The role of cone volatiles and thermogenesis in the pollination of *Encephalartos* cycads with particular reference to *E. villosus*

by

TERENCE NKWANWIR SUINYUY

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Declaration

I confirm that this work is my own except in the instances mentioned below and use of all material from other sources has been properly and fully acknowledged.

Prof. John S. Donaldson conceived the idea of push-pull interaction in the African cycads and I carried out all the studies from data collection to writing of thesis.

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Terence N. Suinyuy

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as a sign of gratitude for their constant and caring love

and to my daughter

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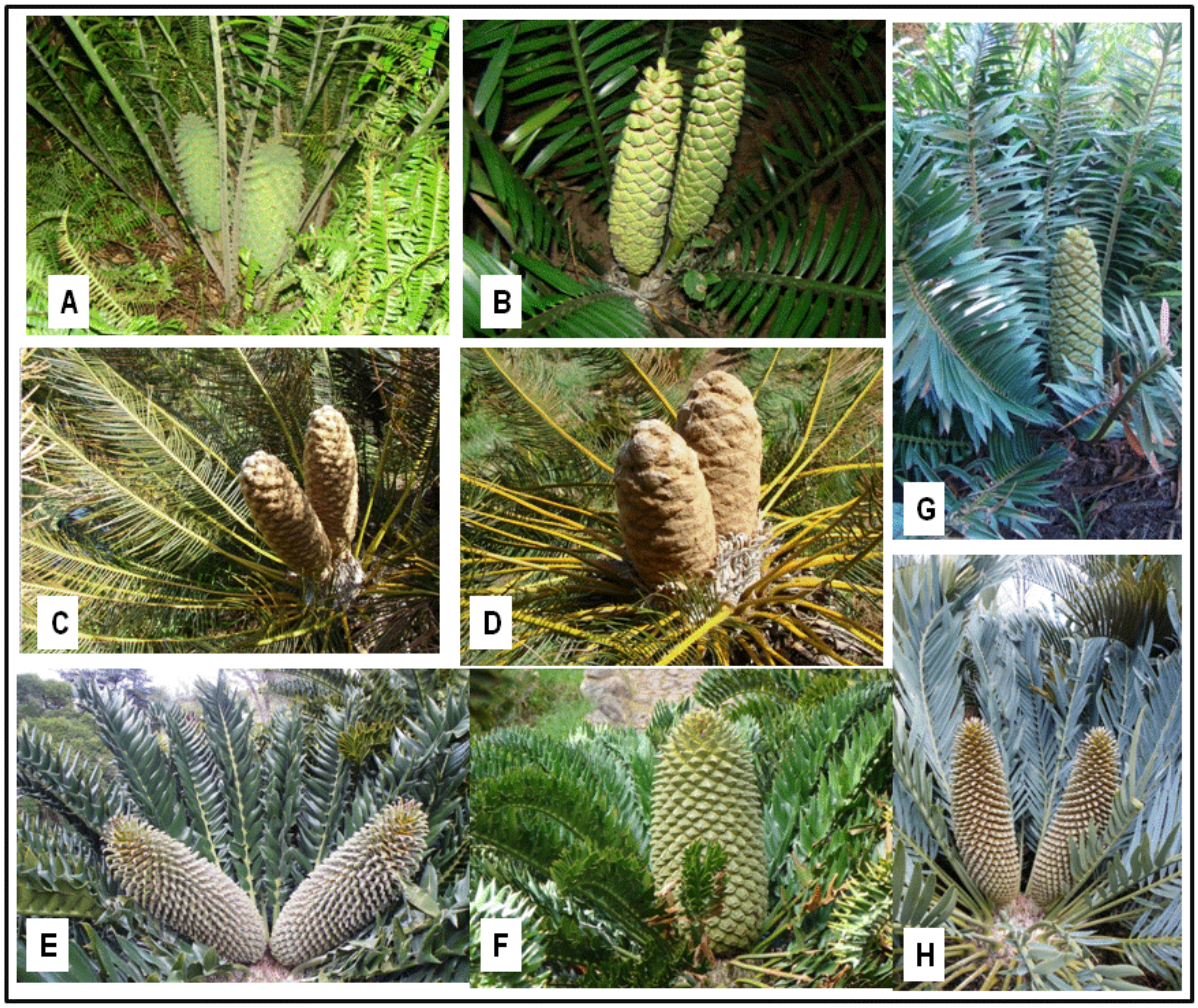
who has been my joy



Frontispiece. Mature *Encephalartos* species. **A**: Female *E. villosus* and, **B**: Male *E. villosus* bearing two cones each; **C**: Male *E. ghellinckii*, and **D**: Female *E. ghellinckii* bearing two cones each; **E**: Male *E. latifrons* bearing two cones, and **F**: Female *E. latifrons* bearing one cone; **G**: Male *E. caffer* bearing one cone, and **H**: Male *E. princeps* bearing two cones.

Summary

Pollination systems of cycads usually involve obligate mutualisms in which the cycad taxa depend on between one and three insect pollinators. Generally, the pollination periods of many cycad taxa are characterised by odour emissions in pollen shedding (male) and receptive (female) cones usually accompanied by heat production. The emission of cone odours and heat production coincide with periods of insect activity on the cones. The simultaneous occurrence and coincidence of both events at the time when insect pollinators are active on cones suggest that cone odour and heat influence insect behaviour. In some highly specialised plant-insect pollinator interactions, the emission of volatile compounds in association with heat production has been shown to influence pollinator behaviour indicating that floral odour and heat production are adaptations for insect pollination. Cone odour and heat in cycads could therefore have the same role as floral odour and heat in some flowering plants.

In most cycad-pollinator interactions, the pollinators use the male cone sporophylls as brood sites and therefore have a strong association with male cones. In contrast to the singular role of attracting pollinators, daily variations in cone volatiles and thermogenesis have been found to attract and repel insect pollinators in *Macrozamia* cycads in a complex push-pull interaction. It is not known if this is pattern of cone odour emission and heating is more widespread in cycads and the roles of cone volatile emissions and heating in the relationship between cycads and their insect pollinators remains an open question. The focus of this study was to investigate cone volatiles and, to a lesser extent, cone heating in *Encephalartos* in order to get a better understanding of their role in pollinator interactions. The widespread species, *E. villosus,* was used as a model species.

The thesis has six chapters, starting with a general introduction to cycad pollination systems in Chapter 1. The chapter outlines what is known about the role of cone volatiles and thermogenesis in cycad pollination, how this relates to comparable systems in angiosperms, and identifies the main questions that are addressed in the four main content chapters.

Chapter 2 comprises a study of the chemical composition, patterns of cone odour emissions and heating in both male and female *E. villosus* at different developmental stages using plants from a few populations in the Eastern Cape (EC) and KwaZulu Natal (KZN) of South Africa. This was to determine whether these patterns were consistent with push-pull interactions such as those observed in *Macrozamia* cycads. The chemical composition was investigated using a gas chromatography-mass spectrometer (GC-MS) and miniature data loggers (ibuttons). The abundance of putative pollinators was also investigated in relation to daily and developmental stages of cone odour and heating for pre-dehiscent, dehiscent, and post dehiscent male cones and pre-receptive, receptive and post receptive female cones. The results show relatively small daily differences in the relative amounts of cone volatiles with larger differences between stages in cone development. However, thermogenesis resulted in significant daily spikes in cone temperature in male cones. The results also revealed unexpected differences in volatile profiles of different *E. villosus* populations suggesting that there may be at least two chemotypes of *E. villosus*.

In Chapter 3, the extent of variation in cone odour chemistry within *E. villosus* populations across its distribution range was investigated further, including the possibility that it might reflect a change in pollinators and even that *E. villosus* could comprise at least two species. Cone odours and associated insects were collected from 10 populations spanning the full extent of the species distribution. The results confirmed that *E. villosus* consists of two main chemotypes but there was no change in insect pollinator composition across the different *E. villosus* populations. The variations do not support the hypothesis that

the current circumscription of *E. villosus* comprises more than one species but it does raise questions about how different chemicals may affect pollinator interactions in different populations.

Tests of the physiological and behavioural responses of beetles to the cone volatiles of *E. villosus* are presented in Chapter 4. The tests comprised olfactometer choice trials using cone sporophylls, gas chromatograph electroanntennogram detection (GC-EAD) tests on known pollinators, choice tests using chemicals to which beetles responded in the GC-EAD, and field tests using traps baited with specific compounds. The results showed that two insect pollinators exhibited physiological responses to (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene. Attraction to (3*E*)-1,3-octadiene was confirmed in olfactometer tests and in field tests. In addition, field tests showed that pollinators, especially from EC populations of *E. villosus* were attracted to 2-isopropyl-3-methoxypyrazine. The results therefore confirm the role of cone volatiles as attractants for pollinators and confirm that there are different attractants for the same pollinator species in different populations of *E. villosus.*

In Chapter 5, I examined variation in scent chemistry among 19 *Encephalartos* species that represented different phylogenetic clades and included species from a range of different habitats with different pollination syndromes. The aim was to determine whether cone volatiles have been conserved across specific lineages in *Encephalartos* or whether shifts in odour composition might reflect adaptive responses to particular pollination syndromes. The study revealed a high diversity and variation in volatiles across the different *Encephalartos* species. There was some evidence that volatile composition had been conserved within lineages, especially in the *E. friderici-guilielmi* clade. However, even in this clade, but particularly in the *E. villosus* clade, the data indicated considerable divergence in volatile composition that may reflect adaptive shifts associated with pollinators. The analysis of pollinator syndromes did not reveal a distinct pattern and changes in cone volatiles may therefore reflect more subtle adaptations to specific

pollinators, which is consistent with the observation that *Porthetes* spp. pollinators have mostly species specific interactions with their cycad hosts.

Finally, in Chapter 6, I summarise the results and ideas presented in the thesis and discuss the main findings in the context of what is currently known about cycad pollinator interactions and its significance in the broader context of chemically mediated plant-insect interactions. I also identify questions for further study.

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Chapter 1. General Introduction

Cycads are a group of dioecious gymnosperms which originated about 300 million years ago during the Permian period and are considered to be the oldest extant seed plants (Norstog and Nicholls, 1997). They were most diverse and widely distributed during the Jurassic. Currently cycads are often rare and comprise one of the most threatened groups of plants (Donaldson, 2003) with their range restricted to tropical and subtropical habitats (Jones, 1993). They may represent the oldest lineage of animal pollinated plants (Terry et al., 2004a).

Although early reports suggested that insects were involved in cycad pollination (Pearson, 1906; Rattray, 1913; Marloth, 1914), cycads were generally considered to be wind pollinated (Chamberlain, 1935) until experimental evidence finally proved the importance of insect vectors (Norstog et al., 1986). Inferences of wind pollination in cycads were based on the resemblance of female cycad cones to conifer cones and the assumption that all gymnosperms were wind pollinated (Chamberlain, 1935). In spite of arguments in favour of wind pollination, there is now evidence from pollination experiments that insect pollination occurs in at least seven of the ten extant cycad genera, i.e. *Bowenia* (Wilson, 2002), *Cycas* (Kono and Tobe, 2007), *Encephalartos* (Donaldson et al., 1995; Donaldson, 1997; Suinyuy et al., 2009), *Lepidozamia* (Hall et al., 2004), *Macrozamia* (Chadwick, 1993, Mound and Terry, 2001; Terry, 2001; Terry et al., 2005), *Stangeria* (Proches and Johnson, 2009), and *Zamia* (Norstog et al., 1986; Norstog, 1987; Tang, 1987a; Norstog and Fawcett, 1989).

Given the prevalence of insect pollination across distinct clades of extant cycads, it has been argued that insect pollination originated early in the evolution of cycads and is likely to be ubiquitous in modern cycads (Norstog and Nicholls, 1997; Stevenson et al., 1998). There is a remarkable similarity at a higher

taxonomic level between pollinator groups from different cycad genera. Weevils (Coleoptera; Curculionoidea) have been identified as pollinators in *Bowenia* (Wilson, 2002), *Cycas* (Raju and Jonathan, 2010; Yang et al., 2010), *Encephalartos* (Donaldson, 1997; Suinyuy et al., 2009), *Macrozamia* (Terry et. al., 2004a and b), *Lepidozamia* (Hall et al., 2004) and *Zamia* (Norstog et al., 1986; Tang, 1987a; Norstog and Fawcett, 1989; Stevenson et al., 1998). Similarly, cucujoid beetles (Coleoptera; Cucujoidea) have been implicated in the pollination of species in three genera, i.e. *Zamia* (Norstog et al., 1986; Tang, 1987a), *Ceratozamia* (Vovides, 1991), and *Encephalartos* (Donaldson et al., 1995; Donaldson, 1997; Suinyuy et al., 2009) and nitidulid beetles in the pollination of two genera, *Cycas* (Kono and Tobe, 2007) and *Stangeria* (Proches and Johnson, 2009). Notable exceptions where pollinator groups may be restricted to only one genus include thrips (Thysanoptera) in the pollination of Australian *Macrozamia* species (Mound and Terry, 2001; Terry, 2001; Terry et al., 2005) and Lepidoptera that have been implicated in the pollination of *Cycas micronesica* (Terry et al., 2009; Marler, 2010). Similarities in beetle pollinators could be used to infer that ancient cycads were pollinated by beetles, but Oberprieler (2004) has noted that beetles associated with cycads are spread across different clades in both curculionid and cucujid phylogenies and he proposed that current pollinator assemblages represent convergent evolution and include species that may have shifted from angiosperm hosts.

One of the key aspects of all known cycad-pollinator interactions is that they involve feeding and larval development in male cones, where the cone tissue acts as a brood site and food for the pollinator larvae (e.g. Norstog and Fawcett, 1989; Donaldson, 1997; Stevenson et al., 1998; Terry et al., 2004; Hall et al., 2005), thus serving as a reward for insect visitation. It has been suggested that insects visiting female cones in search of rewards feed on micropylar exudates which contain sugars and amino acids (Norstog et al., 1986; Norstog, 1987). However, Tang (1987a) showed that the presence of beetles was not correlated

with micropylar droplets in female cones of *Z. pumila.* It thus seems likely that insects generally visit female cones by mistake and do not benefit from any reward, having been attracted by cues that are similar to those of male cones (Tang, 1987a; Terry et al., 2005). Norstog et al. (1986) found that both male and female cones of *Z. furfuracea* emitted a strong odour during pollen shed and receptivity respectively and Tang (1987a and b) found that the odour of both male and female cones of *Z. pumila* at the time of pollination were similar. The fact that male and female cones emit similar odours suggests that the same odour may attract insect pollinators to both sexes thereby facilitating pollination.

Floral volatiles are secondary plant substances that influence the behaviour of insects and other organisms by acting as attractants or repellents (Fraenkel, 1959; Raguso, 2008). It has been hypothesized that floral volatiles originally evolved as part of chemical defence systems that deterred insects from feeding on plant reproductive structures, but as insects co-evolved and became attracted by some compounds, volatiles began to function as attractants for pollinators (Pellmyr and Thien, 1986). It has been proposed that the evolution of such pollinator specific volatiles has resulted in reproductive isolation among closely related plant species (e.g. Gröth et al., 1987; Azuma et al., 1997; Levin et al., 2001) and that these compounds may reflect phylogenetic relationships (e.g. Azuma et al., 1999; Jürgens et al., 2003; Levin et al., 2003). Scent chemistry is therefore likely to be a key mediator of cycad-pollinator interactions and an important aspect of the evolution of this enigmatic plant group.

Role of cone odour and heat production in cycad pollination

Studies of cycad cones have shown that some cycad species release volatile odours during pollen dehiscence in male cones and/ or receptivity in female cones, and that this is often associated with an

increase in cone temperature (Poisson, 1878; Jacot-Guillarmod, 1958; Tang, 1987b). Release of volatiles and heat production coincides with periods of insect activity on cones (Tang, 1987a; Donaldson, 1997; Terry, 2001; Seymour et al., 2004; Suinyuy et al., 2010) and the initial thinking was that cone odour and heat production attract pollinating insects in cycads (Tang, 1987b; Tang, 1993). It was also suggested that thermogenesis may function, inter alia, to volatilize the attractive odours, enhance insect movement between cones, facilitate dehiscence from pollen sacs, and enhance cone elongation (Tang, 1993; Seymour et al., 2004; Terry et al., 2004a).

Similarities between cycad cone odours and angiosperm floral attractants provides some support for the 'pollinator attraction' hypothesis. Convergent functions between floral odours in angiosperms and cone odours in cycads are suggested by the occurrence of compounds that are common to both groups. One example is linalool, which is emitted by *Zamia furfuracea* (Pellmyr et al., 1991; Tang, 1993), *Macrozamia machinii* (Terry et al., 2004a and b) and *Encephalartos natalensis* (Suinyuy et al., 2010), and is also emitted by many flowering plants (Raguso and Pichersky, 1999; Knudsen et al., 2006). The presence of the same volatile compounds in male and female cycad cones, as found in *Zamia pumila* (Tang, 1987a), has also been interpreted as evidence that cone volatiles function to attract insect pollinators. The strongest evidence that cone odours attract insect pollinators has been obtained from experiments. For example, pollen shedding male cones of *Z. furfuracea* attracted *Rhopalotria mollis* beetles (Coleoptera: Curculionoidea) at close range (Tang, 1993). Olfactometer experiments have shown that sporophylls from pollen shedding cones attracted *Cycadothrips chadwicki* pollinators to *Macrozamia lucida* (Terry et al., 2007a and b).

Studies on several Australian cycad taxa suggest that the functions of cone odour and thermogenesis are more complex than simply to attract pollinators. Terry et al. (2004a) observed that

insects leave male cones of *Macrozamia lucida*, *M. machinii*, and *M. macleayi* during peak periods of heat and odour production. This suggested that insect pollinators tend to leave male cones due to heating and the associated increase in metabolic activity and/or an increase in the release of repellent compounds. A series of olfactometer experiments showed that *C. chadwicki* was attracted to *M. lucida* sporophylls early in the day, repelled by sporophyll odours at midday, and attracted again in the late afternoon to early evening (Terry et al., 2007a and b). Further olfactometer experiments using specific compounds showed that *C. chadwicki* was attracted to *β*-myrcene at low concentrations but was repelled by high concentrations of the same compound (Terry et al., 2007a and b). These results corresponded to field observations and showed that insects appeared to be attracted to male cones in the morning when volatile emissions and cone temperature were low, were leaving male cones at midday when volatile emissions and cone temperature were high, and were attracted later in the day when cone emissions and temperatures were low (e.g. Terry et al., 2004a). Terry et al. (2007a and b) referred to this system as a 'push-pull' strategy following the terminology of integrated pest management in which a combination of attractant and repellent factors are used to influence the behaviour of pest and natural enemy populations (Pickett et al., 1997, 2006; Cook et al., 2007).

It is not known whether push-pull pollinator interactions apply generally in cycads. Various studies of cone odours and/or thermogenesis have been undertaken for cycad taxa other than *Macrozamia* (e.g. Pellmyr et al., 1991; Tang, 1987b, 1993; Donaldson, 1997; Azuma and Kono, 2006; Proches and Johnson, 2009; Suinyuy et al., 2010) but these studies have not explored the roles of cone odours and thermogenesis in pollination or have not been detailed enough to test the push-pull hypothesis. Several models for push-pull systems have been proposed. For example, Donaldson (2007) proposed mixed models in which cone volatiles and or heating may attract pollinators in some instances or repel them in

others and highlighted the need for detailed studies that examine both the patterns of cone heating and odour production over the full period of pollination and specifically test insect responses. Various experimental techniques have also enabled researchers to move beyond correlation. These include the use of gas chromatography-electroantennogram detection (GC-EAD), olfactometers, choice tests, and field bioassays (Schiestl and Marion-Poll, 2002). GC-EAD makes it possible to identify specific compounds that trigger a physiological response and to then test the behavioural response to these specific compounds (e.g. Ayasse et al., 2000; Stensmyr et al., 2002; Terry et al., 2007a and b).

In this study, I investigated pollination systems in the African cycad genus, *Encephalartos,* which is regarded as the sister taxon to the Australian genera *Macrozamia* and *Lepidozamia* (Treutlein and Wink, 2002), with particular reference to the role of scent in mediating cone-insect interactions.

Chemical variation of volatile compounds within the *Encephalartos* genus

The chemical composition of floral odour is thought to be important to specific pollinator classes, and some pollination syndromes are characterized by distinctive odour compounds (Kaiser and Tollsten, 1995; von Helversen et al., 2000). Different types of floral odour are associated with adaptation to different pollinator groups (Knudsen and Tollsten, 1993) and odour differences between species are interpreted as cues for attracting distinct pollinators (Dodson et al., 1969; Knudsen and Tollsten, 1993; Raguso and Pirchersky, 1995).These odour compounds can therefore potentially be important in confirming established taxonomic relationships, function as reproductive isolation barriers and delimit species boundaries (e.g. Hills et al., 1972; Thien et al., 1975; Gröth et al., 1987; Azuma et al., 1997; Levin et al., 2001, 2003; Jürgens et al., 2003). However, the effectiveness of floral odour in maintaining reproductive isolation

barriers is weakened when pollination is not specific, when the composition of floral odour compounds overlap between species, and when other cues, such as colour and morphology, are used by pollinators. It is therefore necessary to examine the role of scent within the broader context of cycad pollination ecology.

Previous studies of cone odour showed that *Zamia pumila* and *Z. furfuracea* emit mainly methyl salicylate and (3*E*)-1,3-octadiene respectively (Pellmyr et al., 1991; Tang, 1993) and are pollinated by different species in the weevil genus *Rhopalotria* (Tang, 1987a; Norstog and Fawcett, 1989). This indicates that different compounds may be involved even when pollinators are closely related. In the Australian genus, *Macrozamia,* there are two recognised subgeneric groupings, i.e. sections *Macrozamia* and *Parazamia.* Pollination by *Tranes* weevils (Coleoptera: Curculionoidea) has been recorded in *M. machinii* and *M. parcifolia* both from section *Parazamia* (Forster, 2004) and in which cone odours contain linalool. In contrast, *M. lucida* and *M. macleayi* are pollinated by *Cycadothrips* (Thysanoptera) and *β*-myrcene is a major constituent of their cone odours (Terry et al., 2004a and b). The thrips pollinated species are represented in both section *Parazamia* and section *Macrozamia* (Forster, 2004) indicating that the pollination syndrome in *Macrozamia* is not conserved within a group.

It is not yet known whether cone odours in other cycad groups reflect adaptation to specific pollinators or the extent to which cone odours are conserved within cycad lineages. *Encephalartos altensteinii* and *E. natalensis* which are phylogenetically related and have contiguous distributions (Treutlein et al., 2005), both emit (3*E*)-1,3-octadiene as a major compound (Pellmyr et al., 1991; Suinyuy et al., 2010). Because they emit similar odour compounds and occur in close proximity, they may be expected to attract the same insects. However, they are visited by distinct *Porthetes* species (Downie et al., 2008), suggesting that other compounds may play a role in attraction. However, these studies were conducted on very few species and broader comparative studies are required to determine the extent of variation in odour

chemistry between cycad species and to better understand the role of cone odour in specificity of pollination systems.

There are 37 *Encephalartos* species in South Africa, spanning the coastal and inland areas of the Eastern Cape in the south up to Limpopo province in the north (Norstog and Nichols, 1997; Donaldson, 2003; Hill et al., 2007). Some species occur across a relatively wide range of several hundred kilometers (e.g. *E. altenstenii*, *E. natalensis*, and *E. villosus;* Vorster, 2004) and may occur sympatrically with other species in parts of their range where the potential for hybridization exists and where cues for pollinators may need to be more species specific. One of the most widely distributed species is *E. villosus.* This species is of particular interest because its range overlaps with those of other species, allowing tests of whether scent chemistry is divergent or convergent between species that occur in the same geographical range. Its distribution also makes it an ideal species to examine how interaction with pollinators across its range may have been influenced by either shifts in pollinator interactions or coevolution with specific pollinators.

Surveys of insects on *Encephalartos* indicate that interactions with known pollinator groups range from species specific relationships to more general interactions (Oberprieler, 1995; Downie et al., 2008). The majority of *Porthetes* spp. (Curculionoidea) are host specific or occur on a few closely related species of *Encephalartos* (Downie et al. 2008) whereas species of Erotylidae and *Metacucujus* (Boganiidae) have broader host ranges. If species specific interactions are dependent on specific odour cues, one expectation might be that closely related *Encephalartos* would emit similar odour compounds to attract closely related insects. Closely related host taxa (Ehrlich and Raven, 1964) and chemically similar host taxa (Beccera, 1997) are often colonised by related insect herbivores. The study of odour chemistry in *Encephalartos* therefore provides an opportunity to examine the relationships between different species in relation to their

pollination biology. Although the overall phylogeny of *Encephalartos* is not well-resolved (Treutlein et al., 2005) and precludes detailed analysis of co-evolutionary interactions between plants and pollinators (Downie et al., 2008), there are several well defined clades within *Encephalartos* (Treutlein et al., 2005) and it is therefore possible to study the diversity of cone volatiles and insect pollinators within these clades.

Objectives of the study

The objective of this study was to investigate cone volatiles in *Encephalartos* in order to get a better understanding of their role in pollinator interactions, and to better understand the interaction between cone volatiles and thermogenesis in the pollination of *Encephalartos* cycads. To achieve this, the first step was to test for push-pull interactions in *Encephalartos* by undertaking detailed studies of the chemical ecology of *E. villosus.* Second, to examine variation in scent chemistry within *E. villosus* and across a subset of *Encephalartos* species in order to better understand whether cone odour composition is consistent across the range of a species, whether it has been conserved in specific lineages, and, how shifts in odour composition might be associated with shifts in pollinators.

The study focused on *Encephalartos* for the following reasons:

1. *Encephalartos* cones are usually aromatic and/ or thermogenic at some point in their development (e.g. Jacot-Guillarmod, 1958; Tang, 1987b; Pellmyr et al., 1991; Tang, 1993; Donaldson, 1997; Suinyuy et al., 2010). Insects have been found on male cones mainly at pollen shed when they are thermogenic and/ or emitting odour (Donaldson, 1997; Suinyuy et al., 2010) suggesting that pollinator interactions are influenced by cone odour and thermogenesis.

2. A wide variety of insect species are associated with cones of *Encephalartos* (e.g. Oberprieler, 1995, 2004; Donaldson, 1999). Some are seed parasites (e.g. *Antliarhinus*, Donaldson, 1993a, 1997) and some are involved in pollination, e.g. species of Erotylidae, *Metacucujus* and *Porthetes* (Donaldson et al., 1995; Donaldson, 1997; Suinyuy et al., 2009). This provides a range of interactions with which to test ideas relating to the role of cone volatiles.

3. *Encephalartos* is the second largest extant cycad genus, with over 65 species restricted to Sub-Saharan Africa (Donaldson, 2003). Studies focusing on comparative biology of reproductive systems in this genus might therefore shed light on the diversification of cycads and the interactions that are required for their survival. Most *Encephalartos* species occur in small populations and are threatened with extinction (Donaldson, 2003) and pollinator decline may further endanger their survival (Donaldson, 1999). Existing studies of their pollination systems show they involve obligate mutualisms but it is not clear what factors influence these mutualisms and to what extent factors such as cone odour may be used to enhance pollination of highly threatened taxa. Conservation efforts require information that enhances the welfare of the host plant and that of their pollinators (Spira, 2001).

