential

Plant water relations in the mistletoe-host pairs of the two populations were assessed directly in the field in September 2009. An instantaneous predawn and midday water potential (Ψ) of mistletoe-host leaf pairs was measured using a Scholander pressure chamber (Scholander *et al.* 1965; Tyree & Hammel, 1972). At each site, and for each host species, we selected 15 different host trees infected with one mistletoe and we measured (predawn and midday) a pair of leaves, one from the host and the other from the mistletoe parasitising the host tree. All selected trees were similar in size and all leaves used for the measurements had a similar orientation in the canopy so as to minimise differences in microclimate. To ensure minimal water loss, we immediately measured leaf Ψ . The leaf petiole in the chamber was examined under a simple 10X magnifying lens and readings were recorded with the first observation of water exuded on the surface of the petiole.

Predawn Ψ reflects the plant's water status at their daily maximal ability of water uptake due to lower transpiration caused by nocturnal stomatal closure and lower ambient temperatures (Scholander *et al.* 1965, Bowie & Ward, 2004; Ward *et al.*, 2006). If this predawn Ψ value is very negative, it implies that the plants have little available water to take up and are water-stressed

(Shrestha *et al.*, 2003). Midday Ψ is a measure of maximal water stress of the plants and is more negative due to maximum transpiration at midday (Scholander *et al.*, 1965).

Relative water content

At the same time as the Ψ measurements in the two sites, we investigated the relative water content (RWC) (Koide *et al.*, 1996) of the mistletoes and their hosts, calculated as:

$$\text{RWC} = ((\text{fresh mass} - \text{dry mass}) / (\text{saturated mass} - \text{dry mass})) * 100$$

Fresh mass was measured by removing five replicates of leaves from 15 host trees and their mistletoes at predawn and midday from each site. We weighed the leaf samples immediately in the field (fresh mass) using an electronic digital balance. We then immersed the leaves in distilled water for 12 h in double-sealed petri-dishes. We re-weighed the leaves after we removed excess water from the surface with a paper towel (saturated mass). The leaves were then oven dried in the laboratory at 70 °C for 24 h, after which they were weighed a final time (dry mass).

Statistical analysis

All data were analysed using Genstat version 11 and SPSS 15. Analyses of variance (ANOVA) with *post hoc* Scheffe tests for multiple comparisons were used to estimate the significance of differences between means of nutrient concentration, water potential and relative water contents of the host species and their mistletoes in the two sites. We also used one-way t-tests to test the significance of differences of nutrient N:Ca ratio of the mistletoes from 1. Simple linear regression analyses were used to examine the relationships between predawn and midday Ψ values for mistletoe-host pairs.

Results

Nutrient status

The mean nitrogen (N) concentration did not differ significantly between the two host species at both sites ($F = 1.58$, $P = 0.211$). However, the N concentration and N:Ca of host species and their mistletoes were significantly different from each other in both host species-mistletoe combinations at both sites (range in $F = 25.14 - 58.56$, $P < 0.001$). There was also a significant site \times species interaction of N concentration and N:Ca ratio (N, $F = 6.76$, $P < 0.001$; N:Ca, $F = 30.07$, $P < 0.001$). The *post hoc* Scheffe test revealed that N of the host species, *A. karroo* and *A. caffra*, was not significantly different from each other in either site (Fig. 1a,b). However, mistletoes had significantly higher N content than their host species in both sites (Fig. 1a,b). Thus, the mistletoe:host ratio (N concentration of the mistletoes relative to N concentration of their host trees) was always greater than 1. Moreover, the N concentration of mistletoes parasitising *A. caffra* was higher than that of *A. karroo* in both sites (Fig. 1a,b). The N concentration of mistletoes growing on *A. karroo* was positively correlated with that of their host trees at Highover but not at Mtontwane ($r = 0.54$, $F = 5.45$, $P = 0.036$, error d.f. = 14). However, the N concentration of mistletoes was not significantly correlated with the host *A. caffra* in either site ($P > 0.05$, error d.f. = 14).

The N:Ca ratio of mistletoes growing on *A. caffra* was significantly greater than 1, while mistletoes growing on *A. karroo* had a ratio of N:Ca < 1 in both sites (Highover and Mtontwane with $P = 0.026$ and 0.006 , respectively; error d.f. = 14 for both sites; one-way t-tests) (Table 1). This result indicates that mistletoes that grow on *A. caffra* can access nutrients actively while mistletoes that grow on *A. karroo* predominantly access nutrients passively.

Water potential

Water potential (Ψ) of the host species and their mistletoes in both sites was significantly different among the following factors; time, species, site \times time and the site \times time \times species interactions (Table 2). The mean midday Ψ measurements of the host species and their mistletoes were

significantly lower (more negative) than at predawn for all measurements in both sites. *A. karroo* and *A. caffra* Ψ showed no statistically significant differences from one another except for Highover at midday, where *A. caffra* was more water-stressed than *A. karroo* (Fig. 2). Mistletoes on *A. karroo* had significantly lower Ψ (more negative) than their host trees in three out of four cases except in one case that the predawn *A. karroo* and their mistletoes Ψ did not differ significantly at Mtontwane. At midday, mistletoes on *A. caffra* had significantly more negative Ψ while at predawn the mistletoe on *A. caffra* Ψ were not significantly different from their host trees at both sites (Fig. 2). Additionally, Ψ of the mistletoes and the host species positively correlated at predawn in both sites (Fig. 3), demonstrating that as the host trees become water stressed the mistletoes also experience water stress. There was no significant correlation of Ψ of the mistletoes with either of their host trees at midday in both sites (Table 3).

Relative water content

Relative water content (RWC) of the host species and their mistletoes was significantly different between sites (Highover and Mtontwane) and times (predawn and midday). Both mistletoes and host species in Highover had higher RWC content than at Mtontwane ($F = 13.24$, $p < 0.001$, error d.f. = 239). There was a significantly higher RWC in both mistletoes and hosts at predawn than midday in both sites ($F = 22.61$, $p \leq 0.001$, error d.f. = 239). There was no significant difference in mean RWC for all other combinations in the analysis ($P > 0.05$) (Table 4). There was no significant correlation of RWC of the mistletoes with the host species for predawn and midday at both sites ($P > 0.05$). Similarly, there was no correlation between RWC and Ψ of the mistletoes and the host species at both times and in both sites ($P > 0.05$).

Discussion

The relationships of nutrient and water status and the access of nutrients via passive or active uptake between the two mistletoe-host pairs were examined in the context of host specificity in the hemiparasitic mistletoe, *A. natalitius*. We found that the two most common host species, *A. karroo* and *A. caffra*, were not significantly different from one another in terms of water potential (Ψ) and nutrient status. However, previous studies have demonstrated that the mistletoes are more abundant on *A. karroo* than would be expected by chance (Okubamichael *et al.*, unpublished), demonstrating that there is some degree of host specificity in the mistletoe *A. natalitius*. That the hosts are not implicitly different in terms of water and nutrient status demonstrates that any preference for host species is not based on the availability of nutrients and water in the host. This is contrary to previous findings that the infection intensity of mistletoes is positively correlated with high water status in host trees (Miller *et al.*, 2003). Thus, we suggest that other factors such as chemical compatibility may better explain infection patterns in the context of host specificity.

We note that the relationship between mistletoes and their hosts in terms of nutrient and water status has not been fully and consistently explained by other studies (Glatzel, 1983; Lamont, 1983; Mathiasen *et al.*, 2008; Glatzel & Geil, 2009). In this study, we found that the nutrient content of the mistletoes was higher than their host trees, a result consistent with many similar studies (Lamont, 1983; Panvini & Eickmeier, 1993). There is contrasting evidence by Bowie & Ward (2004), in which N content of the mistletoe was lower than the host tree (*Acacia raddiana*) in the Negev Desert.

The measurements of Ψ showed that mistletoes had more negative Ψ than their host trees in most cases, especially mistletoes growing on *A. karroo*. However, there was no consistent significant difference between Ψ in mistletoes on *A. caffra* and their host trees. The Ψ results of mistletoes on *A. karroo* were consistent with other studies that found that mistletoe Ψ was significantly more negative than the host trees, which is associated with the higher transpiration rate of mistletoes compared with their host trees (Glatzel, 1983; Schulze & Ehleringer, 1984; Schulze *et*

al., 1984). Ehleringer *et al.* (1985) found that the water use efficiency of mistletoes correlated positively with N supply in the nitrogen-fixing host species. It has been demonstrated experimentally that *A. karroo* can fix N (Cramer *et al.*, 2007), and it is equally plausible that *A. caffra* also fixes N.

Predawn Ψ of the mistletoes and the host species were positively correlated, which is consistent with other studies (Schulze *et al.*, 1984; Strong & Bannister, 2002; Miller *et al.*, 2003). Mistletoes also experience water stress when their host plants are water-stressed (Bowie & Ward, 2004). At midday when the host trees experience maximum stress, the mistletoes' Ψ was not correlated with that of their host trees, indicating that mistletoes tend to control their Ψ . This is consistent with other studies in that mistletoes close their stomata to reduce water loss during times of host water stress, maintaining lower Ψ (Glatzel, 1983; Schulze *et al.*, 1984; Ullman *et al.*, 1985; Whittington & Sinclair, 1988).