Encephalartos villosus was used as a model species for detailed studies of scent-mediated cone-insect interactions and geographical variation in scent chemistry. It is the most widespread species of *Encephalartos* in South Africa and grows in the coastal and inland areas of the Eastern Cape, KwaZulu Natal, right up to Swaziland. It is one of the more abundant species in the region so that experiments wont lead to further threat and this also means that cones are more readily available for study. Further, *E. villosus* is a variable species both in foliage and cone characteristics (Goode, 1989) and visited by a variety of insect species so that it is possible to test a range of hypotheses relating to cycad-pollinator interactions.

Finally, the basic pollination ecology of *E. villosus* has been studied (Donaldson, 1997) and it is known to have aromatic and thermogenic cones associated with insect pollination.

The work is presented in five chapters.

Chapter 2 focuses on daily and life cycle patterns in odour emissions and heating in cones of *E. villosus* and their implications for the occurrence of push-pull pollinator interactions. The aim was to determine whether the patterns in odour emission and heating were consistent either with the daily push pull variations observed by Terry et al. (2007) for *M. lucida* or with any of the life cycle related push-pull models suggested by Donaldson (2007).

Chapter 3 examines variation in cone odours of *E. villosus* across its distribution from the southernmost populations in the Eastern Cape (EC) to populations in the north of the KwaZulu Natal (KZN) province of South Africa. The aim was to determine if there was variation in cone volatiles between populations and the implications of this variation for pollinator interactions. The chapter presents data from 10 different populations over a linear range of ca. 900 km.

Chapter 4 presents data on the physiological and behavioural responses of cycad insects to cone volatiles emitted by *E. villosus*. The aim was to determine if cone volatiles consistently attracted insects as expected for the 'pollinator attraction' function for cone volatiles. Also of importance was to determine whether there was any variation in insect response in the different populations. The study combined the use of gas chromatography-electroantennogram detection (GC-EAD), olfactometer experiments, and baited traps to test insect responses.

Chapter 5 represents a study of cone odour variation within the genus *Encephalartos,* with the aim to determine if volatile odour emissions reflect phylogenetic relationships and to establish if there is any

relationship between cone odour chemistry and currently known pollinators. This chapter considers the alternative possibility that similarity in odour composition among unrelated species in the same geographical region reflects convergent evolution driven by adaptation to functionally similar pollinators.

Chapter 6 summarises the results and ideas presented in the thesis and discusses the main findings in the context of what is currently known about cycad pollinator interactions and more general theories regarding floral volatiles and insect pollination.

Finally, two related studies which stemmed from data collected during this thesis are included as appendices.

Chapter 2. Ontogenetic patterns of odour emission and heating in cones of the African cycad *Encephalartos villosus* (Zamiaceae) and its implications for push-pull pollinator interactions

Abstract

Ontogenetic patterns of odour emissions and heating associated with plant reproductive structures may have profound effects on insect behaviour, and consequently on pollination. In particular, emission of specific odour compounds and changes in temperature at different developmental stages may attract or repel insect pollinators in a complex push-pull interaction. The chemical composition and pattern of cone odour emissions and heating of the African cycad *Encephalartos villosus* in the Eastern Cape (EC) and KwaZulu Natal (KZN) of South Africa was investigated using a gas chromatography-mass spectrometer (GC-MS) and miniature data loggers (ibuttons). The abundance of putative pollinators was also investigated in relation to daily and developmental stages of cone odour and heating: for male cones at pre-dehiscent, dehiscent, and post dehiscent stages and for female cones at pre-receptive, receptive and post receptive stages. The relative amounts of compounds in cone odours varied according to developmental stage but did not vary significantly on a daily cycle. The dominant compounds from EC populations were eucalyptol and 2-isopropyl-3-methoxypyrazine whereas (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene were dominant in KZN populations. Heating in male cones was higher at pollen dehiscence than during pre-dehiscence and post dehiscence and showed a strong daily pattern. Daily heating episodes at dehiscence occurred from about 16h00 and reached a maximum at about 18h30 when temperature in several male cones reached an average of between 4.0 and 12.0 °C above ambient. Temperatures of female cones were lower than those of male cones at all life stages and daily heating episodes occurred from about 13h00 and reached a maximum at ca. 13h45 when it was on average between 0.9 and 3.0 °C

above ambient temperature. Insect species abundance on male cones at dehiscence was higher than at the other stages. Furthermore, insect abundance on dehiscent male cones was significantly higher ($P < 0.05$) on the cones in the afternoon than in the morning and evening. Overall, the analyses show strong variation in patterns of cone volatiles and heating at different developmental stages and strong daily patterns in cone heating. Changes in insect abundance during pollination indicate movement over daily and life cycle stages of cone development but do not represent the strong daily push-pull pattern observed in some *Macrozamia* cycads.

Introduction

Floral volatiles, which function as olfactory cues, have been identified as important signals for chemical communication between plants and animal pollinators across a range of plants including archaic angiosperms (Pellmyr and Thien, 1986) and cycads (Pellmyr et al., 1991; Tang, 1993). These olfactory signals are usually characterised primarily by fatty acids derivatives, benzenoids and terpenoids (Thien et al., 2000; Raguso, 2004, 2008; Knudsen et al., 2006). Pellmyr and Thien (1986) noted that some chemical attractants were derived from known herbivore deterrents, which led them to suggest that olfactory attractants could have evolved from herbivore deterrents in circumstances where the interaction could confer a net benefit to the plant because the herbivore also functions as a pollinator.

The chemical compounds found in volatiles of archaic angiosperms are also emitted by cycad reproductive cones (e.g. Pellmyr et al., 1991; Tang, 1993). Generally, cycads are a group of plants where known pollinators are herbivores that feed on cycad cone tissues and where cone tissues serve as larval brood sites (Norstog et al., 1986; Tang, 1987a; Donaldson, 1997; Hall et al., 2004; Terry et al., 2005).

Nursery pollination in cycads seems to have evolved from cycad-herbivore interactions (Donaldson, 1992, 1993a; Oberprieler, 1995). Chemical attraction of cycad pollinators may have arisen from the fact that cycad herbivores have been attracted to specific cues associated with cycad reproductive structures during pollen shed before the evolution of pollinator mutualisms (Donaldson, 2007).

All plant reproductive structures undergo metabolic biochemical activity with heat as a product of these reactions (Seymour and Schultz-Motel, 1997), but thermogenic plants produce large amounts of heat that is not simply a by-product of regular metabolic activity (Seymour and Shultz-Motel, 1997). Periods of heat production normally correspond with the release of volatile compounds and insect activity in receptive female flowers of angiosperms (Seymour and Shultz-Motel, 1997, 1999; Seymour, 1999; Gibernau and Barabé, 2000; Seymour and Baylock, 2000; Seymour and Matthews, 2006) and has been shown to occur in dehiscing male cones of cycads (e.g. Tang, 1987b; Tang, 1993; Seymour et al., 2004; Suinyuy et al., 2010). Heat production is considered to enhance production and volatilization of volatile compounds which attracts insects (Tang, 1993; Seymour and Schultz-Motel, 1997, 1999; Ervik and Barfod, 1999; Seymour and Matthews, 2006).

Until recently, interactions of floral volatiles and heat production were mostly studied with the assumption they function solely to attract pollinators to inflorescences. Terry et al. (2004a) observed that insect pollinators leave male cones of several Australian cycads (*Macrozamia lucida*, *M. machinii*, and *M. macleayi*) during periods of peak volatile emission, which also coincide with peaks in cone temperature as a result of thermogenesis. These results suggested that insect pollinators tend to leave male cones due to heating and the associated increase in metabolic activity and an increase in the release of repellent compounds. A series of olfactometer experiments showed that *Cycadothrips chadwickii* was attracted to *M. lucida* sporophylls early in the day, repelled by sporophyll odours at midday, and attracted to them again in

the late afternoon to early evening (Terry et al., 2007a and b). This behaviour corresponded to daily field observations which showed that insects were present in male cones in the morning when volatile emissions and cone temperature were low, left male cones at midday when volatile emissions and cone temperature were high, and were attracted later in the day when cone emissions and temperature were low (e.g. Terry et al., 2004a). Terry et al. (2007a and b) referred to this system as a 'push-pull' pollination strategy.

Cone volatiles in the African cycad genus *Encephalartos* have been linked to the attraction of pollinators since Pearson's (1906) and Rattray's (1913) interpretations that weevils, such as *Porthetes* spp. (Coleoptera: Curculionidae: Molytinae, initially identified as *Phlaeophagus*), were possible pollinators of *Encephalartos villosus* and *E. altensteinii*. In a detailed investigation of the pollination biology of *E. villosus*, Donaldson (1997) showed that peak temperatures in the evenings in dehiscing male cones coincided with the presence of insects. The simultaneous production of cone volatiles, heat and occurrence of insects on cones, strongly suggests that these physiological phenomena are important signals in regulating insect behaviour.

The pollination period in male and female cones of *Encephalartos* is characterised by fluctuating daily emissions of odour and/ or heat production (Tang, 1987b, 1993; Donaldson, 1997; Suinyuy et al., 2010). During early stages of development, male cones of *Encephalartos* spp. have tightly packed sporophylls that separate a few days before pollination. At the time of pollination, male and female cones may emit similar volatile compounds and male cones typically undergo periods of heating. These changes in the cone coincide with more frequent insect visitation on male cones (e.g. Suinyuy et al., 2010). These observations suggest that volatile emissions are linked to insect behaviour and function in attracting insect pollinators (Tang, 1993).

It has also been suggested that cone heating may enhance release of attractive odours and increase insect movement between cones (Tang, 1993). Despite these suggestions, no studies have explored the roles of cone volatiles and heat production in *Encephalartos* pollination and it is not known whether the push-pull pollination strategy is a general phenomenon in cycads or is confined to Australian *Macrozamia* cycads.

Several possible models of push-pull mechanisms in cycads have been proposed. In the best studied example, push and pull aspects of the cycad-insect interaction are caused by regular daily changes in levels of volatile emissions and heat production (Terry et al., 2004a, 2007a and b) so that periods of attraction alternate with periods of repellence. Donaldson (2007) proposed several alternative models, including mixed models (Table 2.1), based on theoretical considerations of cycad coning behaviour including push-pull interactions associated with changes in volatile emissions and heat production over the developmental cycle of cones, especially male cones which transform from a pre-dehiscent state to dehiscence and subsequent drying or decomposition over a period of two to three weeks. In the mixed models, the prediction is that cone volatiles and /or thermogenesis will actively attract pollinators to male or female cones at certain stages of cone development or some period during the day and repel them at other stages or period of day. There is a need for detailed studies of patterns of cone volatile emission and heat production over the period of pollination in different cycad taxa to determine whether they are consistent with any of the proposed models of push-pull interactions.

Table 2.1: Combination of possible influences of cone volatiles and /or thermogenesis on cycad pollinator behaviour (adapted from Donaldson, 2007).

Male cone		Female cone	
Attraction	**Dispersal**	**Attraction**	**Dispersal**
Active[a]	Active	Active	Active
Active	Active	Active	Passive
Active	Active	Passive	Passive
Active	Passive	Active	Active
Active	Active	Passive	Active
Active	Passive	Passive	Active
Passive[b]	Active	Active	Active
Passive	Active	Active	Passive
Passive	Passive	Active	Active

[a]Active implies cone volatiles and /or thermogenesis influence pollinator behaviour; [b]Passive implies cone volatiles and /or thermogenesis has no influence on pollinator behaviour.

The main aim of this study was to examine patterns of cone odour emissions and heating in both male and female *E. villosus* to determine whether these patterns are consistent with daily push-pull interactions such as those observed in *Macrozamia* cycads (Terry et al., 2004a, 2007a and b) or with the mixed models proposed by Donaldson (2007). This was achieved by measuring odour emissions, heating and insect abundance daily over the lifespan of both male and female cones.

Materials and Methods

Study area

Populations of *E. villosus* occur mainly in patches of Scarp Forest and Northern Coastal Forest (Mucina and Geldenhuys, 2006). The forest patches are imbedded in elements of the Indian Ocean Coastal Belt Biome (Mucina et al., 2006), specifically the KwaZulu-Natal Coastal Belt, Pondoland-Ugu Sandstone Coastal Sourveld, and Transkei Coastal Belt. The study initially focused on populations from Mount Sullivan (MTS) and Ocean View Guest Farm (OVGF), occurring in forests in the Transkei Coastal Belt vegetation type of the Eastern Cape Province (EC), and additional populations in the Kranzkloof Nature Reserve (KKNR) in the KwaZulu-Natal (KZN) Coastal Belt vegetation type. The study also included cultivated plants from Kirstenbosch Botanic Garden in Cape Town, using plants derived from areas in EC, and the Pietermaritzburg campus of the University of KwaZulu Natal with plants derived from unknown localities in KZN (Figure 2.1). Male and female cones of *E. villosus* are produced between the months of January and February. The male cone extends and the sporophylls become separated around March and April when pollen is shed. After pollination, the male cone disintegrates but the female cone remains on the plants until they mature and eventually disintegrate between September and November.

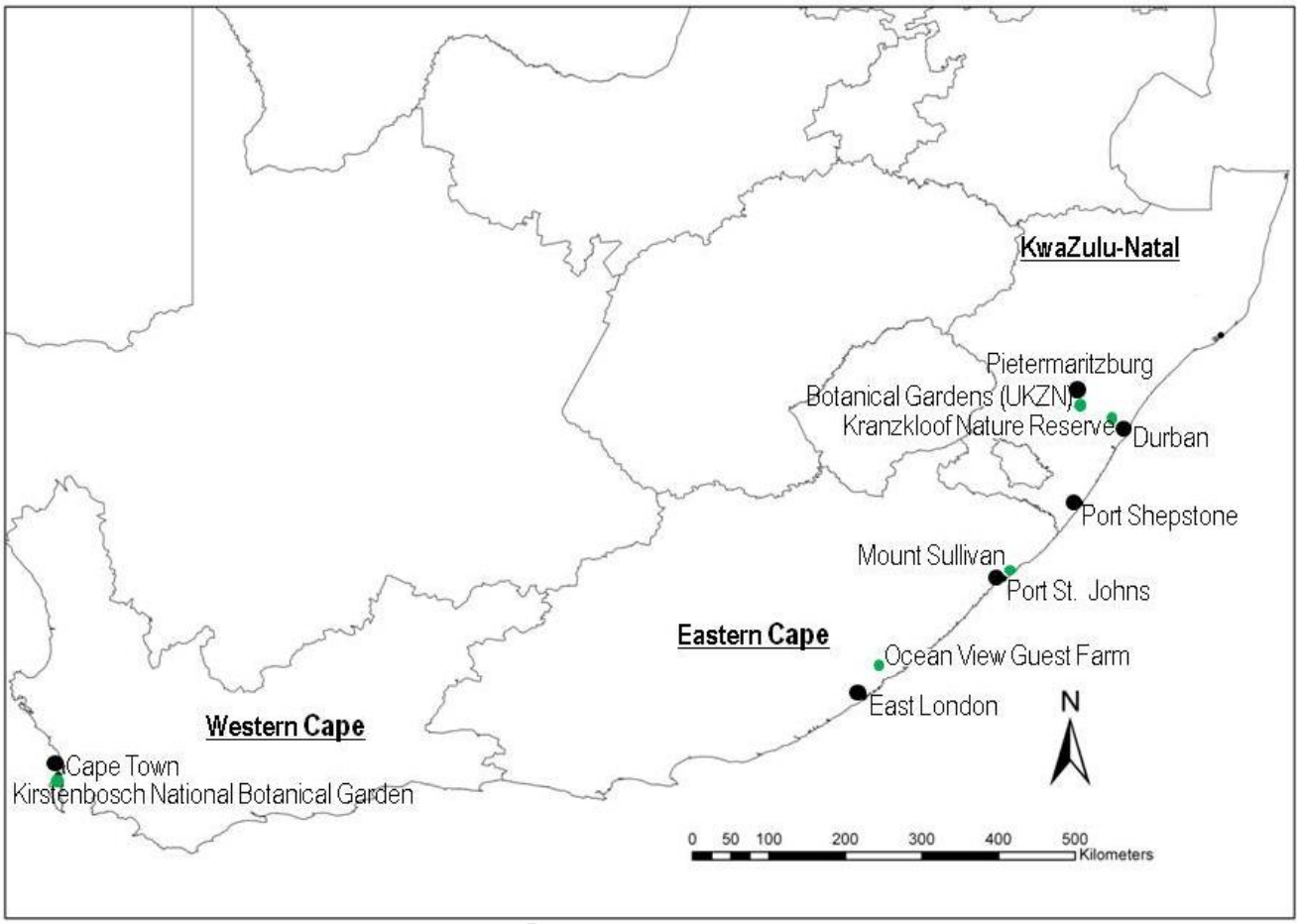

Figure 2.1: Map of South Africa indicating the localities (sites marked in green colour) where studies were conducted.

Developmental stages of *E.* villosus cones

The developmental stages were determined through observation of the cones, starting several weeks prior to pollination. Male cones were classified as pre-dehiscent until the period when the cone elongated and the sporophylls separated (Figure 2.2A); as dehiscent when the cone elongated and there was clear separation between the sporophylls and the microsporangia released pollen (Figure 2.2B); and post dehiscent when the microsporangia ceased shedding pollen, and which was typically when the cone also started to disintegrate (Figure 2.2C).

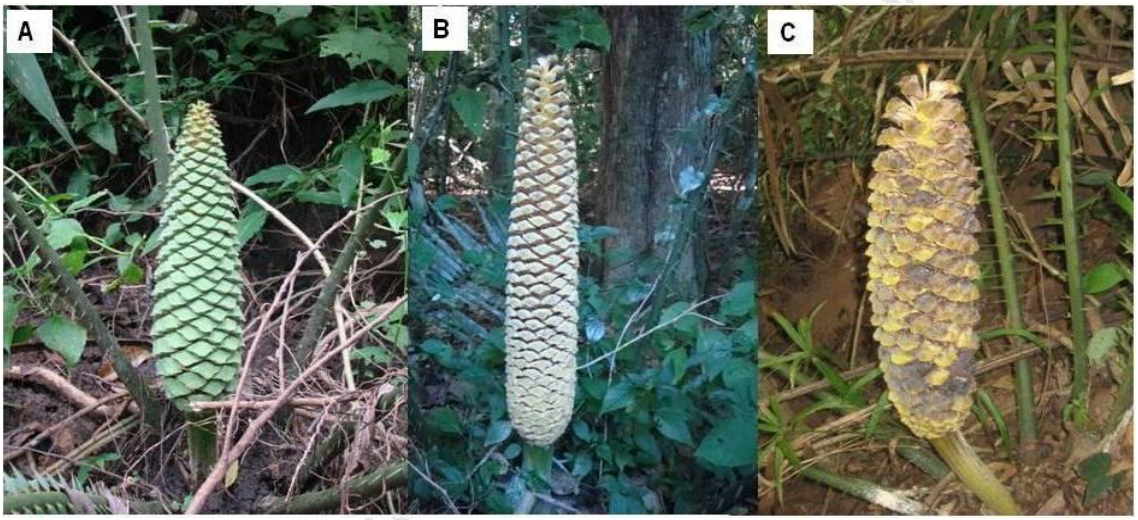

Figure 2.2: Developmental stages in male cones of *E. villosus*; **A**) pre-dehiscent; **B**) dehiscent; **C**) post dehiscent

Female cones were classified as pre-receptive until the cone had reached its full size and the sporophylls on the top half of the cone had separated to reveal small gaps; receptive when the sporophylls were separated; and post receptive when the sporophylls were again tightly closed. At each key developmental stage, cones were sampled for 30 minutes in the morning (09h00 – 11h00),

afternoon (13h30 – 15h30), and evening (17h00 – 19h00). The number of replicates depended on the availability of cones. In the EC, odour samples were collected from five male and four female plants at each developmental stage for each time period (morning, afternoon and evening). In KZN, odour samples were obtained from nine pre-dehiscent male cones, 11 dehiscent and post dehiscent male cones, four pre-receptive female cones, five receptive female cones and three post receptive female cones.

Volatile odour emission

Headspace sampling was used to collect volatiles from male and female cones of *E. villosus*. Polyacetate bags (Nalo Bratfolie Kalle GmbH - Germany) were placed over the entire cone just prior to sampling in order to concentrate the volatile compounds. Air from inside the bags was suctioned for 30 minutes into a chromatoprobe trap using a portable battery operated pump (Spectrex Personal Air Sampler PAS 500, USA) calibrated at 200 ml/ min. Air samples were simultaneously collected from empty polyacetate bags placed way from the plant as controls to identify background contamination. The chromatoprobe trap samples were stored at -20 °C in a sealed vial until analysis.

Chromatoprobe traps were prepared by cutting glass tubes equalling the size of chromatoProbe quartz microvials (length: 15 mm; inner diameter: 2mm). They were then filled with 2mg of a 50:50 mixture of Tenax TA® (Alltech Associates, USA) and activated charcoal (Carbotrap™, Supelco, USA) and closed on both ends with glass wool. The activated charcoal is highly retentive and small quantities can be used due to its high adsorbing capacity (Millar & Sims, 1998; Tholl & Röse, 2006) thus allowing for longer sampling time at a higher flow rate. The adsorbent Tenax TA used in the study is commonly used to trap volatile compounds and has a high thermal stability up to 350 ^{o}C, which allows for thermal desorption in GC analysis (Tholl and Rose, 2006).Before sampling, test runs were conducted for

different time intervals (5, 10, 20 and 30 minutes) at different cone stages to determine the correct sampling time. Gas chromatography-mass spectrometry analyses showed that volatile compounds could be detected mostly from traps that were sampled for 30 minutes. Solvent elution was not a viable option as the solvent masked most of the highly volatile compounds that occur between 5-10 minutes.

Chemical analysis and compound identification

Volatile samples were analysed using a coupled Varian 3800 gas GC (Varian Palo Alto, California, USA) and Varian 1200 mass spectrometer (GC-MS). The GC was equipped with a Carbowax column (DB-wax) of 30m x 0.32 mm internal diameter x 0.25 µm film thickness (Alltech, Deerfield, Illinois, USA). Helium was used as the carrier gas at a flow rate of 1 mL/min. After sampling, traps were placed in a Varian 1079 injector by means of a 'Chromatoprobe' fitting and thermally desorbed. After a 3 min hold at 40 °C, the GC oven was ramped up to 240 °C at 10 °C/min and held there for 12 min. Compound identification was carried out using the NIST05 mass spectral library and comparisons with retention times of chemical standards, where available, as well as comparisons between calculated Kovats retention indices and those published in the literature. A homologous series of alkanes (C8-C20) was used to determine Kovats retention indices. All reference compounds used for retention time comparisons were obtained from Sigma Aldrich Inc. GmbH, Germany, except (3*E*)-1,3-octadiene which was obtained from ChemSampco, USA. Compounds present at higher or similar percentages in controls were considered as contaminants and excluded from the analysis. For quantification of emission rates, known amounts of standards were injected into cartridges and thermally desorbed under identical conditions to the samples.

Temperature measurements

Temperatures of male and female cones of *E. villosus* were monitored *in situ* for each key developmental stage during two reproductive events (2008, 2009). Temperatures were measured using thermocron ibuttons (Fairbridge technology South Africa) set to record the temperature at 10 minutes intervals over at least a 24h period. At the Kirstenbosch Botanic Garden, temperatures were measured for two male and female cones respectively throughout their life cycle during the first reproductive event. During the second reproductive event, cone temperatures were measured for plants growing at the Ocean View Guest Farm (four male cones and two female cones), Mount Sullivan (seven male cones and one female cone), Kranzkloof Nature Reserve (six male cones and four female cones) and the Botanic Garden of the University of KwaZulu Natal, Pietermaritzburg campus (3 male cones and two female cones). Two ibuttons were used for each cone, one inserted between the cone sporophylls to measure cone temperature and the other placed between the leaves of the same cycad to measure ambient temperature. Both ibuttons were positioned in such a way as to avoid direct exposure to sunlight. Thermochron ibuttons have many advantages over other temperature data-logging systems which include, their cost, small size, wireless nature, self-sufficiency (sensor, logger and power unit), long life span and applicability to a vast range of environmental research needs (Hubbart et al., 2005; Hubbart, 2011).

Insect visitors to male and female cones of *E. villosus*

Insects present on male and female cones of *E. villosus* were sampled during all development stages until after pollination. They were sampled at approximately the same times as odour sampling in the morning (09h00 – 11h00), afternoon (13h30 – 15h30) and evening (17h00 – 19h00). The insects were collected from male cones by shaking the cones over a beating sheet. Five male cones were

surveyed for each key developmental stage and each time of day in both EC and KZN. Sampling of insects on female cones was restricted to insects crawling on the surface of the cones as these could be collected without damaging the cone. Insects were recorded on five female cones.

Statistical analysis

The Primer 6 multivariate statistics programme (Clarke and Gorley, 2006) was used to assess the variation in volatiles of *E. villosus* from different developmental stages and times of day. Relative amounts of different compounds in each sample were used for analyses. Non-metric multidimensional scaling (NMDS), based on Bray-Curtis similarities of square root transformed data, was used to detect similarities among samples. The stress value is given to indicate how well the distance matrix is reproduced. The smaller the stress value, the better the fit of the ordination to the reproduced distance matrix (Clarke, 1993). The significant differences in odour samples between developmental stages and between times of the day were assessed by analysis of similarities (ANOSIM) in a two-way crossed layout design (with replicates) (factors: developmental stage; time of day) with 10000 permutations. The ANOSIM calculates the test statistic R as well as the level of significance. The test statistic R is a relative measure of separation between defined groups, based on mean ranks between and within groups. It ranges between 0 and 1. An R-value of zero means no separation, while 1 indicates separation of the groups (Clarke and Gorley, 2006). Separate similarity percentage (one-way SIMPER) analyses were used in Primer 6 to identify the compounds that best explained similarities within and between developmental stages and between times of day from the overall transformed data.

To test for significant differences in the rates of emission of compounds for different cone stages and times of day, data were analysed using Analysis of Variance (ANOVA) in STATISTICA version 7. The first analysis tested the overall differences in the rate of emission of all compounds for different

cone stages and times of day and the second analysis tested differences in rates of emission of individual dominant compounds across all the stages. This was followed by Tukey's Honest Significant Difference (Tukey HSD) method of pair-wise multi-comparison (Zar, 1984). The means are marginal means which takes into account other factors in the model and may lead to equal standard errors.

To test whether there were significant differences among the total number of insects associated with cones at different developmental stages and times of day, data were analysed using the Kruskal-Wallis test (because data were not normally distributed), followed by Tukey's Honest Significant Difference (Tukey HSD) method for pair-wise multi-comparisons (Zar, 1984).

Results

Daily and life-cycle patterns in volatile emissions

Initial analysis of the chemical composition of odours from male and female cones of *E. villosus* populations showed differences between EC and KZN populations. The data for cone odours during different developmental stages are therefore presented separately for EC and KZN in Tables 2.2 and 2.3. The compounds were identified by common names and CAS (Chemical Abstract Service) registry numbers and listed according to estimated Kovats Retention Indices (KRI). They are listed according to classes, which to some degree reflect their biosynthetic origin (Knudsen et al., 2006).

Volatile samples from EC populations

In total, 64 compounds were found in populations from the EC out of which 63 were identified (Table 2.2). The terpenoids were the most numerous compounds (36 identified, as well as one unknown monoterpene and three sesquiterpenes), followed by seven benzenoids, 16 fatty acid

derivatives or aliphatics (six alcohols, five aldehydes, and three aliphatic acids, one ketone, one lactone), and one nitrogen-containing compound. The male cones emitted 50 compounds (30 at pre-dehiscence, 37 at dehiscence, and 32 at post dehiscence stages) whereas the female cones emitted 48 compounds (35 at pre-receptive, 41 at receptive, and 25 at post receptive stages). Sixteen compounds were specific to male cones of which five (3-octanone, 3-octanol, *trans*-Linalool oxide (Furanoid), *cis*-linalool oxide (pyranoid), and borneol) were emitted only at pre-dehiscence; three (2,4-octadienal, *α*-terpinolene and verbenone) only at dehiscence, and two (*trans*-*β*-ocimene and *cis*-*β*-ocimene) only at post dehiscence. The remaining six compounds were recorded from dehiscent and post dehiscent stages. Fourteen compounds were specific to female cones with four of them (1-octen-3-ol, (*E*)-2-decenal, *cis*-linalool oxide (furanoid), and pinocarvone) occurring only at the pre-receptive stage, four others (nonanol, *γ*-butyrolactone, guaiacol, and *α*-curcumene) only at the receptive stage and the remaining six compounds at two or more stages. Out of 64 compounds, 11 compounds occurred in all developmental stages in male and female cones, namely benzaldehyde, *p*-anisaldehyde, *α*-pinene, *β*-pinene, eucalyptol, *α*-irone, 2-isopropyl-3-methoxypyrazine, phenol, camphor, linalool and *α*-terpineol. Other compounds which occurred in high relative amount but did not occur in all the developmental stages were camphene, *α*-terpinene and cymene, heptanal, ethanoic acid, 3-methyl-1-butanol and 1-octen-3-ol.

One-way SIMPER (factor developmental stage) showed that samples from pre-dehiscent male cones were characterised by high relative amounts of benzaldehyde, *β*-pinene, phenol, *α*-irone and eucalyptol explaining more than 79 % of the similarity among samples from this stage. At dehiscence, eucalyptol, *β*-pinene and 2-isopropyl-3-methoxypyrazine explained more than 80 % of the similarity among samples. The post dehiscent stage was characterised by high relative amounts of eucalyptol, 2-isopropyl-3-methoxypyrazine, *β*-pinene, camphene, *α*-pinene, phenol and *α*-terpineol explaining more than 83 % of the similarity. In the female cones, the pre-receptive stage was characterised by high

relative amounts of eucalyptol, phenol, *β*-pinene, benzaldehyde and 2-isopropyl-3-methoxypyrazine explaining more than 82 % of the similarity. At the receptive stage, eucalyptol, 2-isopropyl-3-methoxypyrazine, phenol, *α*-pinene and benzaldehyde explained more than 83 % of the similarity. The post receptive stage was characterised by high relative amounts of eucalyptol, *α*-pinene, *β*-pinene, and 2-isopropyl-3-methoxypyrazine explaining more than 90 % of the similarity. One-way SIMPER, using time of the day as a factor, showed that across all stages, the samples in the morning afternoon and evening were characterised by high relative amounts of eucalyptol, 2-isopropyl-3-methoxypyrazine, benzaldehyde, phenol, *α*-pinene and *β*-pinene accounting for more than 80, 85 and 77 % of the similarity at the different times of the day.

A Bray-Curtis NMDS analysis of odours from EC populations, based on the relative amount of volatile compounds in each sample (Figure 2.3) using a two-way cross design (with replicates) showed a weak but significant separation between developmental stages in male and female cones combined (NMDS stress value 0.17; ANOSIM, R (developmental stage) = 0.173, P = 0.03), but showed no significant separation of samples relating to time of day (ANOSIM, R (time) = -0.082, $P > 0.05$). The significant difference in developmental stage was accounted for by the separation between pre-dehiscent and dehiscent stages in male cones (R = 0.541, $P < 0.01$). The dehiscent male cone samples were in one cluster characterised by the dominance of 2-isopropyl-3-methoxypyrazine and eucalyptol (Figure 2.3). This cluster also included about half the samples of the receptive female cones, as well as nearly half the samples of post dehiscent male and post receptive female cones. Almost all the pre-dehiscent male cone samples and pre-receptive female cones clustered together characterised by *β*-pinene and *p*-anisaldehyde and benzaldheyde (Figure 2.3). This cluster also included some post dehiscent and one dehiscent male cone

Table 2.2: Average relative amounts (%) of odour compounds emitted by male and female cones of *E. villosus* in the Eastern Cape before, during and after pollination in the morning (M), afternoon (A) and evening (E). Compounds are identified by common names and CAS (Chemical Abstract Service) registry number, and listed according to estimated Kovats retention index (KRI) within each compound class. Values are mean percentages of the total peak area. The number of samples in which the compound was identified is given in parentheses.

Cone sex			Male cones									Female cones								
Cone stage			Pre-dehiscent			Dehiscent			Post-dehiscent			Pre-receptive			Receptive			Post-receptive		
Time of day			M	A	E	M	A	E	M	A	E	M	A	E	M	A	E	M	A	E
Number of samples			5	5	5	5	5	5	5	5	5	4	4	4	5	4	4	4	4	4
Number of compounds			25	26	23	31	34	35	28	27	28	25	18	32	40	33	35	24	21	24
Emission rate (ng/cone/hr)			13.75	22.75	51.78	1688.82	2030.20	3698.08	6796.09	2410.37	7428.18	7.23	24.30	29.81	795.31	1842	1633.27	30.70	256.50	76.37
Compounds	CAS	KRI																		
ALIPHATICS																				
Aliphatic acids																				
Ethanoic acid[b]	64-19-7	1467	-	-	0.45 (1)	0.32 (1)	0.09(1)	0.04 (1)	-	-	-	1.87 (1)	-	8.17 (1)	5.88 (1)	-	0.47 (1)	-	-	-
Hexanoic acid[b]	142-62-1	1852	1.25 (1)	2.08 (1)	0.95 (1)	0.44 (1)	0.14 (1)	0.04 (1)	-	-	-	-	-	-	0.09 (1)	-	-	-	-	-
Enanthic acid[b]	111-14-8	1969	0.57 (1)	0.37 (1)	0.81 (1)	0.24(1)	0.48 (1)	0.01 (1)	-	-	-	2.29 (1)	-	0.91 (1)	0.73 (1)	0.81 (1)	0.48 (1)	-	-	-
Aldehydes																				
Hexanal[a]	66-25-1	1125	-	-	-	-	-	-	-	-	-	3.07 (1)	-	tr (1)	tr (1)	tr (1)	0.47 (1)	0.94 (2)	4.76 (1)	5.39 (1)
Heptanal[a]	111-71-7	1209	16.53 (2)	2.88 (1)	17.19 (2)	-	-	-	3.91 (1)	3.05 (2)	0.18 (1)	tr (2)	14.71 (2)	tr (2)	0.61 (2)	0.58 (1)	tr (2)	tr (1)	-	tr (1)
(*E*)-2-Nonenal[b]	18829-56-6	1545	-	-	-	-	-	0.03 (1)	-	-	-	-	-	tr (1)	0.05 (2)	0.12 (1)	0.06 (1)	-	-	tr (1)
2,4-Octadienal[b]	30361-28-5	1610	-	-	-	-	-	0.07 (1)	-	-	-	-	-	-	-	-	-	-	-	-
(*E*)-2-Decenal[b]	3913-81-3	1663	-	-	-	-	-	-	-	-	-	0.61 (1)	-	0.58 (1)	-	-	-	-	-	-
Ketones																				
3-Octanone[b]	106-68-3	1274	-	2.37 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2.2 continued

Cone sex			Male cones										Female cones								
Cone stage			Pre-dehiscent			Dehiscent			Post-dehiscent			Pre-receptive			Receptive			Post-receptive		
Time of day			M	A	E	M	A	E	M	A	E	M	A	E	M	A	E	M	A	E
Number of samples			5	5	5	5	5	5	5	5	5	4	4	4	5	4	4	4	4	4
Number of compounds			25	26	23	31	34	35	28	27	28	25	18	32	40	33	35	24	21	24
Emission rate (ng/cone/hr)			13.75	22.75	51.78	1688.82	2030.20	3698.08	6796.09	2410.37	7428.18	7.23	24.30	29.81	795.31	1842	1633.27	30.70	256.50	76.37
Compounds	CAS	KRI																		
Alcohols																				
2-Methylpropan-1-ol[b]	78-83-1	1127	-	-	-	-	0.14(1)	-	-	-	-	-	-	-	4.17 (1)	3.75 (1)	0.51 (1)	3.83 (1)	4.82 (1)	4.25 (1)
3-Methyl-1-butanol[b]	123-51-3	1225	-	-	-	-	-	-	-	-	-	-	-	-	9.65 (1)	8.33 (1)	-	13.51 (1)	11.82 (1)	11.61 (1)
3-Octanol[b]	589-98-0	1386	1.04 (1)	3.37 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Octen-3-ol[a]	3391-86-4	1456	-	-	-	-	-	-	-	-	-	-	5.98 (3)	0.78(1)	-	-	-	-	-	-
1-Octanol[b]	111-87-5	1563	-	-	-	-	-	-	-	-	-	4.61 (1)	-	1.45 (1)	0.14 (1)	-	0.07 (1)	tr (1)	tr (1)	tr (1)
Nonanol[a]	143-08-8	1668	-	-	-	-	-	-	-	-	-	-	-	-	0.09 (1)	0.45 (1)	0.12 (1)	-	-	-
Lactones																				
γ-Butyrolactone[b]	96-48-0	1659	-	-	-	-	-	-	-	-	-	-	-	-	0.03 (1)	-	-	-	-	-
BENZENOIDS																				
Benzaldehyde[a]	100-52-7	1553	13.61 (5)	15.26 (5)	10.57(5)	0.65 (5)	0.26 (5)	0.16 (5)	12.16 (4)	1.49 (5)	0.54 (3)	7.43 (3)	11.04 (4)	7.82 (3)	11.65 (4)	3.40 (4)	0.57 (4)	2.32 (4)	1.15 (4)	1.07 (4)
Methyl benzoate[a]	93-58-3	1638	0.06 (1)	0.72 (2)	-	-	-	-	0.72 (2)	0.06 (2)	0.01 (1)	-	0.50 (3)	0.13 (1)	-	-	-	-	-	-
Methyl salicylate[a]	119-36-8	1808	-	-	-	-	-	-	-	-	-	tr (1)	-	-	tr (1)	-	-	0.11 (1)	0.01 (1)	0.05 (1)
Guaiacol[b]	90-05-1	1888	-	-	-	-	-	-	-	-	-	-	-	-	0.01 (1)	-	-	-	-	-
Benzyl alcohol[a]	100-51-6	1896	0.99 (2)	1.86 (2)	2.56 (2)	-	-	-	0.39 (1)	0.28 (2)	0.03 (1)	-	4.59 (3)	3.81 (1)	0.01 (1)	-	-	1.46 (1)	tr (1)	0.63 (1)
Phenol[a]	108-95-2	2032	2.89 (4)	2.96 (5)	2.21 (5)	0.1.13 (5)	0.20 (5)	0.26 (5)	2.00 (5)	0.33 (5)	1.38 (5)	5.34 (4)	12.29 (4)	2.81 (4)	13.31 (5)	27.62 (1)	0.72 (4)	1.69 (4)	0.81 (1)	0.84 (1)
p-Anisaldehyde[a]	123-11-5	2061	12.64 (4)	2.08 (4)	8.07 (4)	0.99 (5)	0.52 (5)	0.24 (5)	6.35 (5)	18.06 (5)	0.23 (5)	-	1.65 (3)	0.40 (1)	tr (3)	tr (2)	tr (3)	tr (4)	tr (1)	0.07 (1)

Table 2.2 continued

Cone sex			Male cones									Female cones								
Cone stage			Pre-dehiscent			Dehiscent			Post-dehiscent			Pre-receptive			Receptive			Post-receptive		
Time of day			M	A	E	M	A	E	M	A	E	M	A	E	M	A	E	M	A	E
Number of samples			5	5	5	5	5	5	5	5	5	4	4	4	5	4	4	4	4	4
Number of compounds			25	26	23	31	34	35	28	27	28	25	18	32	40	33	35	24	21	24
Emission rate (ng/cone/hr)			13.75	22.75	51.78	1688.82	2030.20	3698.08	6796.09	2410.37	7428.18	10.08	25.84	90.14	795.31	1842.0	1633.27	30.70	256.50	76.37
Compounds	CAS	KRI																		
TERPENOIDS																				
Monoterpenes																				
α-Pinene[a]	7785-70-8	1095	4.34 (3)	9.43 (3)	9.28 (3)	18.65 (5)	23.23 (5)	14.22 (5)	8.14 (5)	6.30 (5)	11.47 (4)	tr (2)	0.01 (1)	9.42 (2)	5.94 (3)	2.62 (2)	17.60 (3)	tr (4)	34.65 (4)	43.67 (4)
β-Thujene[a]	28634-89-1	1102	-	-	-	tr (1)	tr (1)	tr (1)	-	tr (2)	-	-	-	-	-	-	-	-	-	-
Camphene[a]	79-92-5	1112	-	-	-	8.09 (3)	5.91 (2)	6.23 (3)	0.57 (2)	4.43 (2)	tr (2)	-	-	-	0.43 (1)	0.98 (1)	0.65 (1)	10.31 (1)	3.25 (4)	1.68 (3)
β-Pinene[a]	127-91-3	1194	20.85 (5)	27.68 (4)	22.21 (4)	10.48 (4)	6.53 (4)	9.10 (4)	10.10 (3)	26.42 (5)	3.25 (5)	17.94 (2)	10.28 (2)	15.32 (2)	0.25 (2)	tr (1)	1.07 (2)	tr (2)	23.65 (4)	7.92 (4)
β-Myrcene[a]	123-35-3	1199	tr (1)	-	-	1.00 (3)	tr (3)	0.03 (3)	tr (4)	tr (3)	tr (5)	-	-	-	tr (2)	5.09 (1)	4.18 (2)	tr (1)	tr (2)	tr (2)
Unknown		1213	-	-	tr (1)	tr (1)	0.27 (3)	0.46 (3)	-	-	tr (1)	-	-	-	tr (2)	tr (1)	tr (2)	tr (1)	tr (1)	tr (1)
α-Terpinene[a]	99-86-5	1220	-	-	-	1.20 (5)	5.48 (5)	4.59 (5)	17.87 (3)	tr (3)	17.78 (4)	tr (1)	-	tr (1)	1.01 (3)	3.59 (2)	5.56 (3)	-	-	-
Limonene[a]	138-86-3	1224	-	tr (1)	tr (1)	-	-	-	tr (1)	tr (1)	tr (1)	2.66 (1)	2.92 (1)	10.31 (2)	12.65 (2)	7.44 (1)	tr	tr (1)	tr (4)	tr (4)
Eucalyptol[a]	470-82-6	1231	1.35 (2)	10.21 (2)	15.58(4)	45.37 (5)	48.24 (5)	42.52 (5)	25.60 (5)	22.45 (5)	29.67 (5)	33.66 (2)	15.98 (3)	34.31 (2)	34.49 (3)	20.45 (1)	37.51 (4)	50.07 (4)	11.40 (4)	21.37 (4)
trans-β-Ocimene[a]	502-99-8	1267	-	-	-	-	-	-	0.80 (2)	4.01 (1)	1.45 (2)	-	-	-	-	-	-	-	-	-
γ-Terpinene[b]	99-85-4	1269	-	-	-	0.04 (2)	0.15 (2)	1.06 (3)	3.55 (2)	1.38 (2)	18.78 (3)	-	-	-	-	-	-	-	-	-
cis-β-Ocimene[a]	3338-55-4	1275	-	-	-	-	-	-	-	tr (1)	-	-	-	-	-	-	-	-	-	-
p-Cymene[a]	99-87-6	1294	0.53 (1)	4.63 (1)	-	1.37 (1)	1.29 (1)	1.10 (1)	0.53 (1)	0.16 (1)	0.05 (1)	-	12.93 (3)	-	-	-	-	-	-	-
α-Terpinolene[a]	586-62-9	1304	-	-	-	-	tr (1)	tr (1)	-	-	-	-	-	-	-	-	-	-	-	-
trans-Linalool oxide (Furanoid)[a]	5989-33-3	1453	1.18 (1)	0.51 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2.2 continued

Cone sex			Male cones												Female cones					
Cone stage			Pre-dehiscent			Dehiscent			Post-dehiscent			Pre-receptive			Receptive			Post-receptive		
Time of day			M	A	E	M	A	E	M	A	E	M	A	E	M	A	E	M	A	E
Number of samples			5	5	5	5	5	5	5	5	5	4	4	4	5	4	4	4	4	4
Number of compounds			25	26	23	31	34	35	28	27	28	25	18	32	40	33	35	24	21	24
Emission rate (ng/cone/hr)			13.75	22.75	51.78	1688.82	2030.20	3698.08	6796.09	2410.37	7428.18	7.23	24.30	29.81	795.31	1842	1633.27	30.70	256.50	76.37
Compounds	CAS	KRI																		
Carveol[a]	99-48-9	1461	-	-	-	tr (1)	0.01 (1)	0.03 (1)	tr (1)	-	tr (1)	-	-	-	-	-	-	-	-	-
cis-Linalool oxide (Furanoid)[a]	34995-77-2	1467	-	-	-	-	-	-	-	-	-	-	0.45 (3)	0.07 (1)	-	-	-	-	-	-
α-Irone[b]	79-69-6	1535	14.81 (3)	9.02 (3)	1.22 (3)	1.05 (3)	0.04 (3)	0.16 (3)	0.35 (1)	0.10 (1)	0.19 (1)	tr (2)	-	tr (2)	tr (2)	tr (1)	tr (2)	tr (1)	tr (1)	tr (1)
trans-*Z*-*α*-bisabolene epoxide[c]		1537	-	-	-	-	tr (1)	-	-	-	-	tr (1)	-	0.44 (1)	0.12 (1)	0.16 (1)	0.11 (1)	-	-	-
Camphor[a]	464-48-2	1543	3.50 (2)	1.31 (2)	2.12 (2)	0.48 (3)	0.22 (3)	3.54 (3)	0.02 (1)	-	-	7.47 (1)	-	2.84 (2)	1.05 (2)	0.95 (1)	0.42 (2)	tr (1)	1.26 (1)	0.71 (1)
Linalool[a]	78-70-6	1562	0.83 (2)	0.52 (2)	0.67 (1)	0.05 (3)	tr (3)	0.08 (3)	0.21 (3)	0.20 (4)	0.07 (2)	3.70 (1)	2.32 (3)	0.64 (2)	0.34 (1)	0.47 (1)	0.19 (1)	tr (3)	-	-
cis-*p*-Menth-2-en-1-ol[c]	29803-82-5	1576	0.36 (1)	0.06 (1)	0.01 (1)	-	-	-	0.18 (2)	0.14 (2)	0.16 (1)	-	tr (2)	tr (1)	-	-	-	-	-	-
Pinocarvone[b]	30460-92-5	1597	-	-	-	-	-	-	-	-	-	-	-	0.31 (1)	-	-	-	-	-	-
4-Terpineol[a]	562-74-3	1613	-	-	-	0.10 (4)	0.30 (3)	0.05 (3)	0.21 (2)	0.13 (3)	0.02 (2)	-	tr (1)	-	tr (1)	tr (1)	0.01 (1)	-	-	-
Myrtenal[b]	564-94-3	1650	tr (1)	tr (1)	tr (1)	0.02 (1)	tr (1)	0.05 (1)	-	-	-	tr (1)	-	tr (1)	0.02 (1)	tr (1)	0.06 (1)	-	-	-
Menthol[a]	2216-51-5	1662	-	-	-	-	-	-	-	-	-	2.70 (1)	-	0.50 (1)	0.11 (1)	0.08 (1)	0.05 (1)	0.95 (1)	-	0.34 (1)
α-Terpineol[a]	10482-56-1	1720	0.12 (3)	0.58 (4)	0.57 (3)	0.42 9(5)	0.23 (5)	0.41 (5)	0.67 (5)	0.62 (5)	0.68 (4)	0.37 (2)	0.55 (1)	0.13 (3)	0.14 (3)	0.48 (2)	0.19 (3)	0.19 (1)	tr (1)	0.03 (1)
Borneol[a]	507-70-0	1725	0.05 (1)	0.08 (1)	0.43 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citral[b]	106-26-3	1732	-	-	-	-	-	0.01 (1)	-	0.10 (1)	-	-	-	-	-	-	-	-	-	-
cis-Linalool oxide (Pyranoid)[a]	5989-33-3	1755	0.17 (1)	0.10 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2.2 continued

Cone sex			Male cones									Female cones								
Cone stage			Pre-dehiscent			Dehiscent			Post-dehiscent			Pre-receptive			Receptive			Post-receptive		
Time of day			M	A	E	M	A	E	M	A	E	M	A	E	M	A	E	M	A	E
Number of samples			5	5	5	5	5	5	5	5	5	4	4	4	5	4	4	4	4	4
Number of compounds			25	26	23	31	34	35	28	27	28	25	18	32	40	33	35	24	21	24
Emission rate (ng/cone/hr)			13.75	22.75	51.78	1688.82	2030.20	3698.08	6796.09	2410.37	7428.18	7.23	24.30	29.81	795.31	1842	1633.27	30.70	256.50	76.37
Compounds	CAS	KRI																		
Piperitone oxide[c]	5286-38-4	1760	-	-	-	0.01 (1)	0.01 (1)	0.01 (1)	0.01 (1)	-	0.19 (2)	-	-	-	-	-	-	-	-	-
cis-Geraniol[b]	106-25-2	1803	-	-	-	0.04 (1)	tr (1)	0.59 (1)	tr (2)	0.70 (2)	0.01 (1)	-	-	-	-	-	-	-	-	-
Myrtenol[a]	515-00-4	1815	-	-	-	-	-	-	-	-	-	0.97 (1)	-	0.07 (1)	0.07 (1)	0.19 (1)	0.02 (1)	-	-	-
p-Cymen-8-ol[a]	1197-01-9	1872	-	-	-	0.01 (1)	tr (1)	0.01 (1)	-	-	-	-	-	-	0.08 (1)	-	tr (1)	tr (1)	tr (1)	tr (1)
Verbenone[b]	80-57-9	1889	-	-	-	tr (1)	0.01 (1)	tr (1)	-	-	-	-	-	-	-	-	-	-	-	-
p-Cymen-3-ol[a]	89-83-8	2225	-	-	-	0.02 (5)	0.03 (5)	0.02 (5)	tr (3)	0.01 (3)	tr (4)	-	-	-	0.02 (1)	0.07 (1)	0.05 (1)	-	-	-
p-Cymen-2-ol[a]	499-75-2	2232	-	-	-	0.02 (2)	0.11 (2)	0.07 (2)	0.02 (1)	-	0.28 (2)	-	-	-	0.06 (1)	0.12 (1)	0.07 (1)	-	-	-
Sesquiterpenes																				
β-Caryophyllene[a]	87-44-5	1636	0.39 (1)	0.07 (1)	0.16 (1)	-	-	-	-	-	-	0.25 (1)	-	0.38 (1)	tr (1)	0.02 (1)	tr (1)	-	-	-
α-Armophene[b]	483-75-0	1700	-	-	tr (1)	0.02 (1)	tr (1)	0.04 (1)	-	-	-	0.18 (1)	-	0.14 (1)	0.08 (1)	0.22 (1)	0.08 (1)	-	-	-
α-Curcumene[b]	644-30-4	1796	-	-	-	-	-	-	-	-	-	-	-	-	-	0.23 (1)	0.08 (1)	-	-	-
NITROGEN-CONTAINING COMPOUNDS																				
2-Isopropyl-3-methoxypyrazine[a]	25773-40-4	1452	1.71 (2)	1.72 (2)	4.42 (3)	7.75 (5)	6.53 (5)	14.91 (5)	5.65 (5)	9.57 (5)	13.92 (5)	4.89 (2)	3.62 (4)	0.50 (2)	6.69 (3)	11.79 (2)	28.22 (4)	6.62 (3)	2.05 (3)	0.36 (3)

[a]Identifications based on mass spectrum, kovats retention index and authentic standard. [b] Identifications based on mass spectrum and kovats retention index. [c] Identification based on mass spectrum only. tr = trace amounts (< 0.01 %)

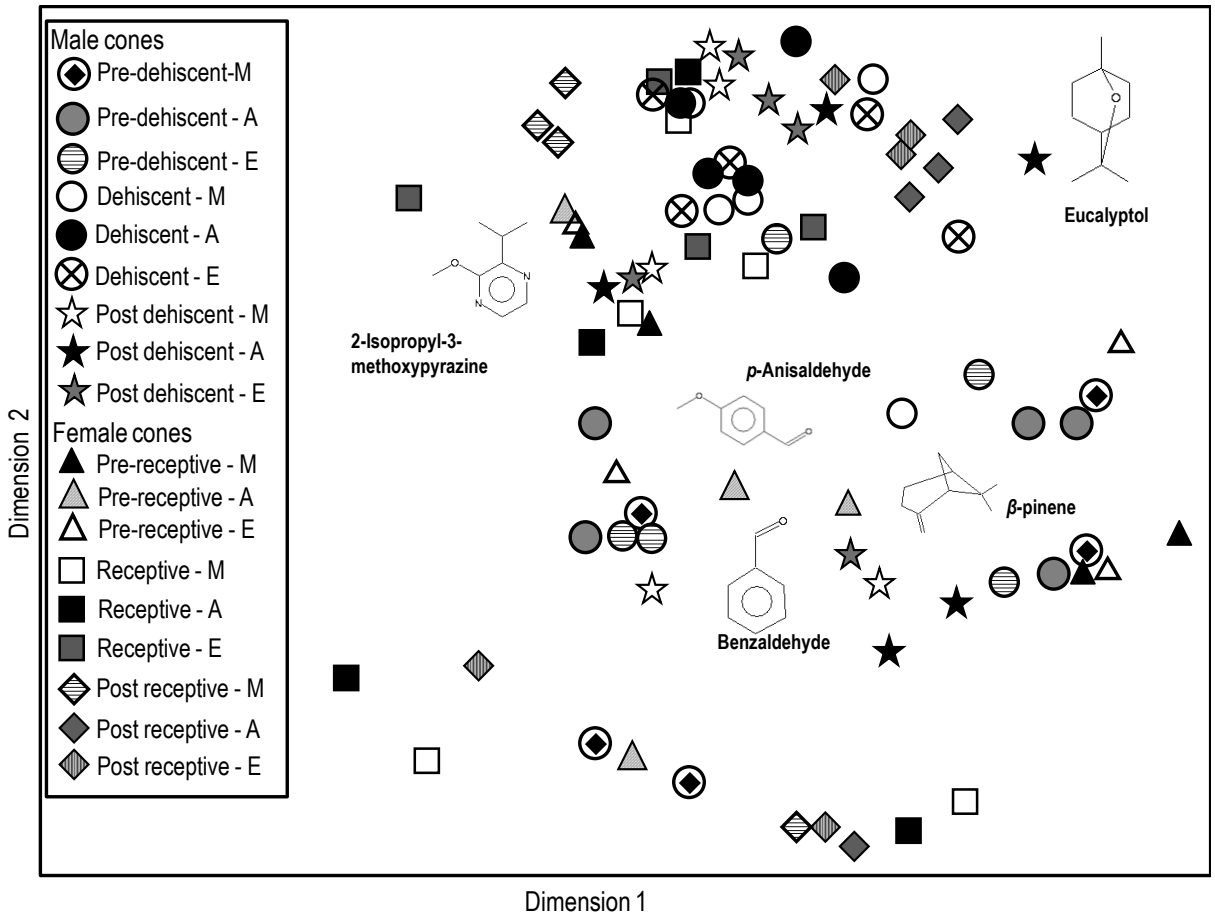

Figure 2.3: Nonmetric multidimensional scaling (NMDS) based on Bray-Curtis similarities of odour compounds of male and female cones of *E. villosus* from EC populations at different stages of development and different times of day - morning (M), afternoon (A) and evening (E). (NMDS 2D stress value = 0.17). The structures and names of the five main compounds dominating the volatile profile of odour at different cone stages and time of day are presented in the figure.