There is also evidence that nutrient acquisition may not be directly coupled with high transpiration in mistletoes (Bowie & Ward, 2004). Experiments under shaded conditions showed that mistletoes maintain more negative Ψ without high transpiration rates so water loss via the stomata is not the only mechanism that maintains lower Ψ (Ackroyd & Graves 1997). In wetter areas where leaf water vapour deficit is likely to be small, mistletoes depend on lower osmotic potential and haustorial hydraulic resistance to maintain lower Ψ (Ackroyd & Graves, 1997; Küppers *et al.*, 1993; Cameron & Seel, 2007). However, this mechanism is not likely to occur in our study area because it is open savanna with high daily temperatures.

In our study, we found that the relative water content of host and mistletoe leaves were higher at predawn than midday. This was expected because trees contain more water during predawn than midday due to stomatal closure and reduced nocturnal temperatures. The host trees and their mistletoes at Highover had higher water content than those at Mtontwane. This can be explained by the fact that Highover lies along the Umkomaas River and host trees probably had

greater access to water than those at Mtontwane. Mistletoes and their host trees did not differ significantly in relative water content in both sites and at both times (predawn and midday). In addition, there were no correlations between water potential measurements and relative water content as recorded in other studies (Bowie & Ward 2004), which deserves further investigation.

In our study, the nutrient contents of the mistletoes that parasitise different host species differs based on the mechanism of nutrient uptake. The N:Ca ratio in mistletoes on *A. karroo* showed that they predominantly use passive uptake while in *A. caffra* they also use active uptake to access nutrients from the phloem. We suggest that the N content of mistletoes in *A. caffra* was higher than in *A. karroo* because mistletoes on *A. caffra* use both active and passive uptake to accumulate more N. Mistletoes on *A. karroo* always maintain significantly lower Ψ than their host trees to promote the passive access of nutrients from the host. However, mistletoes on *A. caffra* showed inconsistent relationships with their hosts' Ψ because they have an active uptake mechanism. Thus, they may not necessarily maintain a lower Ψ than their host trees.

Nutrient acquisition differences between mistletoes growing on related host species may be a reflection of their host compatibility. If mistletoes grow on the more compatible host species, *A. karroo*, they may predominantly use passive uptake which might be easier to maintain and they may control their water use efficiency depending on the supply of nitrogen from their host trees. However, if they grow on the less preferred host, *A. caffra*, they survive with the help of active uptake, which may not be easy to maintain because active uptake requires energy (Lambers *et al.*, 1998). This paradox of different nutrient acquisition mechanisms between ostensibly the same mistletoe populations that parasitise different host species requires further investigation. For example, anatomical studies at the haustorial junction of mistletoes grown on different host species could help to reveal in a more direct way the mechanisms of nutrient uptake (see Calvin & Wilson, 1995).

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Table 1 Mean \pm SE ratio of N of mistletoes/N of host and N:Ca of mistletoes for both sites. The mistletoes accumulated a higher nutrient content than their host trees (N of mistletoe/N of host ratio was always >1). In both sites, mistletoes on *A. caffra* had a N:Ca ratio that was significantly > 1 but not mistletoes on *A. karroo* (see **Results**). Abbreviations; k = *A. karroo*, c = *A. caffra*, mk and mc = mistletoes on k and c respectively

Site	Mistletoes/host species	N of mistletoes/ N of host	Mistletoes	N:Ca mistletoes
Highover	mk/k	1.449 \pm 0.09	mk	0.904 \pm 0.06
	mc/c	1.724 \pm 0.12	mc	1.293 \pm 0.139*
Mtontwane	mk/k	1.268 \pm 0.07	mk	0.885 \pm 0.07
	mc/c	1.164 \pm 0.05	mc	1.294 \pm 0.10*

*indicates the ratio significantly > 1 , p at 0.05 level

Table 2 Analysis of variance of water potential (Ψ) of the two mistletoe-host pair combinations at both times and in both sites. Time, species, site x time and the three way interaction of site x time x species were significantly different. Significant values of p indicated by *

Source of variation	F	p	d.f.
Site	1.48	0.226	1
Time	1370.17	<.001*	1
Species	22.25	<.001*	3
Site x time	4.52	0.035*	1
Site x species	0.67	0.570	3
Time x species	0.85	0.468	3
Site x time x species	3.02	0.031*	3
Error			224
Total			239

Table 3 Linear regression of water potential (Ψ) of the mistletoe-host pairs in both sites. In most cases, Ψ of the host species and their pairs of mistletoes were positively correlated only at predawn but not at midday. Only significant r values are indicated and significant p values are indicated by *. Abbreviations as in Table 1. Error d.f. = 14 in all cases

Site	Time	Species	r	F	p
Highover	Predawn	k x mk	0.76	17.82	<.001*
		c x mc	0.52	4.70	0.049*
	Midday	k x mk		0.68	0.424
		c x mc	0.86	36.30	<.001*
Mtontwane	Predawn	k x mk	0.72	14.28	0.002*
		c x mc	0.91	58.94	<.001*
	Midday	k x mk		0.007	0.954
		c x mc		0.004	0.933

Table 4 The mean \pm SE relative water content (% RWC) of the two mistletoe-host pairs at both times and sites. The predawn and midday (time) and site (Highover and Mtontwane) were significantly different in all cases. For both host species and mistletoes, Highover plants had greater RWC than Mtontwane. Predawn RWC was higher than midday RWC at both sites. Abbreviations as in Table 1

Time	Species	Site	
		Highover	Mtontwane
Predawn	k	88.84 \pm 0.83	81.04 \pm 3.46
	mk	89.60 \pm 1.11	85.41 \pm 1.61
	c	87.20 \pm 1.17	81.64 \pm 5.95
	mc	92.83 \pm 0.77	91.92 \pm 1.32
Midday	k	86.14 \pm 2.66	77.55 \pm 2.46
	mk	81.07 \pm 5.35	73.33 \pm 7.46
	c	82.73 \pm 0.85	76.41 \pm 1.98
	mc	82.21 \pm 4.40	72.03 \pm 4.64

Figure legends

Fig. 1 The mean \pm SE of N of the mistletoe-host pairs. In most cases, the host species were not significantly different from each other but the mistletoes had significantly higher N content in both sites, except for Mtontwane. N of *A. caffra* was not significant different from the mistletoe values at Highover (closed circles) and at Mtontwane (open circles). The mistletoes on *A. caffra* accumulated more N in both sites. Abbreviations; k = *A. karroo*, c = *A. caffra*, mk and mc = mistletoes on k and c respectively.

Fig. 2 Box plots (median, upper and lower quartiles and maximum and minimum values) of water potential (Ψ) of mistletoe-host pairs. The host species (white box) were not significantly different from each other in most cases, but the mistletoes (grey box) had significantly more negative (lower) Ψ at predawn (a and b) in both Highover (a and c) and Mtontwane sites (b and d), but not at midday (c and d). Abbreviations as in Fig. 1.

Fig. 3 Water potential (Ψ) of the mistletoe and host pairs correlated positively at predawn in both Highover (a) and Mtontwane sites (b), but not at midday. Abbreviations, open triangles = mistletoes on *A. karroo* with their host tree pairs, and closed circles = mistletoes on *A. caffra* and their host tree pairs.

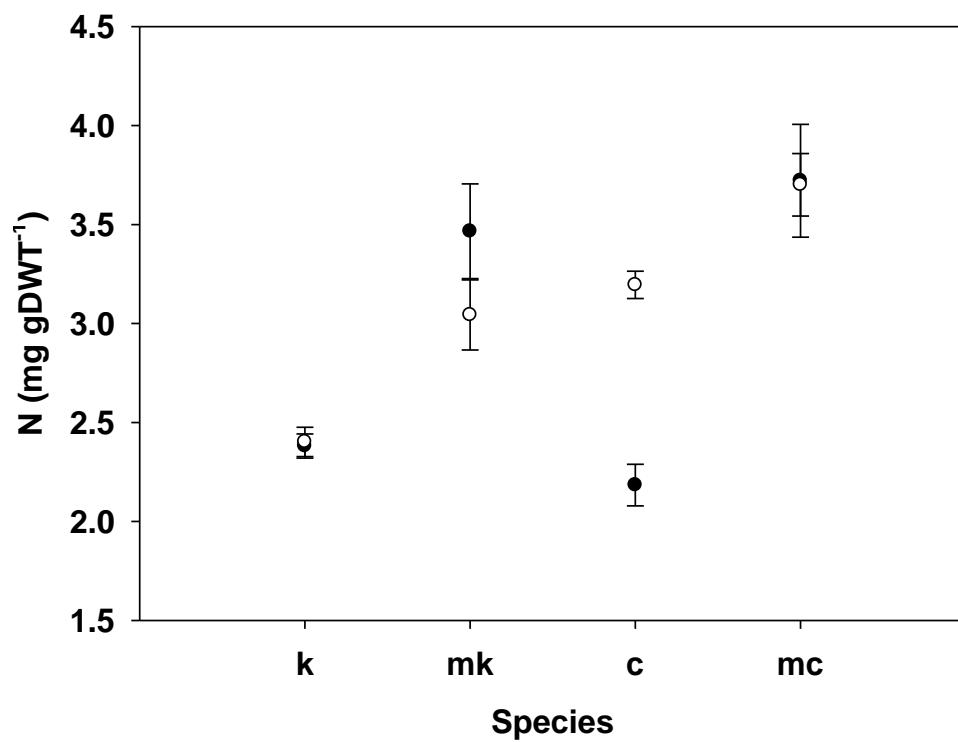


Fig. 1

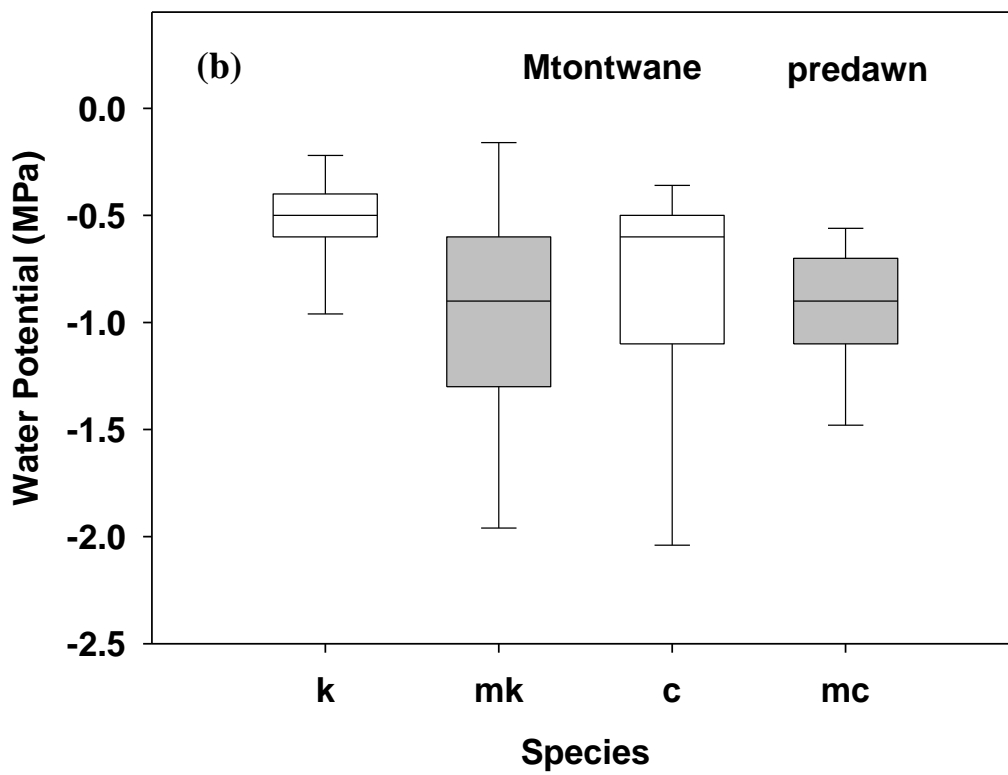
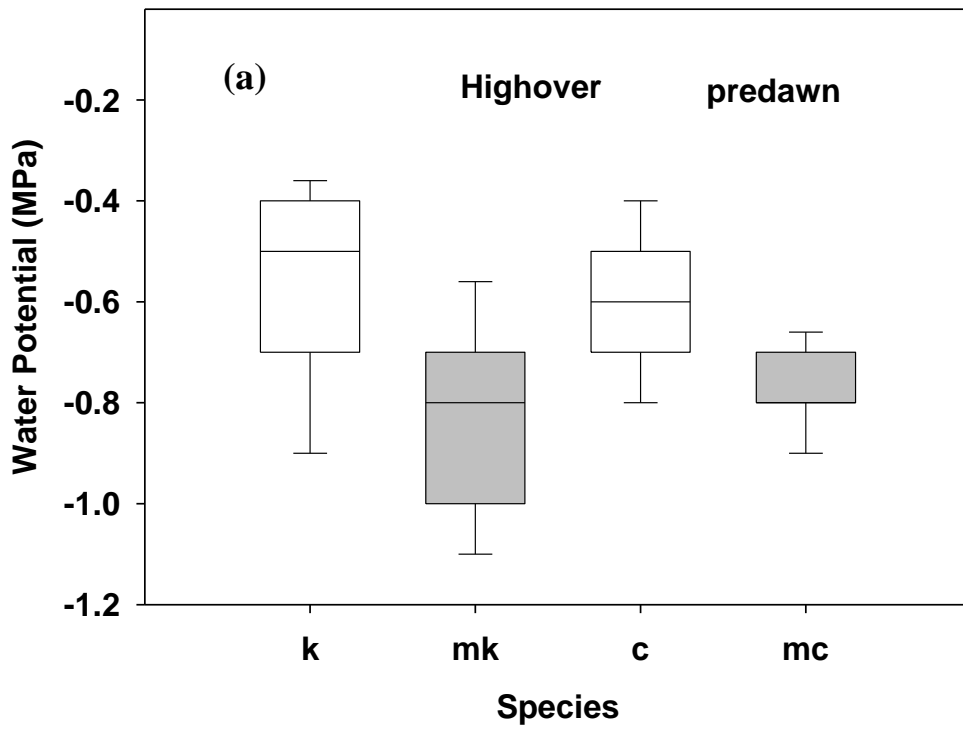


Fig. 2

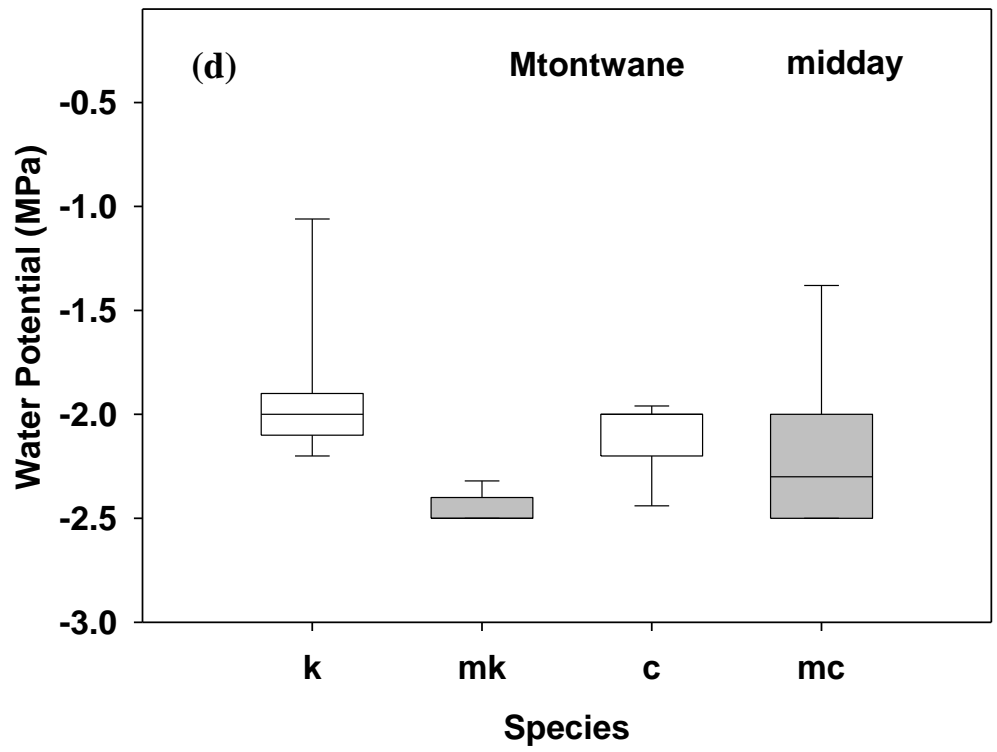
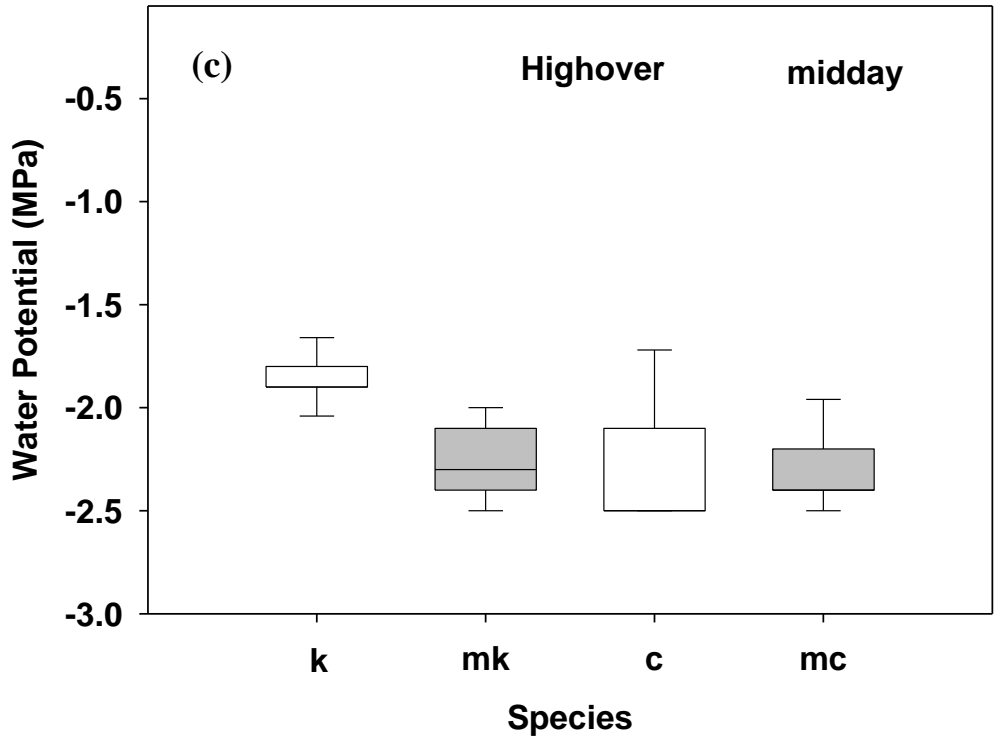