There were changes in the emission rates of volatile compounds at different cone stages and times of day (Table 2.2). In male cones, marginal (model adjusted) mean emission rates per cone per hour were highest in cones at the post dehiscent stage 6140.91 ± 1687.81 ng/cone/hour; (mean ± S.E) and were marginally significantly greater than in cones at the dehiscent (2779.55 ± 1687.81) and pre-dehiscent stages (29.43 ± 1687.81) (ANOVA, $F_{2, 36} = 3.29$; $P = 0.05$). In female cones, the marginal (model adjusted) mean emission rate per cone per hour was highest in cones at the pollen receptive stage (1423.52 ± 454.38 ng/cone/hour) but was not significantly different from emissions in cones at the pre-receptive (20.45 ± 470.33) and post receptive stages (121.19 ± 40.33) (ANOVA, $F_{2,28} = 2.89$; $P = 0.07$). Variation in volatile emission rates between the cone stages was accounted for by a few dominant compounds emitted in almost all cone stages at different emission rates in male and female cones (Figures 2.4A and B; Table 2.2). In male cones, the emission rates of four compounds *α*-pinene, *β*-pinene, eucalyptol and 2-isopropyl-3-methoxypyrazine were highest at the post dehiscent stage, followed by dehiscent and pre-dehiscent stages (Figure 2.4A). There was no variation in the emission rates of benzaldehyde, phenol. Heptanal, *p*-anisaldehyde and *α*-irone.The emission rates of 2-isopropyl-3-methoxypyrazine at post dehiscent (561.57 ± 156.44) was significantly greater than emission rates at dehiscent (379.25 ± 156.44) and pre-dehiscent stage (0.83 ± 156.44) (ANOVA, $F_{2, 36} = 3.34$; $P = 0.05$; Figure 2.4A). The variation in emission rates were not significant for *α*-pinene ($P = 0.09$), eucalyptol ($P = 0.1$) and *β*-pinene ($P = 0.2$) at the different cone stages.

In the female cones, emissions rates at the receptive stage were significantly greater for 2-isopropyl-3-methoxypyrazine (278.91 ± 85.84 ng/cone/hour) (ANOVA, $F_{2, 28} = 3.42$ $P < 0.05$) than at the post receptive (1.32 ± 88.85) and pre-receptive stages (0.26 ± 88.85) (Figure 2.5B). Emission rates were significantly different for *β*-pinene in post receptive cones (19.21 ± 4.38), pre-receptive cones (2.59 ± 4.38)

and receptive cones (0.61 ± 4.23) (ANOVA, $_{F2, 28}$ = 5.52, *P* = 0.01) (Figure 2.4B). Emission rates for *p*-anisaldehyde were significantly different for post receptive, receptive and pre-receptive cones (ANOVA, $_{F2, 28}$ = 7.44, *P* = 0.003). The emission rate for eucalyptol was highest at the receptive stage (779.57 ± 256.76) and was not significantly different from emissions at pre-recptive and post receptive stages (P = 0.07) (Figure 2.4B). Similarly, the emission rate for *α*-pinene was high in receptive cones (136.05 ± 46.50) and not significantly different from emission rates in post receptive (60.21 ± 48.13) and pre-receptive stages (0.54 ± 48.13) (*P* = 0.15, Figure 2.4B). There was no significant variation in the emission rates of benzaldehyde (*P* = 0.1), phenol (*P* = 0.12), and heptanal (*P* = 0.21) among different cone stages. Emissions at different times of the day were not significant for male cones (ANOVA, $F_{2, 36}$ = 5.31, *P* = 0.60) and female cones (ANOVA, $F_{2, 28}$ = 0.23, *P* = 0.80) despite variations in emission rates between time of day (Table 2.2).

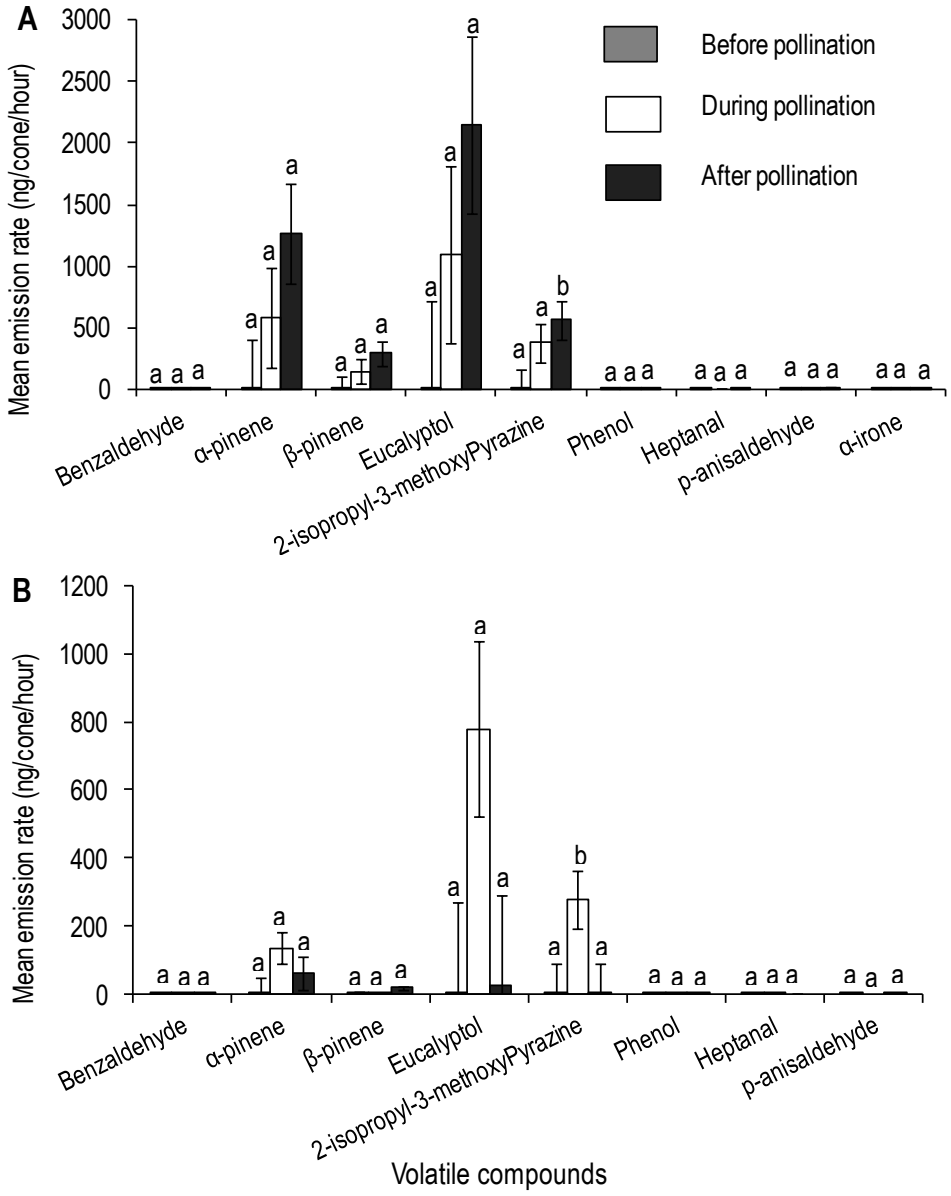

Figure 2.4: Variation of scent emission rates in ng of dominant compounds emitted before pollination, during pollination, and after pollination in: **A**) male cones, (number of samples N = 15, 15, and 15, respectively); and **B**) female cones (number of samples N = 12, 13, and 12, respectively) in the Eastern Cape. Bars represents mean (± 1 S. E). Letters above bars denote homogeneous groups for each compound using Tukey's Honest Significant Difference (Tukey's HSD) test.

Volatile samples from KZN populations

In KZN populations, 70 compounds were recorded out of which 69 were identified (Table 2.3) with terpenoids as the most numerous compounds (36 monoterpenes and one unknown, three sesquiterpenes), followed by fatty acid derivatives or aliphatics (five aldehydes, four alcohols, four unsaturated hydrocarbons, two aliphatic acids, one ketone, and one ester), 12 benzenoids, and one nitrogen-containing compound. The male cones emitted 63 compounds (31 at pre-dehiscence, 42 at dehiscence, and 52 at post dehiscence) whereas the female cones emitted 33 compounds (11 at pre-receptive, 25 at receptive, and 24 at post receptive). In total, 37 compounds were male specific of which two (cresol and *p*-cymen-3-ol) were specific to the pre-dehiscent stage; five (1-nonen-3-ol, *trans*-*β*-ocimene, piperitone oxide, carvone and 2,5-dimethyl pyrazine) to the dehiscent stage, fourteen (grandisol, *p*-methyl-anisole, 1-ethenyl-4-methoxybenzene, guaiacol, 2-phenylethanol, *trans*-linalool oxide (furanoid), carveol, *cis*-linalool oxide (furanoid), pinocarvone, 2,6,6-trimethyl-2-cyclohexene-1-methanol, *trans*-*p*-Menth-2-en-7-ol, *trans*-linalool oxide (pyranoid), *cis*-myrtenol, and an unknown monoterpene) to the post dehiscent stage and the remaining 16 compounds were recorded from all developmental stages. There were eight compounds that were specific to female cones with one (anisole) occurring only at the receptive stage and five others (acetic acid, heptanoic acid, 1-octanol, *α*-irone, and camphor) only at the post receptive stage, one (limonene) at receptive and post receptive stages and citral at pre-receptive and receptive stages . Eight compounds occurred across all the developmental stages, namely benzaldehyde, (3*E*)-1,3-octadiene, (3*E*,5*Z*)-1,3,5-octatriene, 1-octen-3-ol, *β*-myrcene, eucalyptol, *β*-pinene and linalool. Other compounds emitted in high relative amounts but not at all stages included (*E*,*E*,*E*)-2,4,6-octatriene, 1,2-dimethyl-1,4-cyclohexadiene, hexanal, heptanal, 3-octanone, 3-octanol, 2-phenethyl hexanoate, *p*-anisaldehyde, *α*-pinene and camphene.

One-way SIMPER (factor stage) showed that the pre-dehiscent stage was characterised by high relative amounts of (3*E*)-1,3-octadiene, heptanal, (3*E*,5*Z*)-1,3,5-octatriene, *p*-anisaldehdye and hexanal explaining more than 89 % of the similarity in this stage. At the dehiscent stage, (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene explained more than 87.48 % of the similarity within the stage while linalool, 1-octen-3-ol, (3*E*,5*Z*)-1,3,5-octatriene, 3-octanol, (3*E*)-1,3-octadiene and *p*-anisaldehyde explained more than 69 % of the similarity at post dehiscence. In the female cones, high relative amounts of hexanal, 2-phenethyl hexanoate and heptanal explained 92.06 % of similarity in the pre-receptive stage. The receptive stage was dominated by high amounts of (3*E*)-1,3-octadiene, hexanal, heptanal, 1-octen-3-ol and (3*E*,5*Z*)-1,3,5-octatriene explaining more than 92 % similarity within this stage. At the post receptive stage, (3*E*)-1,3-octadiene, limonene, eucalyptol, phenol and benzaldehyde explained more than 81 % of the similarity within the group.One-way SIMPER, using time of the day as a factor, showed that samples from KZN taken in the morning, afternoon and evening were characterised by high relative amounts of (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene, heptanal, 1-octen-3-ol and hexanal accounting for > 70 % of the similarity at different times of the day. A Bray-Curtis NMDS analysis of odour compounds from cones in KZN using a two-way cross design (with replicates) showed a weak but significant separation in samples between developmental stages in male and female cones (NMDS stress value 0.18; ANOSIM, R (developmental stage) = 0.324, $P < 0.01$), and no significant separation in samples between times of day (ANOSIM, R (time) = -0.055, $P > 0.05$) (Figure 2.4). The weak separation can be accounted for by the fact that these cones at all developmental stages emit similar volatile chemicals in similar relative amounts.

Table 2.3: Average relative amounts (%) of odour compounds emitted by male and female cones of *E. villosus* from KwaZulu Natal before, during and after pollen and receptivity in the morning (M), afternoon (A) and evening (E). Compounds are identified by common names and CAS (Chemical Abstract Service) registry number, and listed according to estimated Kovats retention index (KRI) within each compound class. Values are mean percentages of the total peak area. The number of samples in which the compound was identified is given in parentheses.

Cone sex			**Male Cones**									**Female Cones**								
Cone stage			Pre-pollen shed			Pollen shed			Post-pollen shed			Pre-receptive			Receptivity			Post-receptivity		
Time of day			M	A	E	M	A	E	M	A	E	M	A	E	M	A	E	M	A	E
Number of samples			9	9	9	11	11	11	11	11	11	4	4	4	5	5	5	3	3	3
Number of compounds			22	24	29	35	37	38	50	48	50	6	8	11	18	20	23	19	22	17
Emission rate (ng/cone/hr)			1592	1676.45	2016.17	4922.55	9464.99	17528.53	1607.57	944.21	2258.15	1.88	2.83	66.04	63.26	41.6	113.91	465.6	295.42	321.61
Compound	**CAS**	**KRI**																		
ALIPHATICS																				
Unsaturated hydrocarbons																				
(3*E*)-1,3-Octadiene[a]	1002-33-1	1062	29.43 (7)	16.75 (7)	32.77 (8)	49.65 (9)	46.34 (9)	47.72 (9)	13.45 (8)	5.92 (8)	5.11 (8)	tr (1)	4.39 (3)	32.50 (3)	30.20 (5)	45.01 (5)	49.09 (5)	67.70 (3)	42.81 (2)	29.10 (2)
(3*E*,5*Z*)-1,3,5-Octatriene[b]	40087-61-4	1148	17.65 (6)	10.94 (6)	12.71 (6)	30.75 (9)	34.58 (9)	32.16 (9)	11.90 (7)	16.18 (7)	18.29 (7)	-	-	12.32 (2)	2.22 (5)	6.72 (5)	12.26 (5)	3.12 (2)	0.02 (1)	-
(*E*,*E*,*E*)-2,4,6-Octatriene[b]	15192-80-0	1216	0.151)	0.06 (1)	0.03 (2)	0.23 (5)	0.06 (5)	0.07 (4)	3.37 (6)	3.77 (6)	6.70 (6)	-	-	-	tr (2)	tr (4)	tr (4)	tr (2)	-	-
1,2-Dimethyl-1,4-cyclohexadiene[b]	17351-28-9	1217	-	-	-	tr (3)	tr (3)	tr (3)	3.92 (6)	1.67 (6)	8.05 (6)	-	-	-	tr (2)	tr (4)	tr (4)	tr (1)	-	-
Aliphatic acids																				
Acetic acid[a]	64-19-7	1467	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.40 (1)	8.24 (1)	-
Heptanoic acid[a]	111-14-8	1969	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.28 (1)	5.01 (2)
Aldehydes																				
Hexanal[a]	66-25-1	1125	5.71 (4)	4.04 (4)	13.84 (4)	-	-	-	-	-	-	7.87 (2)	33.93 (4)	18.57 (4)	21.89 (7)	11.41 (4)	4.39 (4)	-	tr (1)	0.78 (2)
Heptanal[a]	111-71-7	1209	15.52 (6)	23.70 (6)	13.04 (6)	-	-	-	tr (3)	0.10 (3)	tr (2)	6.20 (1)	24.59 (4)	14.82 (4)	4.61 (4)	6.29 (4)	9.00 (4)	tr (1)	1.63 (2)	3.49 (2)
(*Z*)-2-Heptenal[b]	57266-86-1	1340	tr (1)	tr (2)	tr (1)	tr (1)	tr (1)	tr (1)	tr (1)	-	-	-	-	-	-	-	-	-	-	-
(2*E*,4*E*)-Hepta-2,4-dienal[b]	4313-5-3	1513	0.01 (2)	0.07 (2)	tr (1)	0.01 (2)	0.02(3)	0.01 (3)	-	-	0.01 (1)	-	-	-	-	-	0.47 (1)	-	-	-
2,4-Octadienal[b]	30361-28-5	1610	tr (1)	tr (2)	tr (2)	tr (2)	tr (2)	tr (2)	0.01 (1)	-	-	-	-	-	-	-	0.07 (1)	-	-	-
Ketones																				
3-Octanone[b]	106-68-3	1274	-	-	tr (1)	0.39 (7)	0.84 (8)	0.81 (7)	8.31 (5)	6.91 (3)	7.36 (3)	-	-	-	-	-	-	-	-	-

Table 2.3 continued

Cone sex			Male Cones									Female Cones								
Cone stage			Pre-pollen shed			Pollen shed			Post-pollen shed			Pre-receptive			Receptivity			Post-receptivity		
Time of day			M	A	E	M	A	E	M	A	E	M	A	E	M	A	E	M	A	E
Number of samples			9	9	9	11	11	11	11	11	11	4	4	4	5	5	5	3	3	3
Number of compounds			22	24	29	35	37	38	50	48	50	6	8	11	18	20	23	19	22	17
Emission rate (ng/cone/hr)			1592	1676.45	2016.17	4922.55	9464.99	17528.53	1607.57	944.21	2258.15	1.88	2.83	66.04	63.26	41.6	113.91	465.6	295.42	321.61
Compound	**CAS**	**KRI**																		
Aliphatic alcohols																				
3-Octanol[b]	589-98-0	1386	-	-	0.02 (1)	0.17 (6)	0.29 (8)	0.58 (7)	8.87 (6)	11.70 (6)	10.31 (6)	-	-	-	-	-	-	-	-	-
1-Octen-3-ol[a]	3391-86-4	1456	5.58 (4)	1.66 (3)	1.82 (5)	0.39 (7)	0.77 (8)	1.04 (7)	8.15 (9)	12.37 (9)	11.21 (10)	-	-	1.06 (1)	16.60 (1)	9.53 (2)	8.51 (3)	-	-	-
1-Nonen-3-ol[b]		1461	-	-	-	-	0.02 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Octanol[b]	111-87-5	1563	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.06 (1)	0.20 (1)	-
Esters																				
2-Phenethyl hexanoate[b]	6290-37-5	1270	-	-	-	-	-	-	1.85 (1)	tr (1)	1.53 (1)	57.78 (3)	28.51 (4)	4.03 (4)	4.74 (4)	0.90 (3)	0.52 (3)	-	-	-
BENZENOIDS																				
Anisole[a]	100-66-3	1357	-	-	-	-	-	-	-	-	-	-	-	-	1.96 (1)	2.54 (1)	0.77 (1)	-	-	-
p-Methyl-anisole[b]	104-90-3	1460	-	-	-	-	-	-	0.04 (1)	0.04 (1)	tr (1)	-	-	-	-	-	-	-	-	-
Benzaldehyde[a]	100-52-7	1553	1.15 (7)	5.58 (7)	0.57 (7)	0.02 (9)	0.01 (11)	tr (8)	0.48 (9)	0.40 (9)	0.09 (8)	3.16 (4)	8.57 (4)	0.08 (4)	2.06 (5)	3.39 (5)	0.55 (5)	0.32 (2)	1.47 (3)	0.63 (2)
Methyl benzoate[a]	93-58-3	1638	-	-	-	tr (1)	tr (1)	tr (1)	2.15 (1)	0.01 (1)	tr (1)	-	-	-	-	-	-	-	-	-
1-Ethenyl-4-methoxy-benzene[c]	637-69-4	1703	-	-	-	-	-	-	0.41 (3)	0.31 (3)	0.61 (3)	-	-	-	-	-	-	-	-	-
Methyl salicylate[a]	119-36-8	1808	-	-	-	tr (1)	tr (1)	tr (1)	-	-	-	-	-	-	-	-	-	-	tr (1)	-
Guaiacol[b]	90-05-1	1888	-	-	-	-	-	-	0.67 (8)	0.45 (8)	0.59 (8)	-	-	-	-	-	-	-	-	-
Phenylmethanol[a]	100-51-6	1896	-	-	-	tr (4)	tr (4)	tr (4)	0.22 (2)	0.71 (2)	0.14 (2)	-	-	-	-	-	-	-	-	-
2-Phenylethanol[a]	060-12-8	1933	-	-	-	-	-	-	0.23 (3)	0.18 (3)	0.08 (3)	-	-	-	-	-	-	-	-	-

Table 2.3 continued

Cone sex			Male Cones																	
Cone stage			Pre-pollen shed			Pollen shed			Post-pollen shed			Pre-receptive			Receptivity			Post-receptivity		
Time of day			M	A	E	M	A	E	M	A	E	M	A	E	M	A	E	M	A	E
Number of samples			9	9	9	11	11	11	11	11	11	4	4	4	5	5	5	3	3	3
Number of compounds			22	24	29	35	37	38	50	48	50	6	8	11	18	20	23	19	22	17
Emission rate (ng/cone/hr)			1592.12	1676.45	2016.17	4922.55	9464.99	17528.53	1607.57	944.21	2258.15	1.88	2.83	66.04	63.26	41.6	113.91	465.60	295.42	321.61
Compound	**CAS**	**KRI**																		
Phenol[a]	108-95-2	2032	0.39 (2)	tr (1)	tr (1)	0.08 (4)	tr (3)	0.01 (6)	0.18 (10)	0.09 (10)	0.06 (10)	-	-	-	0.28(2)	0.22 (2)	0.10 (2)	0.21 (2)	3.06 (2)	5.01 (2)
p-Anisaldehyde[a]	123-11-5	2061	4.25 (9)	14.78 (10)	1.88 (9)	0.08 (11)	1.65 (11)	0.02 (11)	0.65 (11)	0.47 (11)	0.31 (11)	-	-	-	tr (1)	tr (2)	tr (2)	tr (1)	tr (2)	tr (2)
Cresol[a]	106-44-5	2099	-	-	0.01 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TERPENOIDS																				
Monoterpenes																				
α-Pinene[a]	7785-70-8	1095	0.01 (7)	1.85 (3)	0.27 (2)	0.49 (3)	0.23 (3)	1.12 (3)	0.58 (4)	1.71 (4)	0.88 (3)	-	-	-	9.67 (1)	tr (1)	2.57 (2)	-	-	-
Camphene[a]	79-92-5	1112	-	13.64 (2)	0.16 (2)	1.86 (2)	0.52 (2)	1.22 (2)	-	4.23 (2)	0.41 (2)	-	-	-	-	-	-	-	-	-
β-Pinene[a]	127-91-3	1194	tr (2)	0.62 (3)	0.40 (2)	0.26 (2)	1.10 (3)	3.54 (3)	1.02 (4)	tr (3)	tr (3)	-	tr (1)	tr (1)	tr (1)	tr (1)	tr (1)	12.18 (2)	tr (2)	1.45 (2)
β-myrcene[a]	123-35-3	1199	0.04 (1)	0.08 (2)	0.01 (2)	0.19 (4)	1.19 (4)	0.26 (4)	0.02 (1)	0.54 (3)	1.05 (3)	-	tr (1)	tr (1)	5.68(2)	tr (1)	0.06 (4)	tr (1)	tr (1)	-
Unknown		1213	-	-	-	-	-	-	0.09 (2)	0.07 (2)	0.05 (2)	-	-	-	-	-	-	-	-	-
α-Terpinene[a]	99-86-5	1220	tr (1)	0.51 (2)	7.14 (2)	0.10 (1)	2.85 (2)	0.80 (2)	4.72 (3)	2.92 (3)	1.51 (3)	-	-	-	-	-	-	-	-	-
Limonene[a]	138-86-3	1224	-	-	-	-	-	-	-	-	-	-	-	-	-	4.65 (1)	-	tr (1)	21.87 (2)	22.12 (3)
Eucalyptol[a]	470-82-6	1231	3.97 (3)	4.45 (3)	4.75 (2)	10.54 (4)	7.47 (4)	7.00 (4)	1.96 (5)	10.50 (5)	11.17 (5)	-	-	-	0.06 (1)	0.03 (1)	tr (1)	7.81 (1)	11.09 (1)	21.77 (1)
trans-β-Ocimene[a]	502-99-8	1267	-	-	-	tr (1)	tr (1)	0.03 (1)	-	-	-	-	-	-	-	-	-	-	-	-
γ-Terpinene[b]	99-85-4	1269	-	-	0.68 (2)	0.29 (2)	0.07 (2)	0.08 (2)	0.01 (2)	0.02 (1)	0.73 (2)	-	-	-	-	-	-	-	-	-
cis-β-Ocimene[a]	3338-55-4	1275	0.77 (1)	tr	-	tr (1)	0.01 (1)	0.03 (1)	tr (1)	tr (1)	tr (1)	-	-	-	-	tr (1)	tr (1)	-	-	-
p-Cymene[a]	99-87-6	1294	3.47 (2)	0.73 (2)	0.44 (2)	1.09 (2)	0.67 (2)	0.97 (2)	0.54 (2)	0.45 (2)	1.71 (2)	-	-	-	-	-	-	-	-	-
α-Terpinolene[a]	586-62-9	1304	tr (1)	tr (1)	0.17 (2)	tr (1)	0.07 (2)	0.10 (2)	tr (1)	0.05 (1)	0.25 (2)	-	-	-	-	-	-	-	-	-