Fig. 2

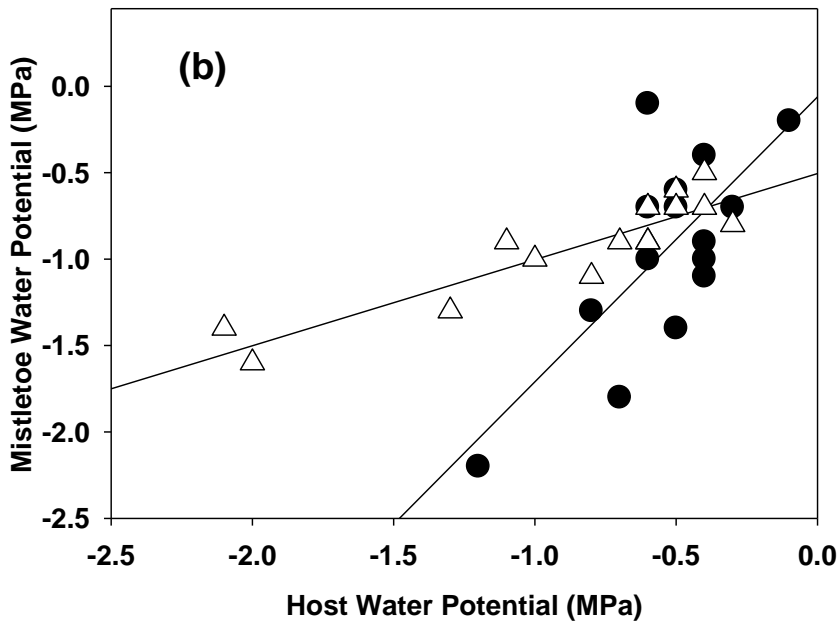
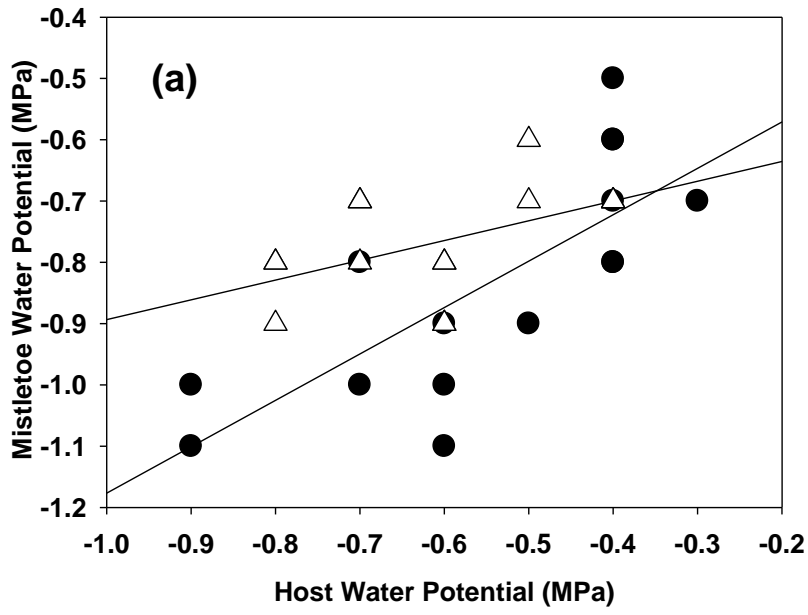


Fig. 3

Chapter 5

Host specificity and bird dispersal in the hemiparasitic mistletoe, *Agelanthus natalitius* (Loranthaceae)

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Abstract We studied dispersal of seeds of the mistletoe, *Agelanthus natalitius*, and evaluated the role of avian dispersal in directing host specificity in this species. We carried out field observations and studied seed retention time in captive birds. We evaluated the germination success of seeds that had been processed by different bird species and estimated dispersal distance of the mistletoe based on bird behaviours and fruit processing. Seven avian species were frequently observed to feed on mistletoe fruits in the field, namely Cape Batis (*Batis capensis*), Cape White-eye (*Zosterops virens*), Red-fronted Tinkerbird (*Pogoniulus pusillus*), Red-winged Starling (*Onychognathus morio*), Dark-capped Bulbul (*Pycnonotus tricolor*), Speckled Mousebird (*Colius striatus*) and Village Weaver (*Ploceus cucullatus*). Of the four bird species used in captive feeding trials, most of the birds removed the pulp-cover of the mistletoe fruits and left the exposed seeds in potentially germinable condition. Red-winged Starlings ingested but then regurgitated the seeds and wiped their bills on a twig to dislodge the sticky viscin-covered seeds individually. Mistletoe seeds obtained from cages of Red-winged Starlings had the highest germination success of all the seeds obtained from the captive bird study. The handling of mistletoe fruits applied by all species of birds both in field and captivity showed that mistletoe dispersal is likely to occur over short distances, although the seeds are likely to be dispersed to safe sites due to direct dispersal on parental host trees. Such autoinfection and local aggregation of the mistletoe may enhance local specialisation.

Keywords dispersal distance, fruit handling, germination, gut retention time, local specialisation

Introduction

Many mistletoes are hemiparasites that depend on host trees for nutrients and water (Kuijt 1969). Mistletoes are capable of infecting a variety of vascular plants and exhibit various degrees of host specificity (Norton and Carpenter 1998; Rödl and Ward 2002). Most mistletoe species produce fleshy fruits and many frugivorous birds are highly specialised for consumption of these fruits and, in turn, disperse them (Restrepo et al. 2002; Aukema 2003). Many studies have found that birds are responsible for the non-random distribution of mistletoes among the available host species (Godschalk 1985; Aukema and Martínez del Rio 2002a,b,c).

Many frugivorous birds and mistletoes are mutualists that are likely to have coevolved (Reid 1987; Reid et al. 1995; Aukema and Martínez del Rio 2002a,b,c). The fruits are often large, high in sugar concentration and brightly coloured (white, yellow, red or purple) to attract birds (Polhill and Wiens 1998). In addition, the mistletoe fruit phenology effectively manipulates bird dispersers by often being available in the winter when few other food sources are available in the ecosystem (Ladley and Kelly 1996; Polhill and Wiens 1998; Watson 2001). Moreover, mistletoe plants have prolonged discontinuous ripening within an individual or asynchrony in peak fruiting time among individuals within species and among species within communities (Davidar 1983; Hawksworth and Wiens 1996; Polhill and Wiens 1998; Watson 2001). The sticky viscin that coats mistletoe seeds also influences the behaviour of birds, as it often creates difficulties for birds to expel seeds that are attached to the bill, abdomen or other parts of the body of a bird (Reid 1991; Aukema 2003). In addition, the viscin contains a mucilaginous pectic material known for its water-holding capacity and ability to withstand repeated drying and rehydration events, which accounts for the ability of seeds to adhere to host twigs before a permanent attachment forms (Paquet et al. 1986).

Birds preferentially perch on and disperse mistletoe seeds to tall host trees and trees that are already parasitised by mistletoes (Reid and Stafford Smith 2000; Aukema and Martínez del Rio 2002b,c; Roxburgh and Nicolson 2005). Birds are also responsible for local aggregation because

they spend most of the time perching in areas that already have an abundant source of mistletoes (Aukema 2004; Ward and Paton 2007; Green et al. 2009). Local aggregation is strongly facilitated by gut passage rate, which in turn depends on the bird's size, physiology, morphology, behaviour and specialisation (Traveset et al. 2001; Levey and Martínez del Río 2001). For example, Mistletoebirds (*Dicaeum hirundinacum*) have a specialised gut with a relatively short alimentary tract, allowing rapid passage of a large number of mistletoe fruits (Richardson and Wooller 1988; Reid 1991). Mistletoe seeds are large and constrain flight by increasing the weight of a bird that has eaten mistletoe fruits. Fast release of mistletoe seeds increases the gut capacity of birds to take and process more fruits at a time (Roxburgh 2007). Such fast processing of mistletoe fruits enhances the dispersal of the seed in a very short time, which implies that dispersal is over short distances, often resulting in direct dispersal on the same host as the parent plant or on neighbouring trees (Roxburgh 2007).

Few studies have investigated the movement behaviour of bird dispersers in the field and related this movement to gut passage rate of mistletoe seeds in order to predict the potential dispersal distance (Ward and Paton 2007, Green et al. 2009). One study examined the dispersal of seeds from the mistletoe *Amyema miquelii* around parent plants (seed shadow) and within a population (seed rain) and they found that there is direct dispersal of the mistletoe to already parasitised host trees (Ward and Paton 2007). In addition, seed rain was positively correlated to areas that have abundant mistletoes (70% of mistletoe seeds were deposited within 100 m of their parent plant) (Ward and Paton 2007). Another study found that Yellow-vented Bulbuls, the primary disperser of the mistletoe *Plicosepalus acaciae*, spent a large portion (up to 93%) of their total time in the *Acacia* trees that serve as hosts to the mistletoe, allowing for direct dispersal of the seeds to appropriate host plants (Green et al. 2009). However, the influence of mistletoe dispersers on mistletoe distribution at scales larger than the individual or local scale is not well understood.

The aim of the study was to investigate avian dispersal in the mistletoe, *A. natalitius*, and evaluate the role that avian dispersal plays in directing local host specificity in this mistletoe. We

investigated avian dispersal in two distinct populations in the two field sites in KwaZulu-Natal, South Africa. We then conducted captivity studies to evaluate the effect of fruit processing by birds on germination success of mistletoe seeds and recorded seed gut retention time to estimate the dispersal distance.

Materials and methods

Study sites and species

The study was conducted in two populations of mistletoes in KwaZulu-Natal, South Africa: Highover (29° 54'S, 30° 05'E) and Mtontwane (28° 80'S, 29° 93'E), about 110 km apart. The predominant vegetation at both sites is *Acacia*-dominated open savanna. The mistletoe *A. natalitius* parasitises at least eleven tree genera in South Africa (Visser 1981). At Highover, the two most common host species were *Acacia karroo* and *A. caffra*, while at Mtontwane, mistletoes parasitised two additional host species, namely *A. nilotica* and *A. tortilis*.

A. natalitius is a deciduous perennial that begins flowering in September and peaks in November (Wiens and Tölken 1979; Visser 1981). Fruiting starts in February and takes about 28 weeks for complete development, making fruits available into the winter months (Visser 1981; Godschalk 1983). *A. natalitius* produces fleshy fruits that turn from green to dark red when fully ripe. They contain a large single seed and embryo that are surrounded by a viscin layer that facilitates adhesion to the bark of host trees after the fruit has been removed.

We measured the mean weight and maximum axis of 100 randomly-selected fresh *A. natalitius* fruits (mean \pm SE = 0.926 \pm 0.013 g and 0.480 \pm 0.002 mm, respectively). We also analysed the sugar composition and content of the mistletoe fruits of the exocarp (outermost layer of the fruit) by high performance liquid chromatography (HPLC) in the Department of Horticulture

at the University of KwaZulu-Natal (UKZN). The fruits contain glucose, sucrose and fructose at mean levels of 9.16, 7.93 and 4.25 mg/g, respectively.

Field observations

Frugivorous birds were monitored for mistletoe consumption during the fruiting season in May 2008. We selected and tagged 33 mistletoe-host pairs in which the mistletoes had ripe fruits. The focal plants were observed for a minimum of 7 h between 07h00 until 17h00 at both sites (a total of over 200 h). Every visit to the focal plants by birds was observed with 10 × 42 binoculars. The birds were followed until they were lost from sight. We recorded visit duration, feeding behaviour, ingestion type (whether the entire fruit or a portion of the fruit had been consumed), regurgitation, wiping of seeds and defaecation of seeds on host branches.

Seed retention time and germination

Retention time of mistletoe seeds was studied in four selected species of frugivorous birds during May-June 2009 in the University of KwaZulu-Natal aviary. All bird species used for the captivity study were observed in the field to ingest fruits of *A. natalitius*. These were the Red-winged Starling (*Onychognathus morio*), Speckled Mousebird (*Colius striatus*), Village Weaver (*Ploceus cucullatus*), and Cape White-eye (*Zosterops virens*). We used five individuals from each species to estimate dispersal distance based on retention time and to evaluate germination success following the processing of fruits. All the birds except the Village Weavers were already in the aviary. Village Weavers were caught one week before sampling and were kept in the aviary. During the process of acclimatisation to captivity, all birds were housed in outdoor aviaries and given a maintenance diet of mixed fresh commercial fruit.

To assess the retention time of seeds within the gut, birds were transferred to an indoor aviary with controlled conditions [12L/12D photoperiod; 25 °C] and housed individually in 45 × 60 × 90 cm cages. For one week prior to the start of the experiment, birds were provided the maintenance diet mixed with mistletoe fruits to allow them to acclimatise to the new food source. Within this period, *A. natalitius* fruits growing on *Acacia karroo* and *A. caffra* in both field sites were bagged using nylon mesh bags (200 mm by 450 mm) prior to hand picking just before the start of the experiment. Fully ripe (dark red) fruits were collected from different trees and stored in paper bags at 4 °C until used within a week for the experiment. On each of the experimental days, 10 ripe mistletoe fruits were strung on a wire and presented to five individual birds housed separately. This was done because mistletoe fruits in a petri dish roll around and this created difficulty for birds in handling. In total, 1000 fruits were presented for the whole experiment (4 species × 5 individuals × 5 days × 10 fruits = 1000).

We videotaped the consumption of mistletoe fruits and recorded retention time (time elapsed from the first intake to the first regurgitation or defaecation) of the caged birds while they were allowed to feed freely for 3 h. The behaviours of the birds and the number of mistletoe seeds defaecated were also noted. If birds ingested and regurgitated more than one fruit or seed at a time, we assumed that the order of ingestion of mistletoe fruits was the same as for the regurgitated or defaecated seeds (see Green et al. 2009). After 3 h, we recovered the mistletoe fruits and seeds from the cages. We then provided the birds with a maintenance diet again. Throughout the experiments, all birds were healthy.

We recorded the condition of fruits as: (1) intact, where the fruits were completely untouched by the birds; (2) partially removed, where only a portion of the exocarp had been removed; (3) or skin-covered seed, in which the exocarp was completely removed but the viscin layer was not exposed and remained covered by the outer skin layer of the seed. We also noted whether fruits were ingested and later either regurgitated or defaecated.

Following the seed retention trials we immediately transferred all mistletoe seeds from the cages to petri dishes for germination. In our previous studies we showed that mistletoes are site-insensitive for germination, thus petri dishes probably did not have any effect on germination (for similar experiments see Yan 1993; Green et al. 2009). As a control we used intact fruits exposed to similar conditions in the laboratory. We finally quantified the germination success after one month. All data were analysed for their frequency differences using chi-square tests in SPSS 15.0.

Results

Field observations of avian dispersers

Twenty-four avian species visited the host-mistletoe pairs, but only seven of these species were observed consuming mistletoe fruits. These were Cape Batis (*Batis capensis*), Cape White-eye (*Zosterops virens*), Red-fronted Tinkerbird (*Pogoniulus pusillus*), Red-winged Starling (*Onychognathus morio*), Dark-capped Bulbul (*Pycnonotus tricolor*), Speckled Mousebird (*Colius striatus*) and Village Weaver (*Ploceus cucullatus*). Dark-capped Bulbuls, Red-fronted Tinkerbirds and Village Weavers were observed to ingest the whole fruits and defaecate the mistletoe seeds (Fig. 1). Red-fronted Tinkerbirds and Red-winged Starlings were observed to regurgitate the seeds on host branches in the field. The other species would consume the fleshy exocarp of the fruit and wipe the seeds on twigs of host trees.

Seed retention time and germination

Birds in captivity did not reliably ingest mistletoe fruits, even though the species had been observed to consume mistletoe fruits in the field. Cape White-eyes did not touch the mistletoe fruits provided in all five days of the feeding trial. Thus, they were excluded from subsequent data analyses.

Village Weavers and Speckled Mousebirds did not ingest whole fruits but rather consumed a portion of the exocarp or completely removed the exocarp without removing the skin that covers the sticky viscin layer of the seed. However, Red-winged Starlings ingested the whole fruits and mainly regurgitated them ($n = 67$) one seed at a time and defaecated very few seeds ($n = 3$) (Fig. 2).

Therefore, all bird species except Red-winged Starlings had no retention time in captivity. The retention time of seeds regurgitated by Red-winged Starlings was 10.1 ± 0.75 min and the data were normally distributed when clustered in 5 min intervals (Fig. 3).

All fruits retrieved from cages were either intact, partially covered by the exocarp, seeds covered by a skin layer or seeds covered with a viscin layer. Germination of the fruits/seeds collected from bird cages was 14 % ($n = 750$). None of the control fruits and the intact fruits obtained from bird cages germinated (Fig. 3). Fruits of mistletoes require at least partial removal of the exocarp to initiate germination (Fig. 3). There was a significant difference in the different actions applied by the bird species to process the mistletoe fruits, which subsequently affected the germination success of the mistletoe seeds (Table 1). The germination success was significantly different among bird species (Table 1). Fruits/seeds obtained from cages of Red-winged Starlings had the highest germination success (32.8 %). The lowest germination success was recorded from fruits collected from Speckled Mousebirds (2.4 %). The regurgitated seeds obtained from Red-winged Starlings had the highest proportion of germination success (81 %). A high proportion (71 %) of the skin-covered seeds also germinated, but would likely not to adhere on a host tree without the exposure of the viscin layer. Partially-removed seeds had the lowest germination success of the seeds retrieved from the captive bird study (12 %).

Discussion

Field observations and the captive studies showed that mistletoe fruits were consumed by a variety of bird species. Many traits of *A. natalitius* fruits make them suitable for consumption by a wide range of frugivores. First, the pulp cover of the fruit is soft which enables it to be easily manipulated, consumed and dispersed by a variety of bird species (see Davidar 1987; Ladley and Kelly 1996 for similar explanation). In addition, the sugar composition and content (sucrose, glucose and fructose) of the mistletoe fruits seems to satisfy a range of sugar preferences of generalist bird species. None of the birds except the Red-winged Starlings ingested whole fruits of the mistletoes in captivity, although many other species were observed ingesting the mistletoe fruits in the field (see Fig. 1). However, in captivity, Red-winged Starlings consumed and mostly regurgitated seeds shortly after ingestion which was also reported in other studies in southern Africa (Godschalk 1983; Polhill and Wiens 1998; Roxburgh 2007). In a similar captive study, caged bulbuls had difficulties swallowing *Melia azedarach* (Meliaceae) fruits in captivity even though they were observed to consume the fruits in field (Voigt et al. unpublished). We suggest that several factors may influence the consumption and ingestion of wild fruits in captivity such as birds' acclimatisation period, fruit chemistry (which may change immediately after picking) and fruit presentation techniques. For example, avian dispersers may depend on the physical presence of the mistletoe and the host tree for perching and feeding cues, but the fruits used in the captivity study lacked any such cues that might aid birds to identify the fruits. Thus, gut passage rate cannot be extrapolated directly to field observations of many species of birds in captivity.