Table 2.3 continued

Cone sex			Male Cones										Female Cones								
Cone stage			Pre-pollen shed			Pollen shed			Post-pollen shed			Pre-receptive			Receptivity			Post-receptivity		
Time of day			M	A	E	M	A	E	M	A	E	M	A	E	M	A	E	M	A	E
Number of samples			9	9	9	11	11	11	11	11	11	4	4	4	5	5	5	3	3	3
Number of compounds			22	24	29	35	37	38	50	48	50	6	8	11	18	20	23	19	22	17
Emission rate (ng/cone/hr)			1592.12	1676.45	2016.17	4922.55	9464.99	17528.53	1607.57	944.21	2258.15	1.88	2.83	66.04	63.26	41.6	113.91	465.60	295.42	321.61
Compound	**CAS**	**KRI**																		
trans-Linalool oxide (Furanoid)[a]	5989-33-3	1453	-	-	-	-	-	-	5.55 (2)	7.23 (1)	0.18 (1)	-	-	-	-	-	-	-	-	-
Carveol[a]	99-48-9	1461	-	-	-	-	-	-	0.01 (1)	tr (1)	0.01 (1)	-	-	-	-	-	-	-	-	-
cis-Linalool oxide (Furanoid)[a]	34995-77-2	1467	-	-	-	-	-	-	0.01 (1)	0.01 (1)	0.01 (1)	-	-	-	-	-	-	-	-	-
α-Irone[b]	79-69-6	1535	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr (1)	tr (2)	tr (2)
Camphor[a]	464-48-2	1543	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.11 (1)	2.69 (2)	6.39 (2)
Linalool[a]	78-70-6	1562	0.66 (2)	tr (3)	1.73 (3)	0.11 (11)	0.29 (11)	0.29 (11)	7.93 (11)	8.80 (11)	7.30 (11)	24.99 (1)	tr (1)	15.95 (1)	tr (1)	tr (1)	0.01 (2)	-	1.96 (2)	2.41 (3)
Pinocarvone[b]	30460-92-5	1597	-	-	-	-	-	-	0.01 (1)	tr (1)	tr (1)	-	-	-	-	-	-	-	-	-
4-Terpineol[a]	562-74-3	1613	-	tr (1)	0.01 (2)	0.04 (4)	0.11 (4)	0.13 (4)	0.16 (2)	0.02 (2)	0.14 (2)	-	-	-	-	-	-	-	-	-
Myrtenal[a]	564-94-3	1650	-	-	-	-	-	-	0.45 (8)	0.32 (8)	0.21 (9)	-	-	-	-	-	-	6.03 (1)	0.64 (1)	0.65 (1)
α-Terpineol[a]	10482-56-1	1720	-	-	-	0.06 (2)	0.18 (2)	0.15 (2)	0.53 (6)	0.24 (7)	0.69 (6)	-	-	-	-	-	0.17 (1)	tr (1)	0.88 (2)	0.45 (2)
2,6,6-trimethyl-2-Cyclohexene-1-methanol[c]	6627-74-3	1720	-	-	-	-	-	-	0.04 (4)	0.45 (4)	0.10 (4)	-	-	-	-	-	-	-	-	-
trans-p-Menth-2-en-7-ol[c]	19898-87-4	1717	-	-	-	-	-	-	0.08 (3)	0.08 (3)	0.16 (3)	-	-	-	-	-	-	-	-	-
Borneol[a]	507-70-0	1725	-	-	-	0.01 (1)	0.04 (1)	0.03 (1)	0.12 (2)	0.02 (2)	0.12 (2)	-	-	-	-	-	-	-	-	-
Citral[b]	106-26-3	1732	-	-	-	-	-	-	-	-	-	-	-	0.68 (1)	0.04 (1)	0.43 (1)	3.86 (1)	-	-	-
trans-Linalool oxide (Pyranoid)[a]	5989-33-3	1755	-	-	-	-	-	-	0.06 (3)	0.09 (3)	0.02 (3)	-	-	-	-	-	-	-	-	-

Table 2.3 continued

Cone sex			Male Cones																	
Cone stage			Pre-pollen shed			Pollen shed			Post-pollen shed			Pre-receptive			Receptivity			Post-receptivity		
Time of day			M	A	E	M	A	E	M	A	E	M	A	E	M	A	E	M	A	E
Number of samples			9	9	9	11	11	11	11	11	11	4	4	4	5	5	5	3	3	3
Number of compounds			22	24	29	35	37	38	50	48	50	6	8	11	18	20	23	19	22	17
Emission rate (ng/cone/hr)			1592.12	1676.45	2016.17	4922.55	9464.99	17528.53	1607.57	944.21	2258.15	1.88	2.83	66.04	63.26	41.6	113.91	465.60	295.42	321.61
Compound	**CAS**	**KRI**																		
Piperitone oxide[c]	5286-38-4	1760	-	-	-	0.04 (1)	-	tr (1)	-	-	-	-	-	-	-	-	-	-	-	-
Carvone[a]	2244-1-6-8	1755	-	-	-	0.04 (1)	tr (1)	tr (1)	-	-	-	-	-	-	-	-	-	-	-	-
cis-Linalool oxide (Pyranoid)[a]	5989-33-3	1781	-	-	-	-	0.01 (2)	0.01 (2)	0.17 (4)	0.03 (3)	0.13 (4)	-	-	-	-	-	-	-	-	-
Myrtenol[a]	515-00-4	1815	-	-	-	-	-	-	0.24 (7)	0.22 (7)	0.11 (6)	-	-	-	-	-	-	-	0.53 (1)	0.61 (3)
Grandisol[b]	30820-22-5	1821	-	-	-	-	-	-	0.27 (3)	0.20 (1)	0.17 (3)	-	-	-	-	-	-	-	-	-
p-Cymen-8-ol[a]	1197-01-9	1872	-	-	0.01 (2)	-	0.01 (3)	0.01 (1)	0.03 (2)	-	0.01 (1)	-	-	-	-	-	-	-	-	-
cis-Myrtanol[c]	514-99-8	1893	-	-	-	-	-	-	tr (2)	tr (2)	tr (2)	-	-	-	-	-	-	-	-	-
p-Cymen-3-ol[a]	89-83-8	2225	-	-	tr (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sesquiterpenes																				
Dihydroedulan I[c]	63335-66-0	1538	11.23 (2)	0.51 (2)	4.20 (2)	2.77 (2)	0.57 (2)	0.79 (2)	3.22 (2)	0.50 (2)	1.57 (2)	-	-	-	-	-	-	-	-	-
(*E,E*) *α*-Farnesene[b]	502-61-4	1760	-	-	-	tr (2)	tr (2)	tr (2)	0.02 (1)	0.01 (1)	0.03 (1)	-	-	-	-	-	-	-	-	-
α-Curcumene[b]	644-30-4	1796	-	-	-	-	tr (3)	tr (3)	0.01 (2)	0.02 (3)	0.04 (3)	-	-	-	-	-	0.03 (1)	-	0.64 (1)	0.14 (1)
NITROGEN-CONTAINING COMPOUND																				
2,5-Dimethyl pyrazine[b]	123-32-0	1336	-	-	-	0.02 (8)	0.01 (8)	0.03 (8)	-	-	-	-	-	-	-	-	-	-	-	-

[a]Identifications based on mass spectrum, kovats retention index and authentic standard. [b] Identifications based on mass spectrum and kovats retention index. [c] Identification based on mass spectrum only. tr = trace amounts (< 0.01 %).

In the male cones, all the developmental stage differences were significantly different with least separation between pre-dehiscent and dehiscent samples (R = 0.192, P < 0.05), and larger separations between dehiscent and post dehiscent samples (R = 0.322, P < 0.01) and between pre-dehiscent and post dehiscent stage (R = 0.308, P < 0.01). In the female cones, the only significant separations were between pre-receptive and post receptive stages (R = 0.784, P < 0.05). Most of the pre-receptive and receptive female cone volatiles overlap with some pre-dehiscent male cones. This group was characterised by heptanal and hexanal emissions. Most dehiscent males were characterised by (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene and overlapped with some pre-dehiscent males as well as some receptive and post receptive female and most post dehiscent samples.

As was the case for EC plants, variation in scent emission rates differed between the different cone stages and times of day in male and female cones of *E. villosus* in the KZN (Table 2.3). In male cones, the marginal (model adjusted) mean emission rates per cone per hour were highest in cones at the dehiscent stage (10638.69 ± 1227.85 ng/cone/hour; mean ± S.E) and were significantly greater than in cones at pre-dehiscent stage (1761.58 ± 1357.44) and post dehiscent stage (1603.31 ± 1227.85) (ANOVA, $F_{2, 92}$ = 17.20; P < 0.001). In female cones, the marginal (model adjusted) mean emission rates per cone per hour were highest in cones at the post receptive stage (360.88 ± 69.52 ng/cone/hour) and were significantly greater than in cones at receptive stage (72.92 ± 53.85) and pre-receptive stage (23.58 ± 60.20) (ANOVA, $F_{2, 27}$= 7.64; P = 0.002). In male cones, rates of odour emission per cone per hour at different times of the day were highest in the evening (7267.62 ± 1272.51) and were marginally significantly greater than emissions in the afternoon (4028.55 ± 1272.51) and in the morning (2707.42 ± 127.51) (ANOVA, F2, 84 = 3.40, P = 0.04) but there were no significant differences in the emission rates of compounds in female cones at different times of the day (P > 0.05).

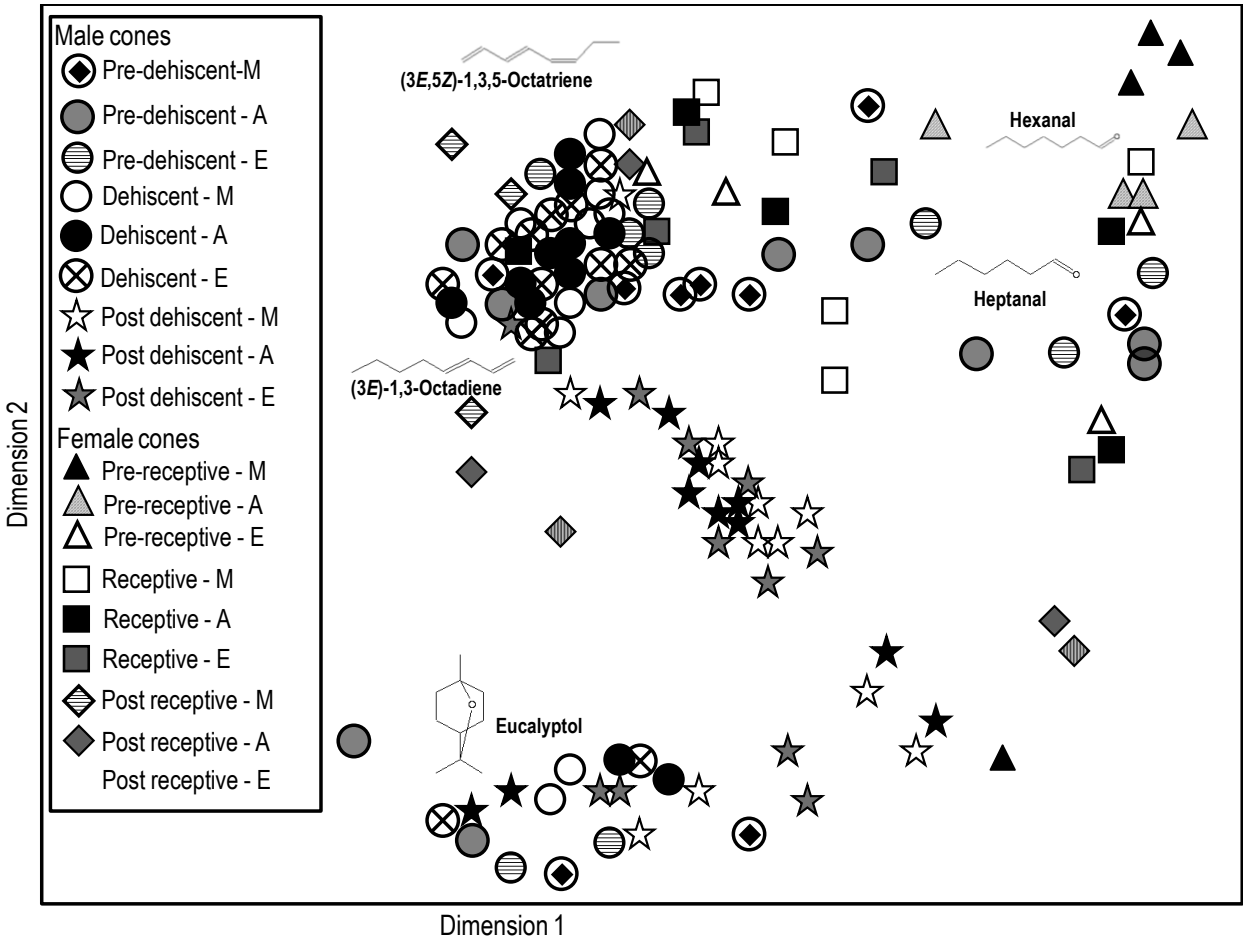

Figure 2.5: Nonmetric multidimensional scaling (NMDS) based on Bray-Curtis similarities of odour compounds of male and female cones of *E. villosus* from the KZN at different developmental stages in the morning (M), afternoon (A) and evening (E). (NMDS 2D stress value = 0.18). The structures and names of the five main compounds dominating the volatile profile of odour at different cone stages and time of day are presented in the figure.

Variation in volatile emission rates between the different cone stages were also accounted for by a few dominant compounds emitted by male and female cones. In the male cones, the emission rate of (3*E*)-1,3-octadiene was highest in cones at the dehiscent stage (5512.89 ± 780.06 ng cone^{-1}hour^{-1}) and was significantly greater than in pre-dehiscent cones (917.163 ± 862.39) and post dehiscent stage (414.25 ± 780.06) (ANOVA, $F_{2, 84}$ = 12.86; P < 0.001) (Figure 2.6A). Similarly, the emission of (3*E*,5*Z*)-1,3,5-octatriene was highest in cones at the dehiscent stage (4307.88 ± 878.36) and was significantly greater than in pre-dehiscent cones (626.11 ± 971.07) and post-dehiscent stage (488.05 ± 878.36) (ANOVA, $F_{2, 84}$ = 5.91; P = 0.04) (Figure 2.6A). In post dehiscent cones, emissions were significantly greater for 1-octen-3-ol (P < 0.001), benzaldehyde (P = 0.003), and linalool (P < 0.001) than in dehiscent and pre-dehiscent cones (Figure 2.6A). Emission of heptanal was significantly greater (P < 0.001) in pre-dehiscent cones than in dehiscent and post dehiscent cones. There were no significant differences in the emission of eucalyptol and *p*-anisaldehyde. In dehiscent cones, 1-octen-3-ol and *p*-anisaldehyde accounted for the marginal significant difference in the emission of compounds at different times of the day. In dehiscent cones, the emission of 1-octen-3-ol was highest in the evening (70.03 ± 12.08) and significantly greater than emissions in the morning (19.02 ± 12.08) and evening (35.38 ± 12.08) (ANOVA, $F_{2, 84}$ = 5.47, P = 0.01). Emission rate of *p*-anisaldehyde was significantly greater in the afternoon (P = 0.02) than in the morning and evening. In the female cones, emission rates of (3*E*)-1,3-octadiene were highest at post receptive stage (206 ± 52.76 ng/cone/hour) and significantly greater than receptive (33.97 ± 40.87) and pre-receptive stages (16.55 ± 45.69) (ANOVA, $F_{2, 27}$ = 4.41, P = 0.02,Figure 2.6B). Emission rates in post receptive cones were significantly greater for benzaldehyde (P = 0.02), linalool (P = 0.05), and eucalyptol (P = 0.02) than in receptive and pre-receptive cones (Figure 2.6B). There were no significant differences in the emission of (3*E*,5*Z*)-1,3,5-octatriene, heptanal, hexanal, 1-octen-3-ol and *p*-anisaldehyde.

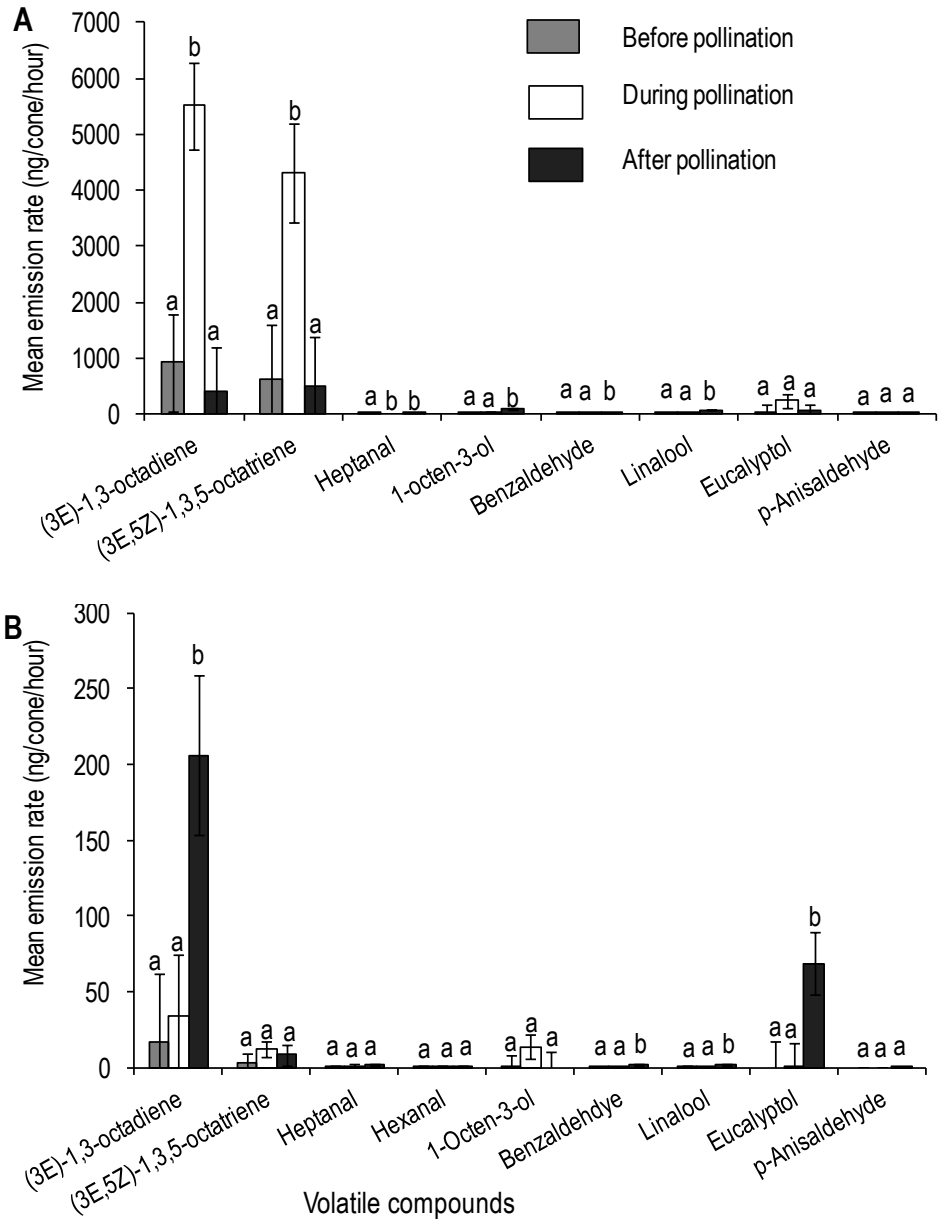

Figure 2.6: Variation of scent emission rates in ng of dominant volatile compounds emitted before pollination, during pollination, and after pollination in: **A**) male cones, (number of samples N = 27, 33, and 33, respectively); and **B**) female cones (number of samples N = 12, 15, and 9, respectively) in KwaZulu Natal. Bars represent mean (± 1 S. E). Letters above bars denote homogeneous groups for each compound using Tukey's Honest Significant Difference (Tukey's HSD) test.

Temperature in male and female cones of *E. villosus*

In male cones, the general pattern of cone heating comprised a consistent rise in cone temperature above concurrent ambient temperature. The cone heating which is due to increase in cone metabolism (Terry et al., 2004a; Roemer et al., 2005) occurred in most male cones during pollen shed, beginning in the late afternoon when air temperatures were cooling (Figure 2.7A-C). In some cones, heating occurred in the late morning to early afternoon when both cone and ambient temperatures increased simultaneously. Such cones sometimes show a bimodal pattern of heating with a first peak in the late morning to early afternoon and a second peak in the evening. In comparison, near ambient cone temperatures were recorded at pre-dehiscent and post-dehiscent stages (Figure 2.7A-C).

Sunrise was at about 06h00 and sunset about 18h00 during the period of measurement. During pollen dehiscence, male cone temperatures monitored between 20 March and 3 April 2009 at the Kranzkloof Nature Reserve were 9.30 ± 1.01 °C (n = 5) above concurrent ambient temperatures. The cones reached their mean maximum temperatures of 31.71 ± 0.42 °C between 17h30 and 19h00 (average 18h22). In PMB, cone temperatures exceeded ambient temperatures by 12.21 ± 2.46 °C (n = 3) and reached maximum temperatures of between 26.7 and 35.7 °C (29.71 ± 0.76 °C) at beween 16h10 and 18h20 (average 17h05). Seven male cones monitored at the Mount Sullivan Area in April 2009 were 7.10 ± 1.28 °C warmer than the ambient temperatures. Cones reached mean maximum temperatures of 29.80 ± 1.65 °C above concurrent ambient temperature at mean time of 18h33. Cone heating due to thermogenesis appeared to be limited to between three and seven days during the dehiscent phase and peaks in cone temperature occurred mostly between 18h00 and 18h30.

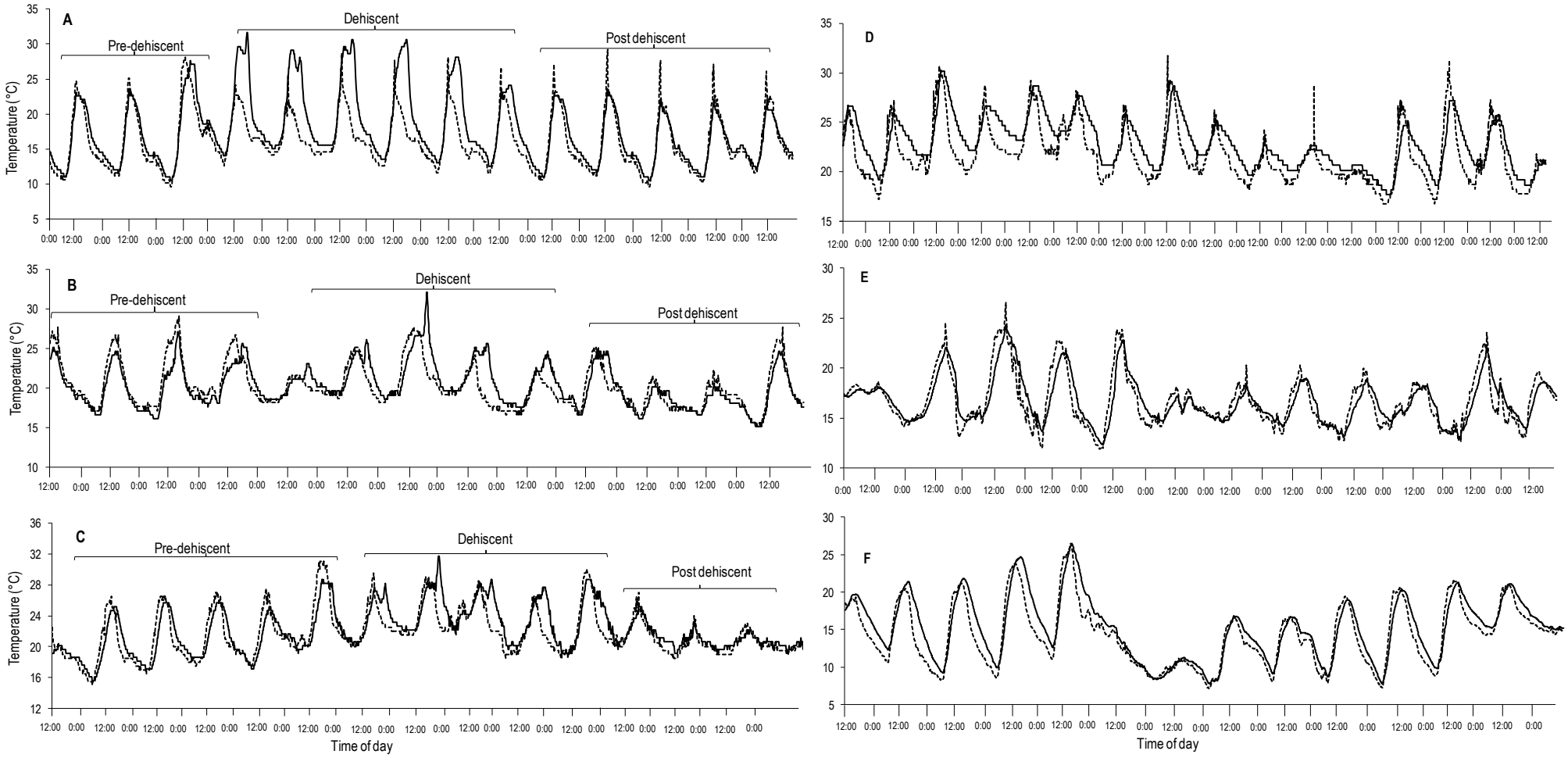

Figure 2.7: Pattern of cone heating in relation to ambient temperature in: **A-C**) three male cones of *E. villosus* over the developmental cycle; **D-F**) three female cones over developmental cycle. Dotted lines = ambient temperature; Smooth lines = cone temperature

However, there were anomalies to this general pattern, both in terms of daily and developmental cycle patterns. In contrast to the typical 18h00-18h30 peak in temperature, earlier peaks (14h00-15h30) were recorded in two cones at the Pietermaritzburg Botanic Garden and Kranzkloof Nature Reserve and double peaks (09h00-13h30; 16h00-17h30) were also recorded in two cones at the Kirstenbosch and Pietermaritzburg Botanic Gardens respectively. In the case of double peaks, the first peak exceeded mean maximum ambient temperatures by between 4.3 °C and 7.0 °C whereas the second peak exceeded ambient temperature by between 7.8 °C and 11.7 °C. Also, at Ocean View Guest Farm, cone heating occurred between 13h30 and 16h00 with the mean cone temperature of 2.83 ±0.45 °C (n = 4) above concurrent ambient temperature.

Despite the absence of thermogenesis in the majority of pre-dehiscent male cones, five male cones (three in EC and two in KZN) did have temperatures above ambient in the pre-dehiscent stage. In these cases, heating began from late morning (between 10h00 and 10h40) and reached a daily mean maximum between 11h50 and 15h07. The cone temperature exceeded mean ambient temperature by between 2.0 °C and 4.5 °C.

In general, there was little or no increase in temperature in female cones throughout the life cycle of the cone (Figures 2.7D-F). In some cases, cone temperatures increased simultaneously with ambient temperature and only rose above concurrent ambient temperature when it started decreasing parallel with it in the evening (Figure 2.7D-E). In contrast to this pattern, a relatively small thermogenic effect appeared to occur in three cones, cone temperature continued to increase up to ca. 2.0 ^{o}C above the maximum ambient temperature over a number of days during the life cycle of the cone (Figure 2.7F) but the increase did not occur as huge temperature peaks observed in male cones temperature. In some cases, measurements

taken in the afternoon registered readings higher than concurrent ambient temperatures by between 1.5 °C and 3.5 °C.

Insect visitors to male and female cones of *E. villosus*

Four species of beetles (Coleoptera) were collected from *E. villosus* male cones in both EC and KZN,- *Antliarhinus zamiae* Thunberg (Curculionoidea: Brentidae), Erotylidae sp. nov. (Cucujoidea), *Metacucujus goodei* Endrödy-Younga (Cucujoidea; Boganiidae), and *Porthetes* sp. (Curculionidae) (Figures 2.8 and 2.9). The *Porthetes* was provisionally named as *P. pearsonii* by Oberprieler (1996), but this remains an unpublished name.

With the exception of small numbers of *A. zamiae* on pre-dehiscent cones, all the beetles were most abundant on dehiscent male cones (Figures 2.8 and 2.9). At pre-dehiscence, *A. zamiae* were observed crawling over the cone surface while Erotylidae and *M. goodei* were mostly located at the base of the cones and *Porthetes* sp. were seen crawling over the cone surface, forcing their way between the tightly packed sporophylls. At dehiscence, the insects present on cones (Erotylidae sp. nov., *M. goodei* and *Porthetes* sp.) were all covered with pollen while moving in between the sporophylls as well as over the cone surface. At post dehiscence, the abundance of all insect species declined and *A. zamiae* was absent.