In our study, we found that birds apply three mechanisms to handle and disperse mistletoe seeds, viz. bill wiping, defaecation and regurgitation. This is similar to other studies that found that birds apply different modes of handling and processing of fleshy mistletoe fruits in which each mode has different implications for dispersal distance and germination success of mistletoe seeds (Reid 1991; Overton 1994; Ladley and Kelly 1996). Above all, our data showed that birds are

important for initiating germination of mistletoes as all intact fruits used as a control and intact fruits obtained from cages failed to germinate. Partially-removed seeds had the lowest germination success. This showed that presence of the exocarp negatively affects germination. Many studies also reported similar results, indicating that the removal of the exocarp is critical for initiating germination of mistletoes (Godschalk 1983; Ladley and Kelly 1996; Roxburgh and Nicolson 2005; Roxburgh 2007).

In this study, we noted that regurgitation by Red-winged Starlings was the most important mode of mistletoe fruit processing. Such processing had positive effects on germination success, which likely caused seeds to be dispersed over short distances. Red-winged Starlings deliberately wiped their bills on a twig in the cage to dislodge the sticky viscin-covered seeds one at a time. This is an important behaviour because mistletoe seeds will experience less post-dispersal density dependent mortality, which should positively affect survival of mistletoe seedlings (Murphy et al. 1993; Roxburgh 2007). Regurgitation was also done on small twigs (10-14 mm diameter), which are an appropriate size for the establishment of mistletoes (Sargent 1995).

The mistletoe fruits in our study contain a very large indigestible seed. Thus, regurgitation ensures fast release and increases space for subsequent intake that maximises the rate of energy intake and minimises the energy cost that is required to transport the seed through the gut (Levey and Grajal 1991; Traveset and Verdú 2002; Roxburgh 2007). For this reason, even birds that pass the seeds through the gut, such as dicaeids, phainopeplas and euphonias, have specialised digestive systems that enable them to process mistletoe fruits quickly (Walsberg 1975; Reid 1989;1990; 1991; Traveset and Verdú 2002). Above all, regurgitation enhances directed dispersal. Local aggregation of the mistletoes is likely to have a role in enhancing local specialisation in mistletoes (Aukema and Martínez del Rio 2002c; Martínez del Rio et al. 1996). In light of this, our findings are consistent with other findings that birds determine the prevalence and infection pattern of mistletoes in a community, which in turn directs local host specificity (Aukema and Martínez del Rio 2002a,b,c).

Those studies that investigated the role of mistletoes in an ecosystem identified them as keystone species, i.e. mistletoes are the main food for their dispersers, pollinator birds and herbivores (Watson 2001; Press and Phoenix 2005). In light of this, consideration of mistletoes as merely destructive pests or redundant species (i.e. species that are either unnecessary or can be replaced in their contribution to ecosystem functioning; Naeem (1998)), underestimates their network of interactions with other organisms and their role as indicators of community integrity and ecosystem health (Mathiasen et al. 2008). In particular, the mistletoes used in our study interact with many species of frugivorous birds and their presence could be essential to maintain biodiversity, particularly of birds, in an ecosystem. On the other hand, avian dispersers facilitate new infection and also facilitate the intensity and rate of infections that negatively affect host trees at an individual and local level (Bowie and Ward 2004). Thus, as a prerequisite to understanding the spread of mistletoes and to manage ecosystems with mistletoes appropriately, we need to understand the dispersal dynamics of mistletoe infection.

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Table 1 Chi-square (χ^2) test of the effect of bird species on mistletoe fruit processing and subsequent effect on germination success of the mistletoe seeds. There was a significant difference in germination success of seeds obtained from different bird species. Bird species showed significant differences in the action they applied in processing mistletoe fruits, which also significantly affected the proportion of seeds that germinated. Sample sizes: bird species (n = 3), action (n = 5), fruits/seeds (n = 750).

Source of variation (Factors)	χ^2	d.f.	P
Bird species versus germination	114.12	2	<0.001
Bird species versus action	198.41	8	<0.001
Action versus germination	236.93	4	<0.001

Figure legends

Fig. 1 Percentage of each bird species that defaecated seeds of *A. natalitius* on a branch of host trees.

Fig. 2 Retention time of regurgitation of *A. natalitius* seeds by Red-winged Starlings in captivity.

Fig. 3 Initiation of germination by Red-winged Starlings (a), Village Weavers (b) and Speckled Mousebirds (c) by removal of the exocarp of the mistletoe fruits in the captive study. Red-winged Starlings ingested the whole mistletoe fruits and mainly regurgitated the seeds and defaecated very few seeds. Intact fruits never germinated. Partially removed fruits had least germination success. A high proportion of the skin-covered seeds germinated.

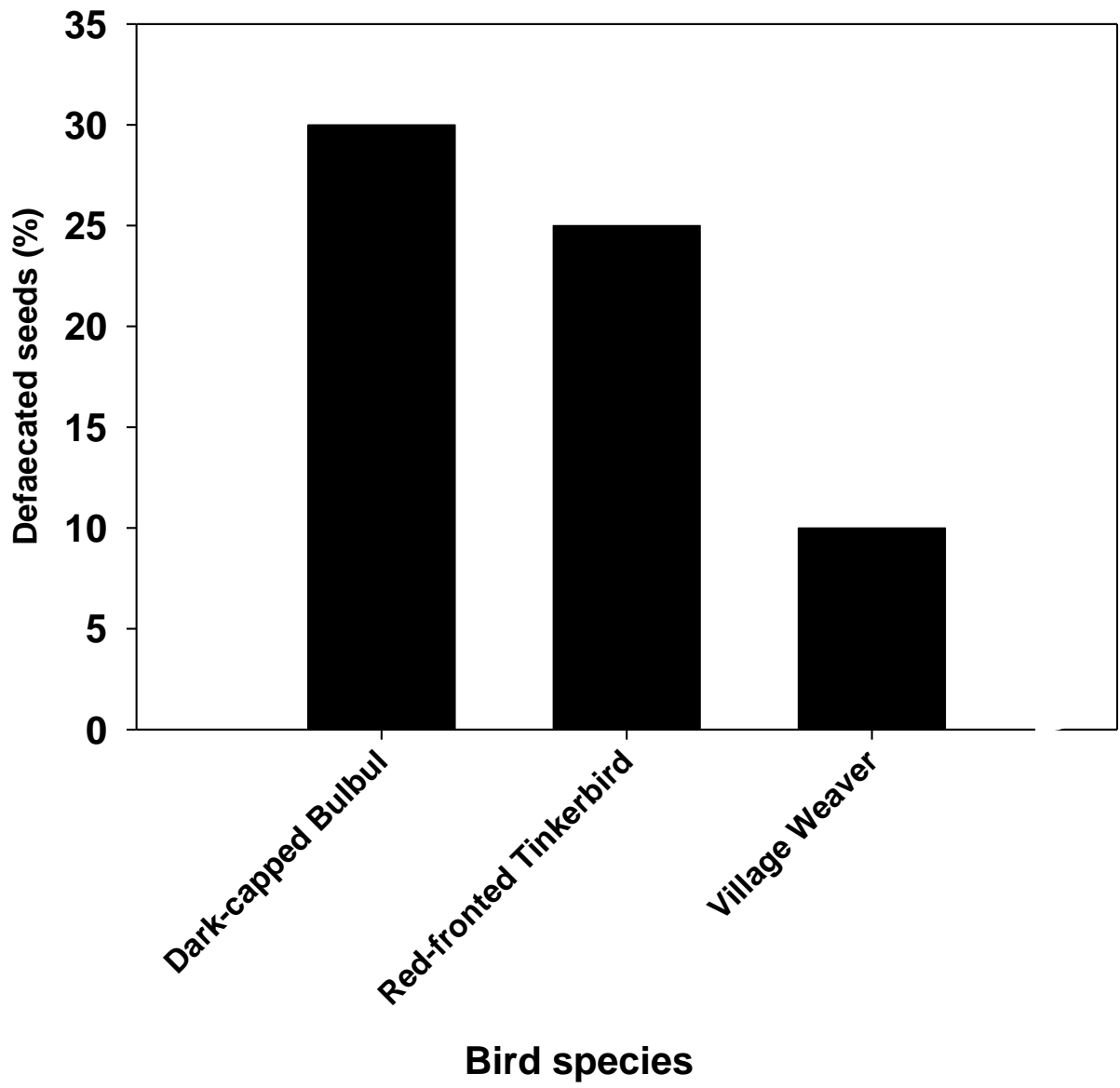


Fig. 1

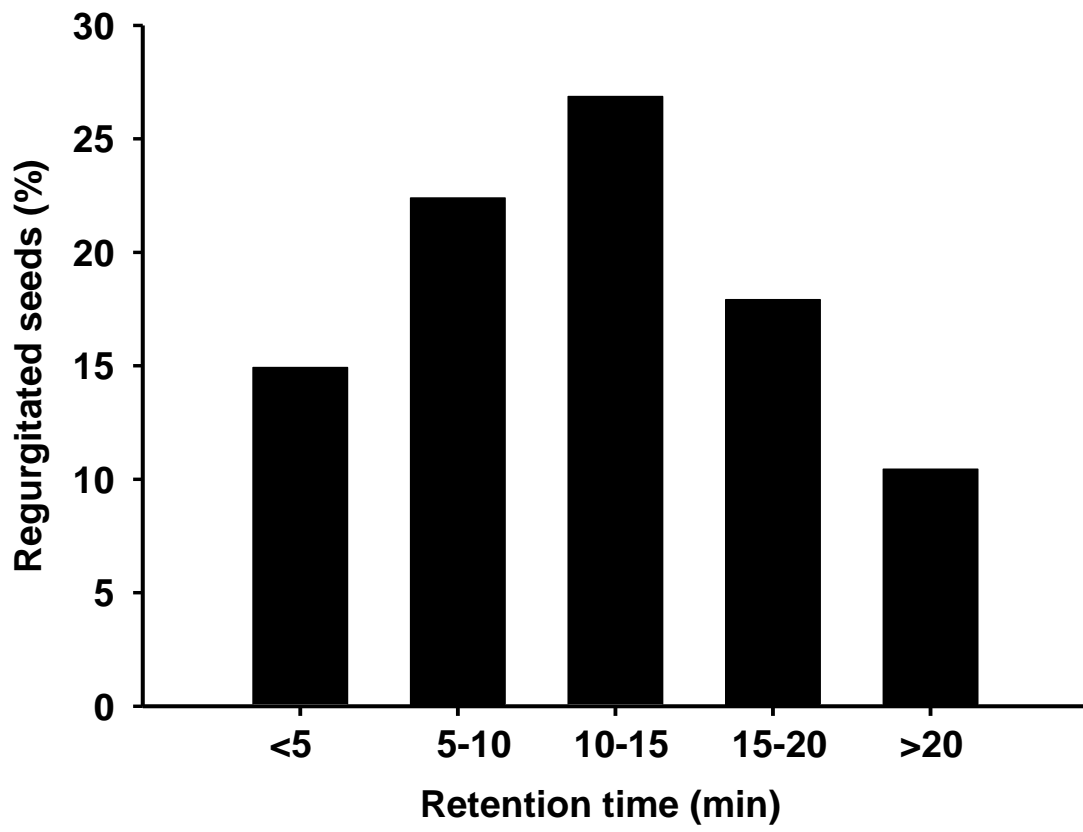


Fig . 2

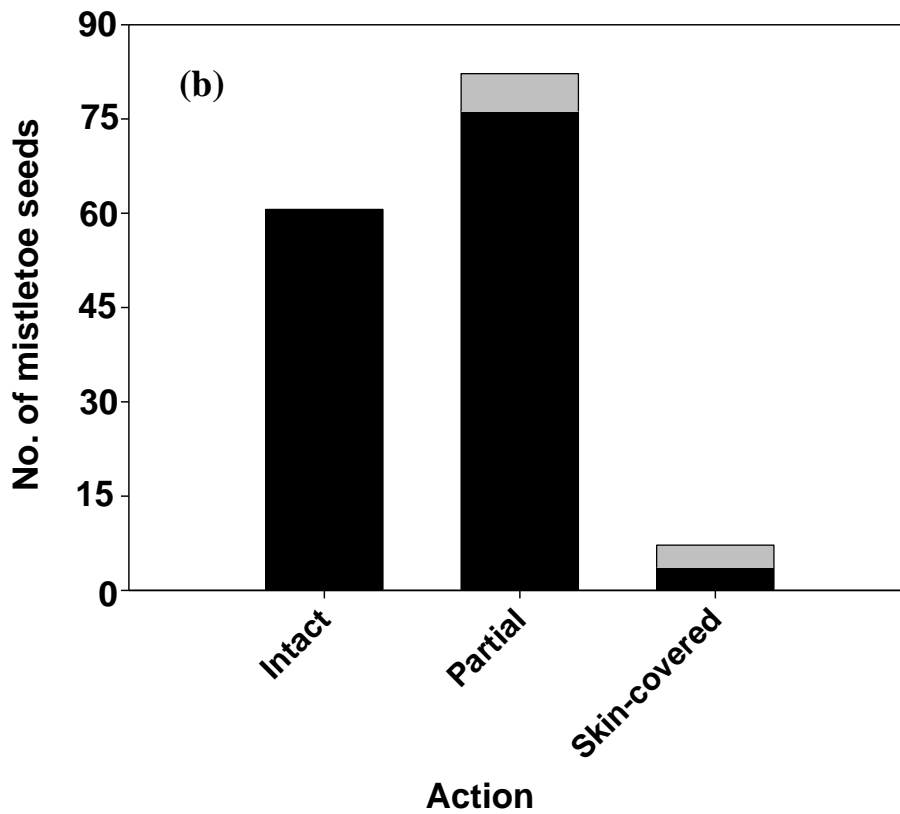
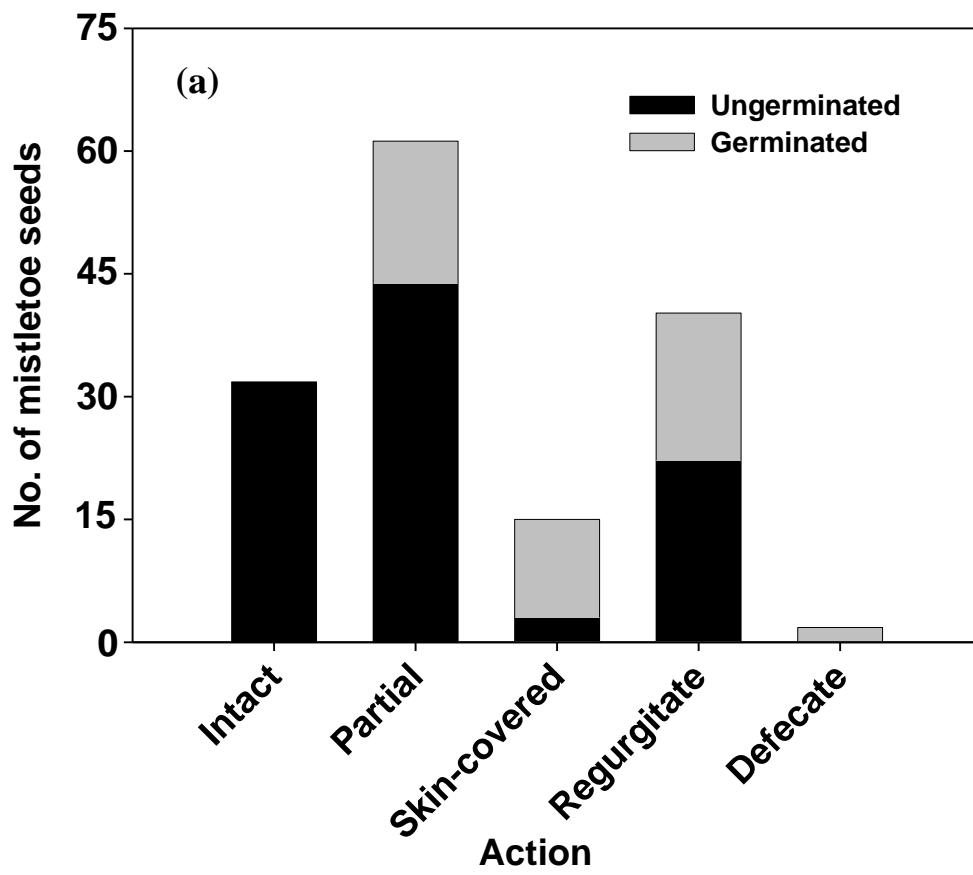