In EC populations, the three beetle species observed on dehiscent male cones (n = 5) showed similar patterns of abundance (Figure 2.8). The relative numbers of beetles were significantly higher in the afternoon than in the evening or morning for Erotylidae sp. nov. (Kruskal-Wallis $\chi^2 = 13.52$, $P < 0.01$, Median = 41, 89, 62 in the morning, afternoon and evening), *Porthetes* sp. (Kruskal-Wallis, $\chi^2 = 30.06$, $P <$

0.001, Median = 97, 292, 185 in the morning, afternoon and evening) and *M. goodei* abundance in the afternoon and evening were significantly higher than in the morning (Kruskal-Wallis χ^2 = 16.16, P < 0.001, median = 28, 69, 43 in the morning, afternoon and evening). There was also a tendency in post dehiscent cones for *M. goodei* to be more abundant in the evening than in the morning or afternoon (Kruskal-Wallis, χ^2 = 13.68; P < 0.001, median = 2, 3, 8 in the morning, afternoon and evening).

The pattern of insect abundance was more variable in KZN populations (Figure 2.9). At dehiscence, there were significantly more individuals of the uncommon *A. zamiae* (Kruskal-Wallis, χ^2 = 6.67, P = 0.01, n = 5) and the more abundant *Porthetes* sp (Kruskal-Wallis, χ^2 = 23.66, P < 0.001, n = 5) during the afternoon than in the evening or morning. In contrast, *M. goodei* was significantly more abundant in the morning and evening than in the afternoon (Kruskal-Wallis, χ^2 = 6.96, P < 0.05, n = 5, Median = 15, 11, 16). The abundance of Erotylidae sp. nov. followed a similar pattern to *M. goodei* although there was no significant difference between times of day.

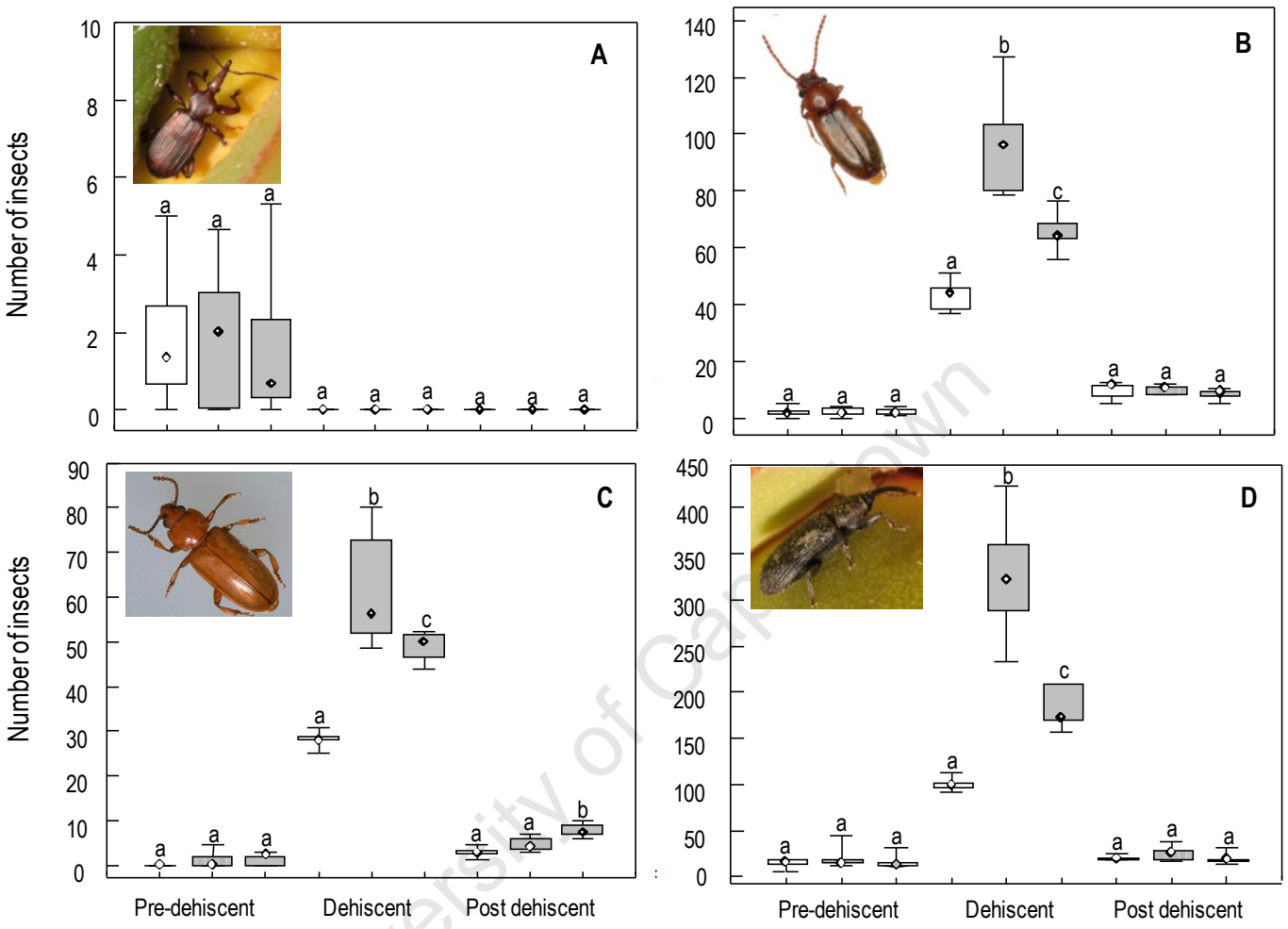

Figure 2.8: Insect abundance in male cones of *E. villosus* in the EC at pre-dehiscence, dehiscence and post dehiscence. **A** = *Antliarhinus zamiae*, **B** = *Metacucujus goodei*, **C** = Erotylidae sp. nov., **D** = *Porthetes* sp. Boxes within each insect group that share letters are not significantly different (Tukey's Honest Significant Difference (Tukey,s HSD) test at $P \leq 0.05$). ◇ = Median, Box = 25 %-75 %, Whisker: Min-Max. ▭: Morning (n = 5); ▭: Afternoon (n = 5); ▭: Evening (n = 5).

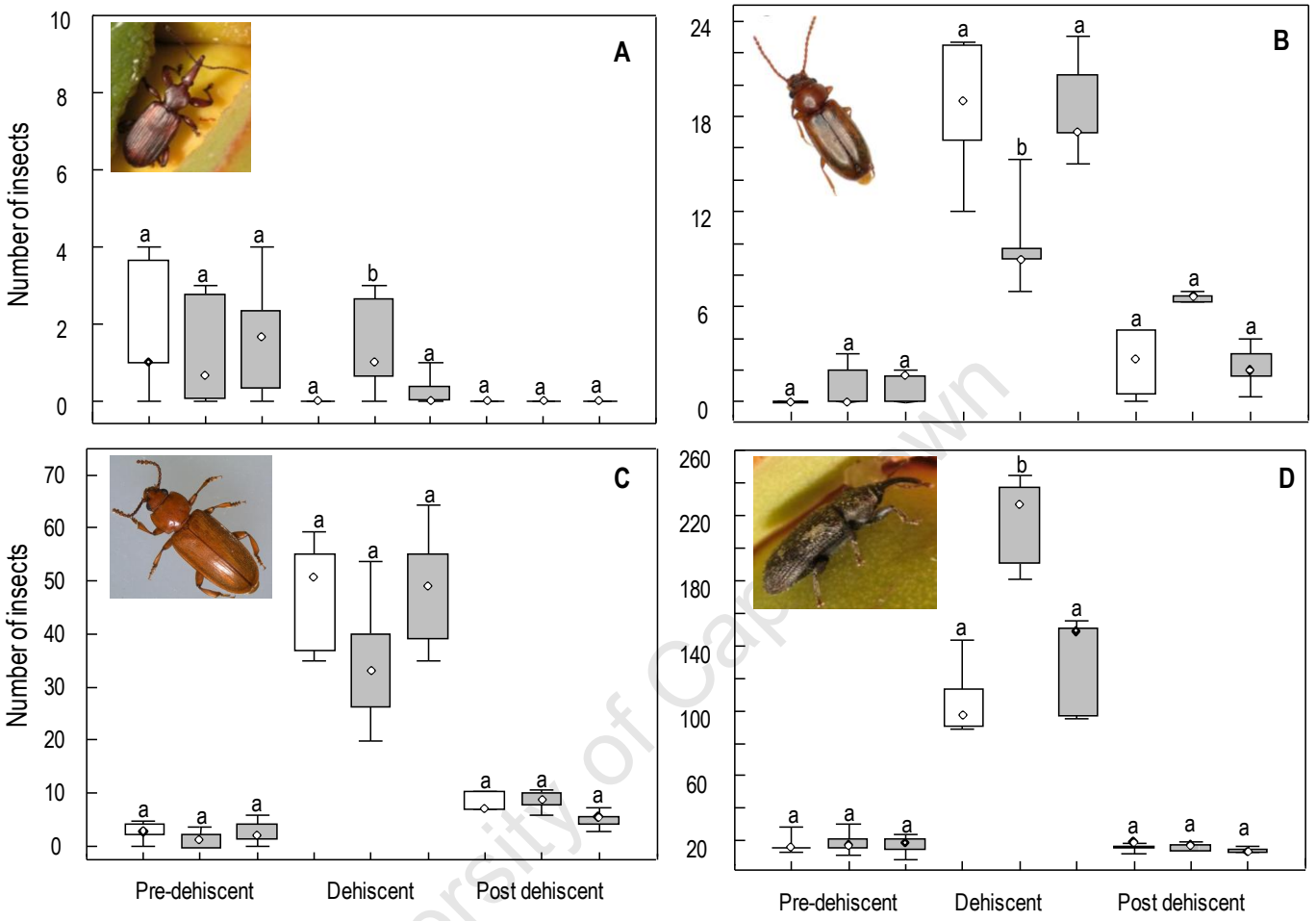

Figure 2.9: Insect abundance in male cone of *E. villosus* in the KZN at pre-dehiscent, dehiscent and post dehiscent. **A** = *Antliarhinus zamiae*, **B** = *Metacucujus goodei*, **C** = Erotylidae sp. nov., **D** = *Porthetes* sp. 1. Boxes within each insect group that share letters are not significantly different (Tukey's Honest Significant Difference (Tukey,s HSD) test at $P \leq 0.05$). ◇ = Median, Box = 25 %-75 %, Whisker: Min-Max., ◇: Morning (n = 5); ◇: Afternoon, (n = 5); ◇: Evening (n = 5).

Discussion

The purpose of this chapter was to determine whether daily patterns of odour and heat production in *E. villosus* are consistent with the type of daily push-pull patterns observed in Australian *Macrozamia* species (Terry et al., 2007a and b) and result in concomitant changes in insect abundance on cones. The study also included possible alternative push-pull models where changes in odour and heat production over the developmental cycle of the cycad cone could result in movement of pollinators between male and female cones (e.g. Donaldson, 2007). The results presented in this chapter show that temperature of male cones follows strong daily cycles during the dehiscent stage when thermogenesis significantly increases cone temperature above ambient temperature (Figure 2.7A-C). However, the composition and emission rates of cone volatiles varies most markedly between different stages of cone development with relatively small differences occurring as part of a daily cycle. Field observations of insect pollinators show similar large differences in abundance between developmental stages with relatively smaller (but significant) differences in abundance on a daily cycle (Figures 2.8 and 2.9). These results are discussed in more detail in relation to daily push-pull interactions (sensu Terry et al., 2007a and b) or possible developmental cycle movement between male and female cones.

The volatile compounds identified in this study are well known compounds that are emitted by cones of cycads (e.g. Pellmyr et al., 1991; Terry et al., 2004a and b; Kaiser, 2006; Suinyuy et al., 2010), as well as many different angiosperm taxa (Knudsen et al., 2006) and seaweeds (Kajiwara et al., 1980; Boland, 1995). Some of the compounds identified in the volatile profile of *E. villosus* are not common in floral scent. For example *α*-irone which attracts euglossine bees (Williams and Whitten, 1983) has been detected in the volatile profile of *Clusia criuva* (Nogueira et al., 2001). Grandisol is an insect pheromone (Francke et al.,

1995) not common in floral scent but its *trans*- isomer fragranol has been recorded in the volatile profile of *Artemisia fragrans* (Bohlmann et al., 1973).

Although the compounds are well known, one of the striking findings in this study was the difference in chemical composition between cone odours of *E. villosus* plants from EC and KZN populations. The odours from EC plants were dominated by eucalyptol and 2-isopropyl-3-methoxypyrazine (Table 2.1), whereas odours from KZN populations were dominated by (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene (Table 2.2). Despite the difference in volatile profile between the populations, the insect species composition did not change suggesting that the insects might be adapted to respond to different chemicals. The variation in volatile profiles of *E. villosus* from a limited sample within the distribution range suggests that there might be a wider variation across the different populations and this forms the basis for Chapter 3 of this thesis. At the same time, the current study was not set up to determine whether differences in chemical composition result in different physiological or behavioural responses in pollinating insects. These responses are examined in greater detail in Chapter 4 using gas chromatography–electroantennogram detection (GC-EAD) and field tests.

Detailed studies of push-pull interactions in *Macrozamia* species have revealed a daily pattern in which the male cones at dehiscence are attractive to pollinators at some stage during the day and repellent at other times (Terry et al., 2007a and b). Terry et al. (2007a and b) determined that the attractive phase for *M. lucida* occurred when the major compound *β*-myrcene was emitted in relatively low concentrations, which coincided with the cool phase in cone heating, whereas the repellent phase occurred when *β*-myrcene was emitted in high concentrations, which coincided with periods of peak cone temperatures. The daily behaviour of pollinators, comprising periods of attraction and repellence, could therefore be linked to clear patterns of volatile emissions and heating in male cones.

In pollen shedding cones of *E. villosus*, the daily pattern of cone volatile emissions indicated that there were no significant differences in the emission rates of the dominant compounds eucalyptol and 2-isopropyl-3-methoxypyrazine emitted by EC plants and (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene emitted by KZN plants. The only distinct daily pattern was evident in cone temperature, with low cone temperatures in the morning, high cone temperatures in the afternoon, and peak cone temperatures in the evening. Despite the apparent uniformity in odour emission rates, there were differences in insect pollinator abundance on dehiscent cones at different times of day. The implication is that insects may be responding to daily patterns of cone heating, albeit on a less dramatic scale than that observed in *Macrozamia* spp. Close observations of dehiscing male cones of *E. villosus* at peak temperatures in the evening indicated that, although insects were generally less abundant than in the afternoon, few insects were leaving the male cones at this time. Relatively few beetles crawled out from between the sporophylls onto the surface of the cone before flying away. This differs from *Macrozamia lucida* and *M. macleayi* (pollinated by thrips *Cycadothrips chadwickii*) and *M. machinii* (pollinated by *Tranes* weevils) in which there was a notable mass movement of pollinators away from male cones at the time of peak odour emission and thermogenesis (Terry et al., 2004a). The daily pattern of insect movement on *E. villosus* in response to variation in cone volatile emissions and cone heating therefore appears to be different to the very clear push-pull interactions observed in *M. lucida and M. macleayi* (Terry et al., 2004a).

Donaldson (2007) proposed several alternative models for push-pull interactions in cycads including ones in which there is active attraction of pollinators at some stages of male cone development followed by 'passive' dispersal associated with declining quality of the cone as a brood site (Table 2.1). In this case, specific male cone attributes that are considered as adaptations to facilitate pollination would be expected to influence only the attraction of pollinators but not their dispersal. A further option of no active attraction

accompanied by active dispersal (Donaldson, 2007) was based on the assumption that pollinators would already be attracted to male cones as brood and feeding sites and that no specific adaptations would therefore be necessary to attract pollinators to male cones. In this case, specific adaptations that influence pollinator behaviour would be expected to have only repellent effects that would enhance dispersal of insect pollinators from male cones. Such adaptation may include increased emission rates of compounds and heat production or diminishing and deterioration of brood sites.

The developmental changes over the life cycle of male cones observed in this study indicate that there is a significant change in the emission rates of cone volatiles (Figures 2.3 - 2.6) and cone thermogenesis (Figure 2.7A-C) at the onset of pollen dehiscence. Some of the developmental changes occurred in cone volatiles that comprised relatively low emission rates of the overall volatile profile. The changes in *E. villosus* cones were associated with a consistent increase in the abundance and activity of all three of the confirmed pollinator taxa on male cones (Figures 2.8B, C, D and 2.9B, C, D). In contrast, activity by *A. zamiae*, which is predominantly an herbivore with a negligible role in pollination (Donaldson, 1997), was restricted to the period preceding pollen dehiscence (Figures 2.8A and 2.9A). These results provide circumstantial evidence that male cone traits that are specific to the period of dehiscence do actively attract pollinators, in contradiction of the model where there is no active attraction (Donaldson, 2007).

Developmental variations in different floral volatiles, which result in the attraction of pollinator insects in one developmental stage and repellence in another stage, have been found in the orchid *Ophrys sphegodes* (Schiestl et al., 1997; Schiestl and Ayasse, 2001). The apparent attraction of insects to *E. villosus* has already been demonstrated but insect activity on male cones of *E. villosus* also showed a dramatic decline following dehiscence, which could indicate that the cone is producing repellent

compounds at the stage following dehiscence. At this stage, most of the insects appeared to be second generation adults that were emerging after undergoing larval development in the male cone sporophylls. The beetles were still covered in pollen and would therefore still be effective pollinators. However, by the time of emergence the male cone had started to disintegrate and the sporophylls were decomposing so that the cone would have been unsuitable as a brood site. This is consistent with the possibility of dispersal associated with decline in the suitability of the cone as a brood site, without any specific adaptation to repel pollinators. A similar phenomenon seems to occur in basal angiosperms, where insects that visit the female flower parts during pollination stay there until the scent emission decreases and temperatures cool down and they will then leave in search of another scented male or female flower part (e.g. Miyake and Yafuso, 2003; Seymour and Matthews, 2006). Although the data for *E. villosus* seem to fit the model of active attraction at dehiscence followed by dispersal associated with deterioration of male cones after dehiscence, this does not explain the daily changes in patterns of insect abundance observed on male cones. Because there was relatively little daily variation in the emission rates of cone volatiles, one implication is that thermogenesis has a strong influence on insect behaviour, especially to disperse pollinators. With the exception of *Stangeria* that does not produce heat (Tang, 1987b; Proches and Johnson, 2009), all cycad genera studied so far produce heat at some point in their developmental cycle. Cone volatile emissions and thermogenesis typically occur together in cycads (e.g. Tang, 1987b, 1993; Pellmyr et al., 1991; Stevenson et al., 1998; Seymour et al., 2004; Suinyuy et al., 2010), a phenomenon that also occurs in basal angiosperms (e.g. Seymour and Schultz-Motel, 1997, 1999; Jürgens et al., 2000; Ivancic et al., 2004; Seymour and Matthews, 2006). In all these systems, floral volatiles and thermogenesis are associated with pollinating insects (Seymour et al., 2004) indicating that insect behaviour is regulated by both cone volatiles and heat. Changes in the chemical concentration (emission rates) may play a major role along with

thermogenesis in mediating insect behaviour. However, because volatile emissions and heat production occur simultaneously, it is difficult to separate their individual or combined effects on pollinators and this requires further experimental tests.

Although changes in emission rates at different times of the day did not seem to affect insect behaviour, the results of this analysis provide a sound basis for further study of the roles of volatile compounds in the pollination of *E. villosus*. The sampling of *E. villosus* for this chapter occurred in a few populations but showed substantial differences in the volatile profile of different populations, especially relating to the presence or absence of 2-isopropyl-3-methoxypyrazine, (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene. This unexpected result is explored in greater detail in Chapter 3. In addition, the analysis identified the compounds that occur specifically during dehiscence when insects were attracted to cones and forms the basis for further studies to identify physiologically active compounds that may influence pollinator behaviour. Results from chromatography–electroantennogram detection (GC-EAD), and field tests to determine behavioural responses are presented in Chapter 4.

Chapter 3. Variation in cone volatile composition within and between populations of *Encephalartos villosus* and its implication for pollinator interactions

Abstract

Variation in traits across species distribution ranges is often indicative of diversifying evolution that can ultimately lead to speciation. Of particular interest is whether traits vary clinally or abruptly, as the latter pattern can be indicative of incipient speciation. Understanding of intra-specific variation in chemical traits is still very rudimentary as studies of population variation have tended to focus on morphology or neutral genetic markers. To address these issues, composition of cone volatile odours was examined in 10 populations of *Encephalartos villosus* across its range in the Eastern Cape (EC) and KwaZulu Natal (KZN) using the headspace sampling method and analysis by gas chromatography-mass spectrometry (GC-MS). Volatiles play a key role in attracting pollinators to cones of *Encephalartos* cycads and may thus reflect local adaptation to pollinators. Volatile compounds from populations in the north of the distribution range were dominated by unsaturated hydrocarbons especially (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene. These compounds were absent in odours from the populations in the southern part of the distribution where a nitrogen-containing compound, 2-isopropyl-3-methoxypyrazine, and the terpenoid eucalyptol were the dominant compounds. Aldehydes, ketones and esters were present more in the northern populations whereas nitrogen-containing compounds were more prevalent in the southern populations. A shift between southern and northern populations seemed to occur at the Umtamvuna river, where populations had odour profiles with components of both the southern and the northern populations. However, one population in the north (Vernon Crookes Nature Reserve) had a quantitatively similar odour profile to the populations in the

extreme south of the range. These results reveal strong inter-population variation in the cone scent of *E. villosus*, including variation in the relative emission of alkenes that play a key functional role in this pollination system. However, pollinator assemblages did not differ across the different chemotypes which suggests that these patterns were produced by coevolution or drift, rather than by pollinator shifts.

Introduction

Floral odour can play a key role in plant-pollinator interactions through its influence on the composition and behaviour of pollinators that use this trait as a cue (e.g. Patt et al., 1995; Dobson et al., 1997; Schiestl and Ayasse, 2001; Dobson, 2006; Raguso, 2008). Odours are usually blends of compounds belonging to several chemical classes, typically fatty acid derivatives, benzenoids, terpernoids and sometimes nitrogen-containing compounds, and may vary in the number, composition, and relative amounts of the different constituents, and in their temporal and spatial emission patterns (Raguso, 2001; Raguso, 2004; Knudsen et al., 2006). The particular constituents and pattern of odour emission comprise the signals that influence the composition and behaviour of pollinators (Dodson et al., 1969; Pellmyr, 1986a; Dobson et al., 1997; Raguso, 2008). In some cases, highly specialised plant-pollinator interactions are mediated by floral odours (Seymour and Shultz-Motel, 1997; Pellmyr, 1992; Bronstein et al., 2006; Jürgens, 2009) indicating that these odours can provide a species specific signal ("private channel") that elicits the required behavioural response from the pollinating organism. The implication is that floral odour should be a species attribute and it has been argued that the evolution of floral odours has played a role in the diversification of both angiosperms and pollinating insects (Pellmyr and Thien, 1986). The expectation would be that changes in floral odours may be associated with shifts in pollinators that may lead to speciation in plants (Johnson, 1996; Johnson and Steiner, 1997).

Although the majority of studies of floral odour have focused on flowering plants, work on cycads shows that cone odour is an important factor influencing pollinator behaviour (Terry et al., 2007a and b). Differences in odour profiles between cycad species are typically associated with differences in pollinators, such as differences between *Macrozamia* spp pollinated by either thrips (*Cycadothrips*) or weevils (*Tranes*) (Terry et al., 2004a and b). The expectation may therefore be that the volatiles which influence pollinator behaviour will also be invariant species-wide attributes in cycads. To date there have been no studies of volatiles across the full distribution range of any cycad species to test this hypothesis.

Preliminary results reported in Chapter 2 showed that *E. villosus* plants originating from populations in the Eastern Cape (EC) had different odour profiles to those from KwaZulu Natal (KZN). Cone odours from EC populations were characterised by eucalyptol and 2-isopropyl-3-methoxypyrazine whereas cone odours from KZN plants were characterised by (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene (Chapter 2). These differences appear to be inconsistent with the model of a species-wide odour profile and raise questions about possible shifts in pollination systems across the range of *E. villosus* and the relative influence of different compounds on the behaviour of pollinating insects (this is dealt with in Chapter 4).

In the work reported in Chapter 2, cone odours were obtained from a limited sample because variation across the range had not been expected. As a result, it was not possible to determine if variation was clinal or abrupt or was associated with different insect visitors, and it was therefore not possible to test different hypotheses regarding the underlying reason for variation in odour profiles. The aim of the study outlined in this chapter was to determine the extent of variation in cone odour chemistry across the full distribution range of *E. villosus* and to test two possible hypotheses to explain this variation.

The hypotheses were 1) that changes in volatile composition reflect a change in pollinators and may indicate that the current circumscription of *E. villosus* comprises at least two cryptic species; and 2) that variation is due to geographical separation between populations so that populations that are furthest apart emit distinct volatile compounds.

Materials and methods

Plant material and locality

Encephalartos villosus is distributed in a relatively narrow band along the east coast of South Africa (Figure 3.1) with a linear distance of ca. 900 km between the southern-most and northern-most populations. There is some morphological variation in *E. villosus*: plants from the Eastern Cape tend to have shorter heavily spine leaflets and cones with heavily toothed edges while those from KwaZulu-Natal tend to have longer almost entire leaflets and cones with lightly toothed edges (Goode, 1989; Jones, 1993). Across its distribution, *E. villosus* occurs in patches of Scarp Forests including Eastern Scarp Forest, Pondoland Scarp Forest and Transkei Coastal Scarp Forest (Mucina and Geldenhuys, 2006). The forest patches are embedded within three different vegetation types, the KwaZulu Natal Coastal Belt, Pondoland-Ugu Sandstone Coastal Sourveld, and Transkei Coastal Belt (Mucina et al., 2006). Volatile odour samples were collected from 10 localities (Figure 3.1) spread across the range of the species, including populations near to the southern and northern limits of its distribution. In total, samples were obtained from 59 male plants and 14 female plants. Sampling intensity for each locality depended on the availability of cones as *E. villosus* does not cone regularly and cones may be scarce or absent in particular populations (Donaldson, 1997). Male and female cones that were sampled were either shedding pollen or receptive to pollen.

Volatile collection and chemical analysis

As described in detail in Chapter 2, headspace sampling was used to collect volatiles from male and female cones during pollen release and receptivity respectively. Volatiles were collected during daylight between 09h00 and 18h00. Volatile samples were analysed using a coupled Varian 3800 gas chromatograph (Varian Palo Alto, California, USA) and Varian 1200 mass spectrometer (GC-MS).

The Primer 6 programme (Clarke and Gorley, 2006) was used to assess the variation in cone odours of *E. villosus* from different populations (see Chapter 2). Simple Mantel tests were performed using the 'zt' software to determine whether cone odour composition is correlated with the actual geographic distances between populations (Bonnet and Van de Peer, 2002). The odour similarity matrices were calculated using the Bray-Curtis similarity coefficient (Clark and Warwick, 2001). The geographic distance matrix was calculated from actual geographic distances between the different populations following Hughes et al. (2006). Mantel tests with 10,000 permutations were performed for the complete data set.