Fig. 3

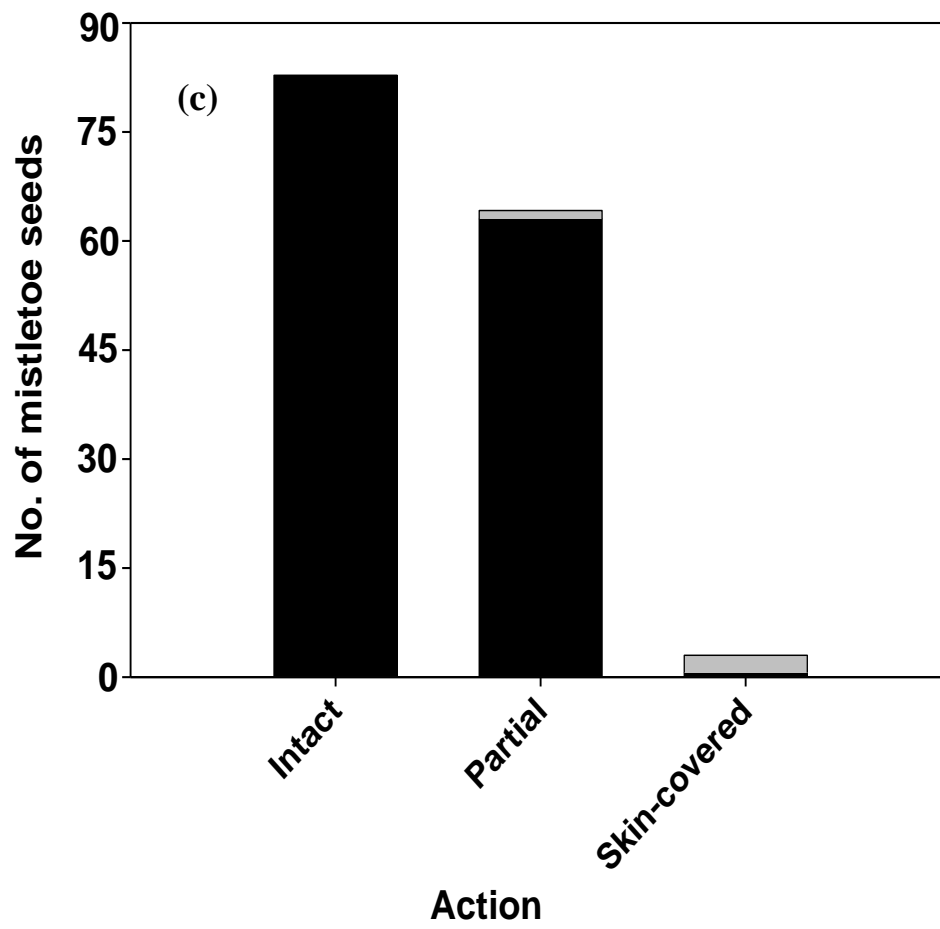


Fig. 3

Chapter 6

Conclusions

Mistletoes are parasitic flowering plants and are ecologically important components of woodlands and forests. Most mistletoe species do not cause damage at the ecosystem level (see e.g. Wiegand et al. 1999), but they can harm host trees (Ward et al. 2006, Mathiasen et al. 2008). In particular, high infection by mistletoes can be responsible for the mortality of host trees. On the other hand, mistletoe berries form a mainstay of the diet of many frugivorous birds in winter when there is very little food available in the ecosystem and also serve as a food for herbivores (Watson 2001, Restrepo et al. 2002). Thus, investigating the unique interactions of hosts, birds and mistletoes provides a tremendous opportunity to study ecology, plant-animal interactions, coevolution, and parasitism and dispersal patterns. Particularly, the evaluation and determination of the role of mistletoes at the ecosystem level deserves further research.

Many mistletoe populations that are morphologically identical may show host preference and may differentially parasitise hosts along a geographic gradient (Rödl and Ward 2002). There may be tight genotypic coupling between mistletoes and their host *Acacia* genotypes (Rödl and Ward 2002). In light of this, many studies have highlighted the importance of host specificity in mistletoes (Mathiasen et al. 2008, Rödl and Ward 2002). Genetically-based differences in the ability to infect and grow on different hosts may lead to the evolution of host races and subsequently to sympatric speciation. Consequently, detailed examination of host race evolution in mistletoes can provide insight into its potential as a pathway for speciation.

In this study, we quantified and determined the mechanisms that are likely to cause local specialisation in the hemiparasitic mistletoe, *Agelanthus natalitius*. *A. natalitius* showed a high degree of host specificity on the most abundant host species, *Acacia karroo*, at both study sites in KwaZulu-Natal, South Africa. These findings are consistent with previous studies that found that

some mistletoe species become specialised on the most frequently-encountered host species (Norton and Carpenter 1998, Norton and de Lange 1999). Compatibility with the most abundant host species allows the mistletoes to interact with the most frequently encountered host species. Thus, host population demographics are important factors to consider in the study of mistletoe host specificity.

Mistletoe distribution was positively correlated with tree size. This can be due to the perching behaviour of birds on tall trees or, more simply, that old trees had more time to be visited by birds and exposed to infection (Aukema and Martínez del Rio 2002, Aukema 2004). Accumulation of mistletoe infections as a tree ages is important, because autoinfection of host trees can be quite high (Overton 1994). We also noted that mistletoe infection prevalence (the number of infected trees per host species) may not be a direct measure of the degree of host specificity. Instead, parasitic load (number of mistletoes per host species) should be taken into account (Overton 1994, Roxburgh and Nicolson 2005). For example, *L. leucocephala* was represented by only two trees in the study sites and both were infected. If prevalence was considered, it would be 100%, which fails to explain host specificity. We also found that many individual trees carried no infection and only a few carried most mistletoes. Thus, mistletoe distribution was highly aggregated, which is consistent with results from other studies (Overton 1994, Donohue 1995, Aukema and Martínez del Rio 2002, Aukema 2004). Mistletoes were more abundant in open areas and in open canopies within their host trees. Similarly, in a greenhouse experiment, mistletoes were more successful where there was less shade. There is similar evidence from other studies that mistletoes are abundant in savannas where shade is minimal (Dean et al. 1994).

Our results showed that germination was site- and substrate-insensitive. It has been shown elsewhere that mistletoe seeds removed from the exocarp immediately germinate at high rates, independent of the substrate (Yan 1993, Rödl and Ward 2002). This is unlike root-parasitic plants that use molecular cues released from the host (host-derived chemicals) to identify their hosts before initiating germination and haustorium development (Yoder 1999, Tomilov et al. 2004).

However, our study clearly showed that the hypocotyls (which subsequently form the haustorium) grew longer when they were placed on their source host species within their locality. This suggests that haustorium formation is the basis for determining local host specificity in mistletoes. Most likely host specificity is mediated through chemical interactions at early developmental stages, but this requires further research.

There is evidence that mistletoes are most successful on hosts that supply high levels of nitrogen (Dean et al. 1994, Bowie and Ward 2004, Ward et al. 2006). It has also been shown that host species that maintain high field water potentials are more likely to support mistletoes than those with low field water potentials (Bannister et al. 1999, Miller et al. 2003). However, in our study for both host species (*A. karroo* and *A. caffra*) at both study sites, there were no significant differences in host nutrient and water content between the two most common host species. This probably implies that host quality in terms of nutrients (especially N) and water may not direct host specificity in this mistletoe species. However, mistletoes accumulated a higher nutrient content than their host trees, which was similar to the results from many other studies (Lamont 1983, Panvini and Eickmeier 1993, Bowie and Ward 2004).

Mistletoes maintain lower (more negative) Ψ as compared with the host trees; a condition that enables mistletoes to exploit the xylem of host trees passively. At predawn, Ψ of the mistletoes and the host species were positively correlated, which is consistent with the widely held belief that mistletoes always maintain lower Ψ by continuous opening of their stomata. However, we found that at midday when the host trees experienced minimum water potential, the mistletoes tended to reduce their water Ψ . This probably reduced water loss, thus the mistletoes no longer had a positive correlation with the Ψ of their host trees. This challenges the concept that mistletoes open their stomata continuously to absorb nutrients by mass flow (Schulze et al. 1984, Strong and Bannister 2002, Bowie and Ward 2004).

We also investigated the mechanism by which this mistletoe species accesses nutrients, i.e. from the xylem (passive nutrient uptake) or phloem (active nutrient uptake) of the host trees. We

used the N:Ca ratio of mistletoes as an indirect index of the mechanism of nutrient uptake (Panvini and Eickmeier 1993, Bowie and Ward 2004) because N is highly mobile and Ca is a large molecule and phloem-immobile. In our study, mistletoes growing on *A. caffra* actively accessed nutrients from the phloem of host trees. However, mistletoes growing on *A. karroo*, passively accessed nutrients from the xylem of host trees. We suggest that mistletoes use passive uptake when they grow on the more compatible host species, *A. karroo*, which doesn't require energy. Thus, the nutrient uptake mechanism might be easier to maintain and could direct host specificity. However, mistletoes on the less preferred host, *A. caffra*, use active uptake which requires energy. This paradox warrants further research to explain the differences in nutrient acquisition of the same mistletoe populations that parasitise related host species. In light of this, we have started to investigate the cytology of the infected twig at the junction of the host and the parasite by means of scanning electron microscope studies. This should provide direct evidence of mistletoe nutrient access, i.e. whether it is from the xylem or phloem of the host trees. This has never been directly investigated in mistletoes.

We observed seven bird species frequently feeding on *A. natalitius* fruits. Although the consumption of fruits by birds in captivity is highly reduced compared to field observations, most selected birds were also effective in removing of the pulp-cover of mistletoe fruits. The handling of mistletoe fruits by all species of birds showed that mistletoe seeds are most likely dispersed over short distances, albeit to safe sites. Most of all, Red-winged Starlings in captivity ingested whole fruits, regurgitated seeds and deliberately wiped their bills to dislodge the sticky viscin-covered seeds one at a time (see also Roxburgh 2007). Of the species used in our captivity studies, Red-winged Starlings are potentially the most effective disperser of mistletoe fruits. Birds are responsible for direct autoinfection and local aggregation of the mistletoes that likely have a role in enhancing local specialisation. However, the mechanisms of long-distance dispersal are poorly understood in hemiparasitic mistletoes, thus research to investigate long-distance dispersal in mistletoes will have great value.

In conclusion, host demography (especially host abundance), enhanced by host compatibility and bird dispersal, contribute to host specificity. However, host quality in terms of nutrient and water contents had no effect in determining host species in this mistletoe. We have already performed pilot genetic studies using allozymes to examine the genetic composition of *A. natalitius* growing on different host species and at different sites to investigate the development of putative host races. We have found several enzyme systems with possible variation that can explain population differences. Using this method, we will be able to determine whether there are genetic differences between mistletoes growing on the same trees, genetic differentiation on different host species within a site, and genetic differentiation among sites.

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