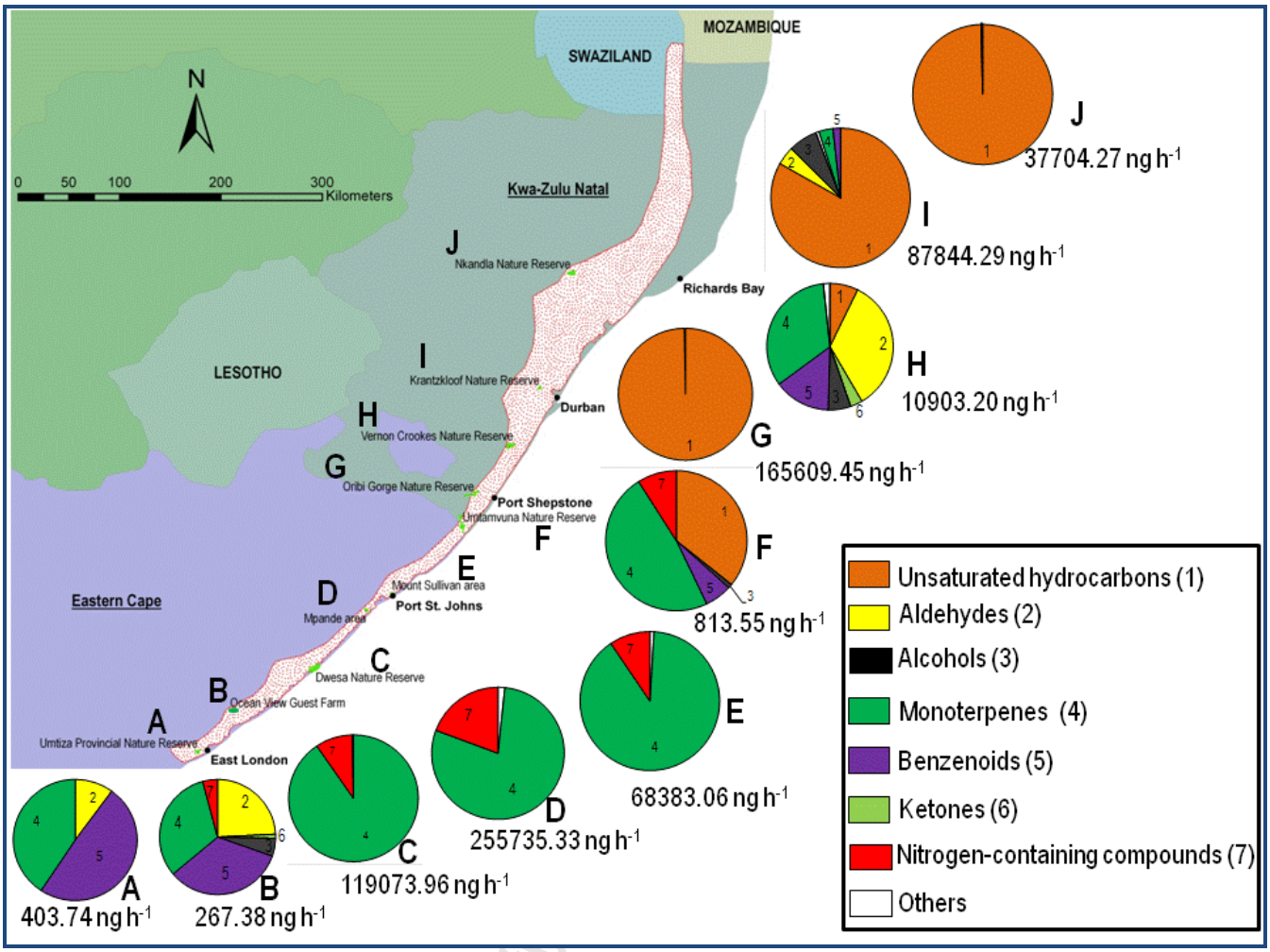

Figure 3.1: Geographic variation in cone odour composition in ten populations (green patches with labels) from south to north across the range of *E. villosus* (dotted area) in South Africa. Total emission rates for each population are shown next to each graph. Numbered pie slices refer to specific compound classes in the legend. Compound class 'Others' contains all compounds and compound classes that contribute below 2 % to the total emission. Pie charts depict percentage of total emission for each population. Eastern Cape populations are; A: Umtiza Nature Reserve (UNR); B: Ocean View Guest Farm (OVGF); C: Dwesa Nature Reserve (DNR); D: Mpande area, Port St. Johns (MPD); E: Mount Sullivan area, Port St. Johns (MTS); KwaZulu Natal populations are: F: Umtamvuna Nature Reserve (UMNR); G: Oribi Gorge Reserve (OGNR); H: Vernon Crookes Nature Reserve (VCNR); I: Kranzkloof Nature Reserve (KKNR); J: Nkandla Forest Reserve (NFR)

Insect visitors to male and female cones

To determine which insects visited *E. villosus*, male and female cones were sampled during pollen shed and pollen receptivity. Insects were collected from cones at the same time that the volatiles were sampled. To survey insects from male cones, a beating sheet was placed under the cone, which was then tapped to dislodge all the insects onto the sheet. The insects were recorded and stored in alcohol. Only insects crawling on the surface of female cones were sampled as these could be collected and stored in alcohol without damaging the female cone.

Results

Compound class patterns in cone odours of *E. villosus*

The chemical composition of cone odours emitted by male and female *E. villosus* is given in Table 3.1. The compounds are identified by common names and CAS (Chemical Abstract Service) registry numbers and listed according to the estimated Kovats retention index (KRI) and in chemical classes which to some degree reflect their biosynthetic origin (Knudsen et al., 2006). In total, 89 compounds were detected in all the odour samples and 87 were identified (Table 3.1). The identified compounds included 20 fatty acid derivatives (four unsaturated hydrocarbons, six aldehydes, two ketones, six alcohols and two esters), nine benzenoids, 54 terpenoids (48 monoterpenes and six sesquiterpenes) and four nitrogen-containing compounds.

The populations of *E. villosus* can be distinguished clearly from the composition of volatile emissions at the level of compound class. Although monoterpenes were present in almost all *E. villosus* populations,

they dominated the volatile profiles of Umtiza Nature Reserve (UNR), Ocean View Guest Farm (OVGF), Dwesa Nature Reserve (DNR), Mpande area (MPD) and Mount Sullivan area (MTS) populations in the EC. Unsaturated hydrocarbons were confined to Umtamvuna Nature Reserve (UMNR), Oribi Gorge Nature Reserve (OGNR), Vernon Crookes Nature Reserve (VCNR), Kranzkloof Nature Reserve (KKNR) and Nkandla Forest Reserve (NFR) populations from KZN and dominated these volatile profiles. The most dominant compound classes were monoterpenes and unsaturated hydrocarbons which contributed 43.71 and 31.04 % of the total emissions respectively while benzenoids, aldehydes and nitrogen-containing compounds contributed 9.96, 7.18 and 5.11 % of the total emissions and sesquiterpenoids were the least emitted compounds proportionally.

Different populations showed different patterns of volatile emissions at the level of compound class, with the highest diversity recorded in VCNR, followed by KKNR and OVGF, and UMNR (Figure 3.1). In the VCNR population, monoterpenes and aldehydes accounted for almost equal amounts (33.17 and 34.15 %) of total volatile emissions whereas unsaturated hydrocarbons accounted for 83.21 % of the total emissions in the KKNR. In the OVGF, benzenoids and monoterpenes comprised 33.51 and 31.96 % of the total emissions whereas in the UMNR, monoterpenes comprised 50 %, unsaturated hydrocarbons 35.93 %, and nitrogen-containing compounds comprised 9.0 % of the total emissions. Compound class diversity was low in UNR, DNR, MPD, MTS, OGNR and NFR (Figure 3.1). In the UNR population, cone volatile composition was dominated by benzenoids (49.23 % of total emission), monoterpene (40.65 %) and aldehydes (10.12 %) whereas monoterpenes and nitrogen-containing compounds dominated total emissions from DNR (78.94 and 19.37 %), MPD (90.43 and 9.30 %) and MTS (89.58 and 9.44 %) and unsaturated hydrocarbons comprised 99.60 and 99.73 % of the total emission in NFR and OGNR.

General overview of pattern of volatile emissions

The number of compounds emitted by male cones varied markedly between populations, ranging from 16 in UNR to 45 in KKNR (Table 3.1). In female plants, as few as 10 compounds were emitted in the MTS population and up to 24 compounds in the KKNR (Table 3.1). In male and female plants, the most commonly occurring compounds found in the majority of populations were *p*-anisaldeyde (all 10 populations), benzaldehyde, eucalyptol and linalool in nine populations, *β*-pinene and *α*-terpinene in eight populations, and phenol and *α*-pinene in seven and six populations respectively. Fatty acid derivatives in the EC populations were composed of only six compounds as compared to 19 compounds in the KZN populations (Table 3.1). Worthy of notice is that all the nitrogen-containing compounds were pyrazine derivatives (Table 3.1).

Table 3.1: Average relative amounts (%) of compounds in the cone odours of *E. villosus* from 10 populations across the species range in the Eastern Cape and KwaZulu Natal. Compounds are identified by common names and CAS (Chemical Abstract Services) registry number, and listed according to the Kovats retention index (KRI) within each compound class. The number of samples in which the compound was identified is given in parentheses. [a]Identification based on mass spectrum, kovats retention index and authentic standard. [b]Identification based on mass spectrum and kovats retention index. [c]Identification based on mass spectrum only. tr = trace amounts (< 0.01 %). [F]Female plants, [M]Male plants, UNR: Umtiza Nature Reserve; OVGF: Ocean View Guest Farm; DNR: Dwesa Nature Reserve; MPD: Mpande area; MTS: Mt Sullivan area; UMNR: Umtamvuna Nature Reserve; OGNR: Oribi Gorge Nature Reserve; VCNR: Vernon Crookes Reserve; KKNR: Kranzkloof Nature Reserve; NFR: Nkandla Forest Reserve

Population and cone sex			UTI_M	OV_M	OV_F	DNR_M	MPD_M	MTS_M	MTS_F	UMNR_M	OGR_M	VCR_M	KKR_M	KKR_F	NFR_M
Number of samples			5	6	4	8	5	8	5	4	6	5	8	5	4
Number of compounds			16	18	17	39	40	20	10	21	22	38	45	24	15
Enission rate (ng cone^{-1} hr^{-1})			80.74	26.49	27.60	14884.25	51147.07	8478.34	111.27	203.39	27601.58	2180.64	10929.64	81.44	9426.07
Compound	CAS	KRI													
ALIPHATICS															
Unsaturated hydrocarbons															
(3*E*)-1,3-Octadiene[a]	1002-33-1	1062	-	-	-	-	-	-	-	7.04 (4)	9.76 (6)	0.02 (5)	64.65 (8)	46.74 (5)	47.53 (4)
(3*E*,5*Z*)-1,3,5-Octatriene[b]	40087-61-4	1148	-	-	-	-	-	-	-	30.65 (4)	89.53 (6)	0.02 (5)	33.10 (8)	11.56 (5)	51.52 (4)
(*E*,*E*,*E*)-2,4,6-Octatriene[b]	15192-80-0	1216	-	-	-	-	-	-	-	-	0.36 (6)	17.29 (5)	tr (4)	tr (4)	0.22 (4)
1,2-Dimethyl-1,4-cyclohexadiene[b]	17351-28-9	1217	-	-	-	-	-	-	-	-	0.08 (4)	6.01 (5)	tr (3)	tr (4)	0.41 (4)
Aldehydes															
Hexanal[a]	66-25-1	1125	-	-	-	-	-	-	tr (2)	-	-	-	-	3.51 (5)	-
Heptanal[b]	111-71-7	1209	10.12 (4)	30.50 (5)	14.70 (2)	-	-	-	-	-	-	23.65 (5)	-	7.51 (4)	-
(*Z*)-2-Heptenal[a]	57266-86-1	1340	-	-	-	-	-	-	-	-	-	-	tr (1)	-	-
(*E*)-2-Octenal[b]	2548-87-0	1446	-	-	-	-	-	-	-	-		1.02 (5)	-	-	-
(2*E*,4*E*)-Hepta-2,4-dienal[b]	4313-5-3	1513	-	-	-	-	-	-	-		tr (4)	-	0.01 (2)	0.69 (1)	-
2,4-Octadienal[b]	30361-28-5	1610	-	-	-	-	-	-	-		tr (5)	-	0.01 (2)	0.09 (1)	-
Ketones															
3-Octanone[b]	106-68-3	1274	-	1.97 (2)	-	-	-	-	-	-	-	2.33 (5)	0.06 (6)		-
2,2,6-Trimethyl-6-vinyldihydro-2H-pyran-3(4H)-one[c]	33933-72-1	1489	-	-	-	-		-	-	-	-	0.06 (3)	-	-	-

Table 3.1 continued

Population and cone sex			UTI_M	OV_M	OV_F	DNR_M	MPD_M	MTS_M	MTS_F	UMNR_M	OGR_M	VCR_M	KKR_M	KKR_F	NFR_M
Number of samples			5	6	4	8	5	8	5	4	6	5	8	5	4
Number of compounds			16	18	17	39	40	20	10	21	22	38	45	24	15
Emission rate (ng cone^{-1} hr^{-1})			80.74	26.49	27.60	14884.25	51147.07	8478.34	111.27	203.39	27601.58	2180.64	10929.64	81.44	9426.07
Compound	CAS	KRI													
Alcohols															
(Z)-3-Hexen-1-ol[b]	928-96-1	1390	-	-	-	-	-	-	-	0.41 (4)	-	-	-	-	-
3-Octanol[a]	589-98-0	1386	-	3.67 (3)	-	-	-	-	-	-	0.02 (5)	2.74 (5)	0.35 (6)	-	-
1-Octen-3-ol[a]	3391-86-4	1456	-	-	7.13 (4)	-	-	-	-	0.66 (3)	tr (5)	2.80 (5)	0.35 (6)	16.60 (3)	0.02 (4)
Lavandulol[b]	498-16-8	1690	-	-	-	tr (1)	tr (4)	-	-	-	-	-	-	-	-
1,7-Octadien-3-ol[c]	30385-19-4	2086	-	-	-	-	-	-	-	-	-	0.61 (5)	-	-	-
Esters															
2-Phenethyl hexanoate[c]	6290-37-5	1270	-	-	-	-	-	-	-	-	-	-	-	1.89 (4)	-
Methyl 2,4-hexadienoate[c]	1515-80-6	1358	-	-	-	-	-	-	-	-	-	1.12 (5)	-	-	-
BENZENOIDS															
Anisole[a]	100-66-3	1357	-	-	-	-	-	-	-	-	-	-	0.03 (1)	1.17 (1)	-
Benzaldehyde[a]	100-52-7	1553	15.78 (5)	14.62 (6)	16.17 (4)	0.02 (8)	0.63 (5)	-	-	1.03 (4)	0.01 (6)	2.72 (5)	0.01 (8)	2.89 (5)	0.03 (4)
1-Isopropyl-2-methoxy-4-methylbenzene[b]	1076-56-8	1616	-	-	-	tr (4)	-	-	-	-	-	-	-	-	-
Methyl benzoate[a]	93-58-3	1646	1.02 (5)	0.65 (3)	0.70 (4)	-	tr (2)	-	-	-	-	-	tr (1)	-	-
Methyl salicyclate[a]	119-36-8	1808	-	-	-	-	-	-	-			-	tr (1)	-	-
α-Methyl-benzyl alcohol[b]	98-85-1	1832	-	-	-	-	-	-	-	-	tr (5)	0.23 (5)	-	-	-
Benzyl alcohol[a]	100-51-6	1896	1.12 (4)	4.51 (6)	8.44 (4)	-	0.01 (1)	-	-	-	tr (2)	-	tr (4)	-	-
Phenol[a]	108-95-2	2032	2.04 (5)	1.54 (5)	1.47 (4)	-	0.13 (3)	0.03 (8)	0.93 (3)	-	tr (3)	-	tr (3)	0.14 (2)	0.01 (4)
p-Anisaldehyde[a]	123-11-5	2061	29.27 (5)	15.17 (6)	2.28 (4)	0.23 (7)	0.92 (5)	0.98 (8)	tr (2)	5.62 (4)	0.03 (6)	7.46 (5)	0.02 (8)	tr (2)	0.09 (4)

Table 3.1 continued

Population and cone sex			UTI_M	OV_M	OV_F	DNR_M	MPD_M	MTS_M	MTS_F	UMNR_M	OGR_M	VCR_M	KKR_M	KKR_F	NFR_M
Number of samples			5	6	4	8	5	8	5	4	6	5	8	5	4
Number of compounds			16	18	17	39	40	20	10	21	22	38	45	24	15
Enission rate (ng cone^{-1} hr^{-1})			80.74	26.49	27.60	14884.25	51147.07	8478.34	111.27	203.39	27601.58	2180.64	10929.64	81.44	9426.07
Compound	CAS	KRI													
TERPENOIDS															
Monoterpenes															
α-Pinene[a]	7785-70-8	1095	tr (4)	-	-	7.35 (8)	23.65 (4)	13.96 (8)	24.31 (5)	-	-	0.10 (2)	0.04 (1)	4.42 (1)	-
β-Thujene[a]	28634-89-1	1102	-	-	-			0.19 (2)		-	-	-	-	0.32 (1)	-
Camphene[a]	79-92-5	1112	-	-	-	7.92 (8)	10.50 (4)	9.50 (8)	25.08 (4)	-	-	-	-	-	-
Unknown		1154	-	-	-	0.29 (7)	0.09 (3)	-	-	0.10 (3)	-	-	-	-	-
β-Pinene[a]	127-91-3	1194	34.05 (4)	0.01 (4)	10.11 (2)	16.55 (8)	2.20 (3)	5.91 (5)	5.89 (4)	3.01 (4)	0.15 (5)	-	0.07 (1)	tr (1)	-
β-Myrcene[a]	123-35-3	1199	tr (4)	-	-	-	-	0.31 (4)	tr (4)	tr (3)	-	-	0.07 (2)	1.54 (4)	-
Unknown		1213	-	-	-	10.33 (4)	2.46 (5)	-	-	6.54 (4)	-	-	0.02 (3)	-	-
α-Terpinene[a]	99-86-5	1220	-	-	-	24.97 (8)	11.26 (5)	11.29 (7)	-	21.56 (4)	-	-	tr (1)	-	-
Limonene[a]	138-86-3	1224	-	-	7.29 (2)	-	0.51 (5)	tr (3)	tr (2)	-	-	-	-	1.69 (1)	-
Eucalyptol[a]	470-82-6	1231	2.55 (5)	11.87 (5)	11.55 (3)	13.88 (8)	18.62 (5)	38.22 (8)	40.04 (5)	1.82 (4)	-	1.32 (5)	0.76 (2)	0.02 (1)	0.05 (2)
trans-β-Ocimene[a]	502-99-8	1267	0.83 (2)	-	-	-	-	tr (1)	-	1.06 (4)	-	-	0.02 (1)	-	0.08 (2)
γ-Terpinene[a]	99-85-4	1269	-	-	-	2.66 (8)	3.29 (5)	2.41(6)	-	1.52 (4)	-	-	-	-	-
cis-β-Ocimene[a]	3338-55-4	1275	-		-	-	-	tr (1)	-	-	0.05 (4)	-	0.02 (1)	tr (1)	-
p-Cymene[a]	99-87-6	1294	1.56 (3)	4.30 (2)	12.72 (3)	5.14 (8)	5.25 (5)	-	-	8.21 (4)	-	-	-	-	-
α-Terpinolene[a]	586-62-9	1304	-	-	-	0.71 (8)	-	-	-	-	-	-	-	-	-
Allo-ocimene[b]	673-84-7	1391	-	-	-	tr (1)	-	-	-	-	-	-	-	-	-
(3*E*,5*E*)-2,6-Dimethyl-1,3,5,7-octatetraene[c]	460-01-5	1419	-	-	-	tr (1)	-	-	-	-	-	-	-	-	-
Perillene[b]	539-52-6	1428	-	-	-	0.01 (3)	tr (4)	-	-	-	-	-	-	-	-
trans-Linalool oxide (Furanoid)[a]	5989-33-3	1453	-	1.41 (2)	-	0.01 (3)	-	-	-		-	-	0.03 (1)	-	-

Table 3.1 continued

Population and cone sex			UTI_M	OV_M	OV_F	DNR_M	MPD_M	MTS_M	MTS_F	UMNR_M	OGR_M	VCR_M	KKR_M	KKR_F	NFR_M
Number of samples			5	6	4	8	5	8	5	4	6	5	8	5	4
Number of compounds			16	18	17	39	40	20	10	21	22	38	45	24	15
Enission rate (ng cone^{-1} hr^{-1})			80.74	26.49	27.60	14884.25	51147.07	8478.34	111.27	203.39	27601.58	2180.64	10929.64	81.44	9426.07
Compound	CAS	KRI													
cis Linalool oxide (Furanoid)[a]	34995-77-2	1467	-	-	0.56 (4)	0.01 (5)	tr (1)	-	-	0.17 (2)	-	0.24 (5)	0.04 (2)	-	-
Nerol oxide[b]	1786-08-9	1488	-	-	-	0.01 (3)	0.01 (1)	-	-	-	-		-	-	-
Camphor[a]	464-48-2	1543	-	-	-	tr (2)	0.01 (3)	-	-	-	-	-	-	-	-
Linalool[a]	78-70-6	1562	0.37 (2)	1.68 (5)	3.44 (4)	0.02 (8)	0.17 (5)	-	-	0.50 (4)	0.01 (6)	20.93 (5)	0.02 (8)	0.02 (2)	0.01 (4)
4-Isopropyl-1-methyl-2-cyclohexen-1-ol[a]	29803-82-5	1576	0.57 (5)	0.69 (3)	0.01 (3)	tr (2)	tr (4)	-	-	-	-	-	-	-	-
Camphene hydrate[c]	465-31-6	1615	-	-	-	-	-	-	-	-	-	0.02 (1)	-	-	-
4-Terpineol[a]	562-74-3	1613	-	-	0.28 (1)	0.17 (4)	0.42 (4)	-	-	0.12 (2)	-	0.15 (5)	tr (2)	-	-
p-Menth-1-en-4-ol[c]	20126-76-5	1618	-	-	-	0.16 (4)	-	-	-	-	-	-	-	-	-
β-Cyclocitral[b]	432-25-7	1647	-	-	-	tr (7)	-	-	-	0.26 (4)	tr (5)	0.15 (5)		-	-
Myrtenal[a]	564-94-3	1650	-	-	-	-	tr (2)	-	-			0.29 (5)	0.02 (6)	-	-
β-Citral[b]	106-26-3	1703	-	-	-	tr (1)	0.01 (3)	-	-	-	-	-	-	-	-
α-Terpineol[a]	10482-56-1	1720	0.72 (5)	0.93 (4)	1.56 (2)	0.11 (6)	0.22 (5)	3.68 (7)	-	0.03 (2)		1.42 (5)	0.02 (2)	0.24 (1)	-
trans-p-Menth-2-en-7-ol[c]	19898-87-4	1717	-	-	-	-	-	-	-	-	-	-	0.02 (4)	-	-
Borneol[a]	507-70-0	1725	-	0.47 (3)	-	tr (1)	0.01 (4)	-	-	-	-	0.30 (5)		-	-
α-Cyclogeraniol[c]	6627-74-3	1737	-	-	-	-	-	-	-	-	-	0.89 (5)	0.13 (4)	-	-
Citral[b]	5392-40-5	1732	-	-	-	-	tr (4)	0.02(2)	-	-	-	-	-	0.31 (1)	-
trans-Linalool oxide (Pyranoid)[a]	5989-33-3	1755	-	0.22 (2)	-	-	-	-	-	-	-	0.12 (5)	0.01 (3)	-	-
Piperitone oxide[c]	5286-38-4	1760	-	-	-	-	-	0.02 (1)	-	-	-	-	-	-	-
cis-Linalool oxide (Pyranoid)[a]	5989-33-3	1781	-	-	-	-	-	-	-	-	-	-	0.05 (4)	-	-
cis-Geraniol[b]	106-25-2	1803	-		-	0.02 (1)	0.19 (4)	0.43 (6)	-	-	-	-	-	-	-
Dihydro-*β*-ionone[c]	17283-81-7	1807	-	-	-	-	-	-	-	-	tr (4)	-	tr (1)	-	0.01 (2)

Table 3.1 continued

Population and cone sex			UTI_M	OV_M	OV_F	DNR_M	MPD_M	MTS_M	MTS_F	UMNR_M	OGR_M	VCR_M	KKR_M	KKR_F	NFR_M
Number of samples			5	6	4	8	5	8	5	4	6	5	8	5	4
Number of compounds			16	18	17	39	40	20	10	21	22	38	45	24	15
Enission rate (ng cone^{-1} hr^{-1})			80.74	26.49	27.60	14884.25	51147.07	8478.34	111.27	203.39	27601.58	2180.64	10929.64	81.44	9426.07
Compound	CAS	KRI													
Myrtenol[a]	515-00-4	1815	-	-	-	-	-	-	-	-	-	0.79 (5)	0.01 (6)	-	-
Nerol[b]	106-24-1	1817	-	-	-	0.05 (3)	-	-	-	-	-	-	-	-	-
Grandisol[b]	26532-22-9	1821	-	-	-	-	-	-	-	-	-	1.08 (5)	0.03 (3)	-	-
p-Cymen-8-ol[b]	1197-01-9	1872	-	-	-	0.01 (7)	0.01 (5)	-	-	-	-	-	tr (1)	-	-
α-Ionone[b]	127-41-3	1875	-	-	-	-	-	-	-	-	tr (4)	1.54 (5)	-	-	0.01 (4)
exo-2-Hydroxycineole[b]	92999-78-5	1877	-	-	-	tr (3)	-	-	-	-	-	0.12 (5)	-	-	-
cis-Myrtanol[c]	514-99-8	1893	-	-	-	-	-	-	-	-	-	0.31 (5)	-	-	-
Dihydro-*α*-ionone[c]	31499-72-6	1989	-	-	-	-	-	-	-	-	tr (2)	0.24 (5)	-	-	-
p-Cymen-3-ol[a]	89-83-8	2225	-	-	-	0.02 (3)	0.03 (4)	0.45 (7)	-	-	-	1.23 (5)	-	-	-
p-Cymen-2-ol[b]	499-75-2	2232	-	-	-	0.02 (3)	0.03 (4)	0.03 (1)	-	-	-	-	-	-	-
Sesquiterpenes															
Dihydroedulan I[c]	63335-66-0	1538	-	-	-	tr (3)	tr (2)	-	-	-	tr (2)	0.02 (5)	-	-	-
α-Bergamotene[b]	17699-05-7	1604	-	-	-	-	tr (1)	-	-			0.22 (5)	-	-	-
β-Caryophyllene[b]	87-44-5	1636	-	-	-	-	-	-	-	-	-	-	tr (1)	-	0.01 (2)
β-cubebene[b]	13744-15-5	1738	-	-	-	-	-	-	-	-	-	-	tr (1)	-	0.01 (2)
(*E*,*E*)-*α*-Farnesene[b]	502-61-4	1760	-	-	-	-	tr (2)	-	-	-	-	0.22 (5)	tr (1)	-	-
α-Curcumene[c]	644-30-4	1796	-		-	tr (4)	tr (3)	-	-	-	tr (4)	0.21 (5)	tr (3)	0.04 (1)	-
NITROGEN-CONTAINING COMPOUNDS															
2,5-Dimethylpyrazine[b]	123-32-0	1336	-	-	-	tr (3)	0.01 (5)	-	-	-	-	-	0.03 (6)	-	-
2-methoxy-3-methylpyrazine[c]	2847-30-5	1392	-	-	-	-	tr (3)	-	-	-	-	-	-	-	-
2-Isopropyl-3-methoxypyrazine[a]	25773-40-4	1452	0.01 (5)	5.80 (6)	1.60(4)	9.31 (8)	19.32 (5)	12.58 (8)	3.75 (5)	9.66 (4)	-	-	-	-	-
2-sec-Butyl-3-Methoxypyrazine[c]	24168-70-5	1519	-	-	-	0.01 (7)	0.04 (5)	-	-	-	-	-	-	-	-

Cone odour variation between populations

In addition to eucalyptol, one-way SIMPER (factor population) showed that benzaldehyde, heptanal, *β*-pinene, linalool and *p*-anisaldehyde were important components defining the emissions of UNR, OVGF and VCNR populations, explaining between 37 and 73 % of the similarity among samples in these populations. In contrast, 2-isopropyl-3-methoxypyrazine, *α*-pinene, camphene and *α*-terpinene characterised the emissions of DNR, MPD and MTS populations explaining between 56 and 80 % of the similarity among individuals in the different populations. The samples from OGNR, KKNR and NFR were characterised by high relative amounts of (3*E*,5*Z*)-1,3,5-octatriene and (3*E*)-1,3-octadiene which was responsible for over 90 % similarity among samples in these populations. The similarity among samples from UMNR, which occurs at the boundary between EC and KZN, was explained by the presence of (3*E*,5*Z*)-1,3,5-octatriene, otherwise found in KZN populations, as well as *α*-terpinene and 2-isopropyl-3-methoxypyrazine which occur in the EC.

These volatile variations contribute to the distinct separations of clusters of *E. villosus* samples from different populations in the two-dimensional representation of the NMDS (Figure 3.2). Consequently, the NMDS analysis of cone odour compounds of *E. villosus* (Figure 3.2), showed a significant separation between the different populations (NMDS stress value 0.12; one-way ANOSIM, factor population: Global $R = 0.835$, $P < 0.01$). Out of 45 pair-wise comparisons between the different populations, significant separations were found between 17 populations despite a majority of them having high *R* values (Table 3.2). The clustering of populations based on chemical profiles (Figure 3.2) tended to follow the overall geographic pattern of separation of EC and KZN populations.The only exceptions were individuals from VCNR in KZN in which volatile were characterised by heptanal, linalool, *p*-anisaldehyde and benzaldehyde and had closer affinities to populations from the south of the species range (OVGF and UNR, Figure 3.2).

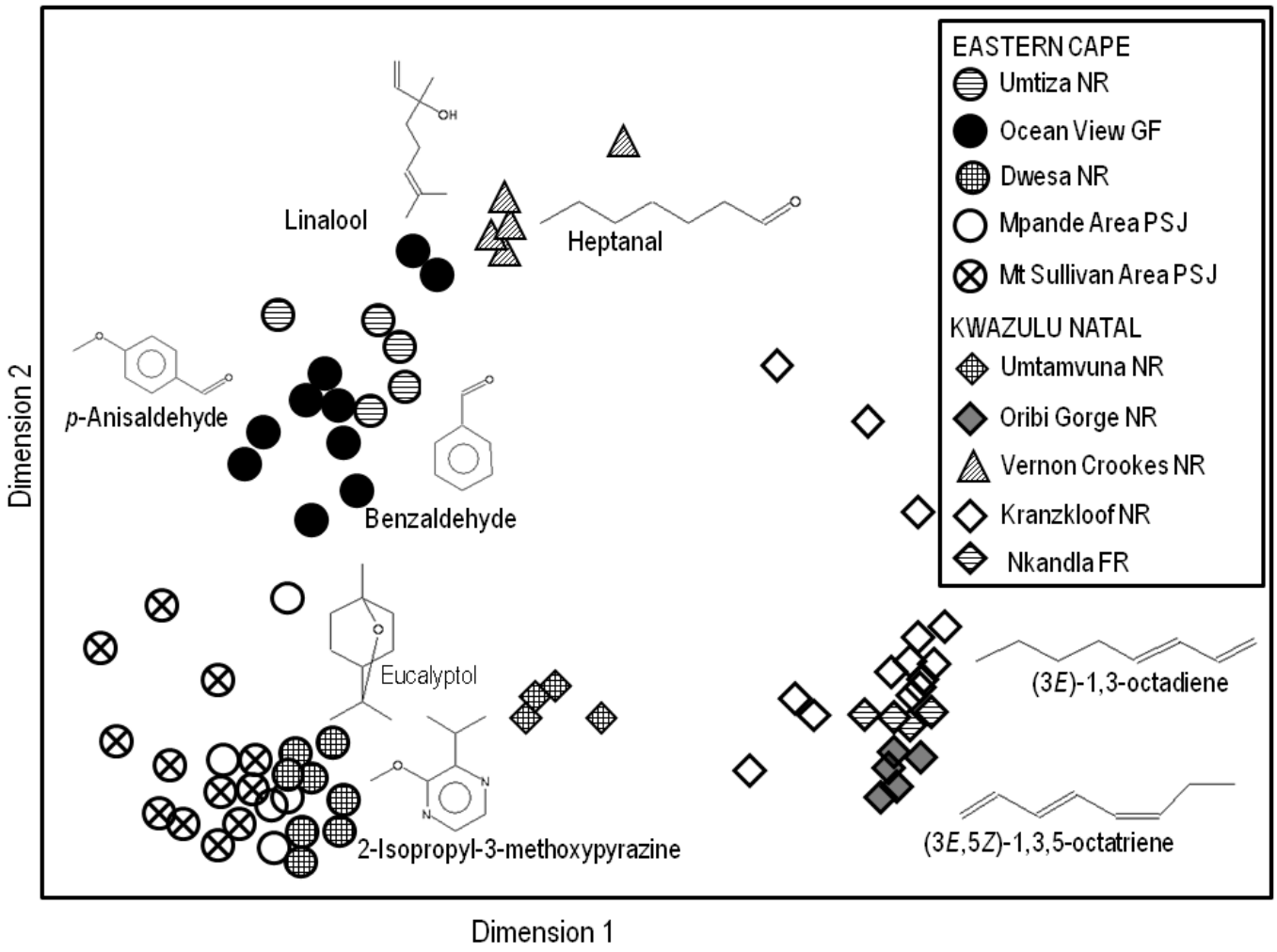

Figure 3.2: Non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarities showing geographic variation in cone odour composition comprising 88 compounds from 73 samples in 10 populations across the distribution range of *E. villosus* from the south in the EC to the north in KZN. (2D stress value: 0.12; ANOSIM Global *R* (population) = 0.835, $P < 0.01$). The structures and names of the eight main compounds dominating the volatile profile of cone odour at different *E. villosus* populations are presented in the figure.

Table 3.2: Test statistics (R) of pair-wise comparisons (ANOSIM) between populations of *E. villosus.*

	UNR[a]	OVGF	DNR	MPD	MTS	UMNR	OGNR	VCNR	KKNR	NFR
UNR										
OVGF	0.41									
DNR	1.00	**0.98**[b**]								
MPD	0.97	**0.92***	0.41							
MTS	**1.00****	**0.97****	0.42	0.13						
UMNR	1.00	0.96	1.00	0.88	**0.95***					
OGNR	1.00	**1.00****	**1.00***	1.00	**1.00***	1.00				
VCNR	0.90	**0.76***	1.00	1.00	**1.00****	1.00	1.00			
KKNR	**1.00****	**0.98****	**1.00****	**1.00****	**1.00****	0.70	0.44	**1.00****		
NFR	1.00	1.00	1.00	1.00	**1.00***	1.00	1.00	1.00	-0.08	

[a]Names of populations are the same as in Table 3.1.

[b]Bold values indicate populations that are significantly different. ** $p < 0.01$; * $p < 0.05$

The lack of significant differences in pairwise comparison between populations with high *R* values (Figure 3.2) might be explained by the fact that individuals from these populations had similar volatile compounds.

Mantel tests were performed in order to compare cone odour matrices calculated using the Bray-Curtis similarity coefficient (Clark and Warwick, 2001) with the matrix of geographic distance (km) between populations. The results showed a significant correlation ($r = 0.39$; $P = 0.001$) between similarity in cone volatiles and geographic distance across the full range of *E. villosus*. When the data were analysed in subsets, changes in volatile composition were strongly correlated with distance between populations in EC ($r = 0.78$; $P = 0.008$) and might be indicative of restricted gene flow between the populations. There was no correlation between volatile composition and geographic separation in KZN populations ($r = 0.05$; $P = 0.51$). This is indicated by the lack of variation in the dominant compounds across the populations especially at OGNR, KKNR and NFR (Figure 3.1).

Insect pollinators on male and female cones of *E. villosus*

At pollen shed, male cones of *E. villosus* in all the populations were visited by an undescribed species of *Porthetes* (Figures 3.3c; Table 3.3). Oberprieler (1996) gave the latter species a manuscript name of *P. pearsonii* but the description has not been formally published. Two other beetle species (Coleoptera) namely an undescribed Erotylidae sp. nov. and *Metacucujus goodei* Endrödy-Younga (Boganiidae) (Figure 3.3a and b), were collected from all the populations except UMNR and NFR (Table 3.3). Their absence from UMNR and NFR might have been due to the small number of cones (four male cones) in each of these populations. *Porthetes* sp. was observed actively moving in between the sporophylls, along the cone axis and on the cone surface. Erotylidae sp. nov. and *M. goodei* were observed actively moving in between the sporophylls and along the cone axis. Female cones of *E. villosus* in the three populations sampled were visited by Erotylidae sp. nov. and *Porthetes* sp., (the same taxa as on male cones) (Table 3.3) and *Antliarhinus zamiae* (Figure 3.3 d-e; Table 3.3). Female *A. zamiae* were observed piercing the cone sporophylls with their rostrums and crawling over the cone surface. Similarly, the male *A. zamiae* were crawling on the cone surface and some were forcing their way in between the tight megasporophylls. In some cases male and female *A. zamiae* were observed mating on the cone surface (Figure 3.3 f). A few Erotylidae sp. nov. and *Porthetes* sp. were actively crawling on the female cone surface and some were found forcing their way in between the tightly packed megasporophylls.

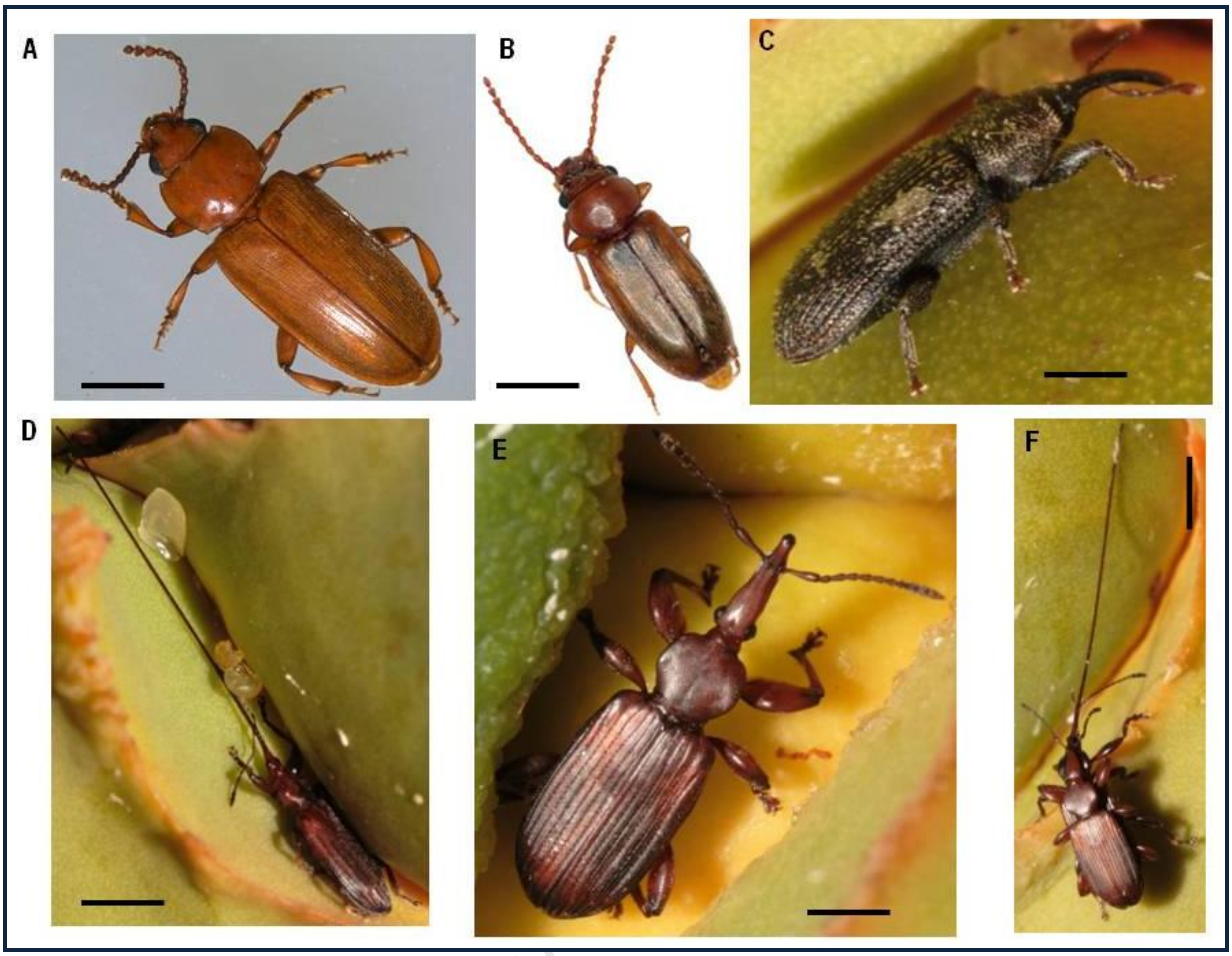

Figure 3.3: Insects which visit male and/or female cones of *Encephalartos villosus*. **A**) An undescribed Erotylidae sp. nov. (male and female cones); **B**) *Metacucujus goodei* (male cones); **C**) *Porthetes* sp (male and female cones); **D**) female *Antliarhinus zamiae* (female cones); **E**) male *Antliarhinus zamiae* (female cones); **F**) male and female *Antliarhinus zamiae* mating. Scale bars = 1000 µm

Table 3.3: Mean (± S.E) number of insects of different species collected from male and female cones of *Encephalartos villosus* at different populations

Population[a]	Cone sex	No. of cones	Insect species and number of individuals			
			Antliarhinus zamiae	Erotylidae sp. nov.	*Metacucujus goodei*	*Porthetes* sp.
UNR	Male	5	-	1.4 ± 0.5	1.8 ± 0.7	7.0 ± 0.9
OVGF	Male	5	-	2.6 ± 1.5	2.0 ± 0.9	15.8 ± 3.6
	Female	5	15.5 ± 3.0	2.8 ± 1.2	-	1.4 ± 0.6
DNR	Male	4	-	86.0 ± 23.0	60.8 ± 11.3	168.0 ± 36.3
MPD	Male	4	-	101.0 ± 16.8	47.8 ± 9.6	191.8 ± 9.1
MTS	Male	5	-	11.0 ± 3.4	7.4 ± 2.3	37.6 ± 9.7
	Female	3	18.0 ± 5.3	1.0 ± 0.6	-	3.0 ± 1.0
UMNR	Male	4	-	-	-	22.5 ± 4.3
OGNR	Male	5	-	43.4 ± 4.3	31.2 ± 3.1	93.4 ± 3.5
VCNR	Male	4	-	64.5 ± 4.8	31.7 ± 3.4	186.3 ± 15.5
KKNR	Male	5	-	34.4 ± 7.2	16.6 ± 4.3	213.2 ± 21.1
	Female	4	17.8 ± 2.0	2.5 ± 0.6	-	11.0 ± 1.8
NFR	Male	4	-	-	-	65.5 ± 6.6

[a]Names of populations are the same as in Table 3.1.

Discussion

The results presented in this chapter confirm preliminary findings from a smaller set of populations (Chapter 2) that there is considerable variation in the chemical composition of cone volatiles between populations of *E. villosus*. The more extensive dataset collected and analysed in this chapter shows a complete shift from dominance of monoterpenes in the southern part of the range (e.g. DNR, MPD and MTS around Port St Johns) to dominance of unsaturated hydrocarbons in the northern part of the range (e.g. KKNR, NFR) and overlap of volatile composition in the centre of the range (e.g. UMNR) (Fig 3.1).

Studies of geographical variation in plant traits have shown several outcomes, including lack of structured variation across the range (Svensson *et al.,* 2005), clinal variation (Knudsen 2002), and discrete or saltational variation reflecting adaptation to different pollinators (Schlumberger & Raguso, 2008). The Mantel test for *E. villosus* data provides statistical evidence for volatile profiles being associated with geographic separation, while the cluster analysis indicates that this variation is more consistent with discrete or saltational changes, than with clinal changes across the range. Further studies are required to explore genetic structure across the different populations of *E. villosus* that may be linked to differences in cone volatiles.

This study provides the first detailed investigation of geographic variation of volatile composition in *E. villosus* and is the first detailed geographic analysis of volatile composition in any cycad species. Out of 87 identified compounds, only five compounds occurred in high relative amounts (≥ 30 %) in at least one population (Table 3.1). The majority of the compounds were emitted in small relative amounts ranging from trace amounts to just above 20 %. Terpenoids particularly monoterpenes are the most numerous compounds in the volatile blend of *E. villosus* and some of them together with some benzenoids occur in almost all the populations (Table 3.1). Their occurrence in almost all populations suggests that they could

be critical compounds that serve different functions and require further investigation. The unsaturated hydrocarbons are few but include the most abundant compounds (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene emitted by plants from KZN populations. These compounds have been recorded in the volatile profile of few plants and have been identified as possible insect attractants (Skubatz *et al.,* 1996).The unsaturated hydrocarbons are thought to have the same biosynthetic pathway normally derived from decarboxylation of fatty acids and then dehydrogenation of 1-octen-3-ol (Larsen and Frisvad, 1995).

Four different pyrazines occurred in varying amounts mostly in plants from the EC populations with 2-isopropyl-3-methoxypyrazine as a dominant compound. Generally, pyrazines have distinct sensory properties and have been associated with warning signals, alerting signals to predators, aggregation pheromones of insects, and oviposition stimulants (Rothschild et al., 1984; Moore et al., 1990; Abassi et al., 1998). Pyrazines have been recorded in the volatile profile of some plants (Kaiser, 2006; review by Knudsen et al., 2006; Suinyuy et al., 2010) and have been identified as possible insect attractants (Ervik et al., 1999).The different pyrazines could therefore fullfil different functions in cycads. Also, different pyrazine formations have been linked to different biosynthetic pathways and the variation observed might be as a result of that. For example, 2-sec-butyl-3-methoxypyrazine is suggested to be formed from leucine (Belitz et al., 2009) while the synthesis of 2-isopropyl-3-methoxypyrazine is from the initial condensation of the amino acids valine and glycine (Cheng et al., 1991).

Although *E. villosus* exhibited geographical variation in cone volatile emissions, the study showed that eight of the 88 compounds occurred in almost all the populations (Table 1) and may be of critical importance in influencing insect behaviour since the same insect assemblages occurred in all populations (Table 3.3).It is noteworthy that the pattern of geographical variation in *E. villosus* cone odour is explained best by changes in only two dominant compounds, 2-isopropyl-3-methoxypyrazine and (3*E*)-1,3-octadiene.

The nitrogen-containing compound 2-isopropyl-3-methoxypyrazine occurs in all southern populations from UNR to UMNR and increases in relative amounts from UNR to MTS. In contrast (3*E*)-1,3-octadiene occurs in all northern populations starting from UMNR and increasing along the northern gradient to NFR (Figure 3.1).

Samples from UMNR in the centre of the distribution range were characterised by compounds from both the southern and northern populations. The UMNR is situated in the eastern part of the Umtamvuna river gorge and occurs within the Maputaland-Pondoland center of endemism. Pondoland is dominated by ancient outcrops of nutrient-poor quartzite sandstone that seem to have acted as edaphic barriers to plant migration (Carbutt and Edwards, 2001) and support a resident flora that is apparently trapped by these barriers (Barnes et al., 2001). However, it is not clear as to why this pattern of odour compounds occur in the UMNR. But the results and the edaphic barrier theory of Carbutt and Edwards (2001) are congruent with the suggestion that there is a biogeographical barrier around the Umtamvuna area which may prevent migration of gene flow across boundaries and thus influence the volatile composition of the resident plants.

The VCNR population, sandwiched between OGNR and KKNR, has a suite of volatile compounds similar to that of plants from the KKNR but present in different relative quantities (Figure 3.1). The dominant compounds are monoterpenes, aldehydes and benzenoids and are closest to those emitted by plants from the Eastern Cape region (Figure 3.1). This suggests that these compounds occurred more widely across the range, but that only plants from the VCNR population have retained and expressed the genes for biosynthesis of all the volatile compounds once present in the different populations. Long distance dispersal of seeds from the southern part of the range is highly unlikely to account for the volatile components of the VCNR population. There is no known long distance dispersal mechanism and cycad dispersal is typically within a short distance of the parent plant (Donaldson, 1995; Snow and Walter, 2007). Long range pollen

dispersal (> 5km) also seems unlikely as Donaldson (1997) discovered that *Porthetes* sp., Erotylidae sp. nov., *M. goodei*, and *A. zamiae* lost a substantial amount of pollen within a few hours after they left the pollen shedding cones of *E. villosus* and that plants situated > 5km from source populations never had insect pollinators present (Donaldson unpublished data).

Intra-specific variation in plant morphology is well documented, but an increasing number of studies are revealing variation in chemical traits (e.g. Azuma et al., 1997, 2001; Dötterl et al., 2005; Chess et al., 2008; Jhumur et al., 2008; Schlumpberger and Raguso, 2008; Soler et al., 2011). Hypotheses for intraspecific trait variation include phenotypic plasticity, neutral processes such as drift, adaptive processes such as coevolution or pollinator shifts, and local hybridization. The evidence for each of these hypotheses is weighed up in relation to the geographical variation in the cone odour of *E. villosus*.

Phenotypic plasticity seems highly unlikely to account for the geographical odour patterns observed in *E. villosus* as plants from populations in EC which have been growing under different environmental conditions in the Kirstenbosch Botanic Garden in Cape Town for close to 100 years emitted the same volatile compounds as those from the natural populations (Chapter 2).

Drift seems also unlikely to account for variation in the major compounds as these have been shown to play functional roles in pollinator attractions (Chapter 4). However, neutral and adaptive processes could apply to different compounds. For example, geographic variation in the volatile profile may occur only in compounds that are not used by pollinators to find and locate host plants (e.g. Dötterl et al., 2005; Schiestl, 2005; Füssel et al., 2007). Pollinator shifts have been invoked for cases where there are different pollinators in different geographic areas and these can result in quantitative shifts involving changes in assemblages for plant species which are visited by a number of different pollinators (Pellymr, 1986b;

Schlumpberger and Raguso, 2008), or involve complete transitions (Johnson and Steiner 1997). However, variation in floral traits can also occur without pollinator shifts (Ellis and Johnson, 2009). This was evidently the case for *E. villosus* which showed no change in beetle species composition across the distribution range. The same insect visitors were recorded from all *E. villosus* populations (i.e. *A. zamiae*, Erotylidae nov. sp, *M. goodei* and *Porthetes* sp.) despite the difference in volatile compounds. A recent molecular study to determine phylogenetic relationships concluded that the *Porthetes* sp. from different *E. villosus* populations across the range of distribution as well as from the related *E. umbeluziensis,* comprised a single species (Downie et al., 2008). This suggests that changes in cone volatiles are not associated with different pollinator assemblages. However, it is still possible that there has been localised co-evolution between these insects and *E. villosus* which is not reflected in molecular markers or morphology of the beetles. Performing scent bioassays at different sites will aid in understanding whether beetles, which are ostensibly the same species, exhibit regional differences in volatile preferences that could account for the geographical variation in the cone odours of *E. villosus*.

Hybridization among different *Encephalartos* species may account for some of the geographical variation in scent of *E. villosus*. It is interesting that *E. altensteinii* and *E. natalensis* which are morphologically similar and closely related species (Treutlein et al., 2005) occur around the Umtamvuna river and other *Encephalartos* spp overlap with *E. villosus* in its distribution range. These cycads cone the same time as *E. villosus.* They emit similar odour compounds (3*E*)-1,3-octadiene (e.g. Pellmyr et al., 1991; Suinyuy et al., 2010) as *E. villosus* north of Umtamvuna river and are visited by undescribed Erotylid beetles and *M. goodei* which are pollinators of *E. villosus* (Donaldson, 1997). If (3*E*)-1,3-octadiene is the major attractant in *E. villosus* it could attract closely related pollinators from other *Encephalartos* species. If there are no other barriers and backcrosses occur, gene flow across species boundaries is possible. Stökl

et al. (2008) showed that overlap of flowering times in *Ophrys iricolor* and *O. lupercalis,* which emit the same compounds and attract the same insects *Andrena nigroaenea* and *A. flavipes,* results in extensive hybridization and introgression. Natural hybrids have been found to occur between *E. villosus* and *E. senticosus* (previously considered part of *E.lebomboensis*) (Dyer, 1965; Giddy, 1984; Vorster, 1986) which emit the same dominant compound (Chapter 5) and occurs around the Jozini area, and between *E. villosus* and *E. altensteinii* near East London (possibly around the Buffalo river) in the Eastern Cape where they occur in sympatry (Dyer, 1965; Giddy, 1984; Vorster, 1986) and emit similar minor volatile compounds (Chapter 5).The variation in volatile compounds in *E. villosus* could therefore be as a result of introgression of odours traits through hybridization with other *Encephalartos* spp. Alternatively, the dominance of (3*E*)-1,3-octadiene among cycads in KwaZulu-Natal may represent convergent evolution resulting from adaptation to a similar local suite of insects. Investigations of volatile compounds and pollinators are required for other *Encephalartos* species to determine how they vary in relation to *E. villosus* across a wide geographic area (Chapter 5).

In conclusion, the widespread cycad *E. villosus* consists of a number of geographically structured chemotypes. The discontinuities between these chemotypes are not pronounced enough to justify recognition of distinct taxa, nor does it appear from preliminary investigations that these chemotypes are associated with different pollinators. However, the patterns suggests ongoing evolutionary diversification in *E. villosus* which makes this species suitable for further microevolutionary studies on the role that insect pollinators played in the evolution of *Encephalartos.*

Chapter 4. Physiological and behavioural responses of insects to volatile compounds emitted by cones of the African cycad *Encephalartos villosus*

Abstract

Interactions between cycads and their insect pollinators are generally considered to be mediated by cone volatiles. The functional significance of both the blend and individual volatiles emitted from cones of the African cycad *Encephalartos villosus* was investigated in both field and laboratory investigations. This cycad species is pollinated by insects which also use the cones as larval brood sites. In olfactometer experiments, the pollinator *Porthetes* sp. (Coleoptera: Curculionidae) responded positively to cones of *E. villosus* at all life stages. Using gas chromatography-electroantennogram detection (GC-EAD), it was shown that the antennae of both Erotylidae nov. sp. and *Porthetes* sp. responded to two unsaturated hydrocarbons (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene which are present in plants from KwaZulu-Natal populations. In field bioassays, in which several compounds were presented individually in bucket traps, beetles were strongly attracted to individual compounds (3*E*)-1,3-octadiene in KwaZulu-Natal and by 2-isopropyl-3-methoxypyrazine in the Eastern Cape. This is consistent with the dominance of (3*E*)-1,3-octadiene in the volatile blend emitted by *E. villosus* plants in KwaZulu-Natal and 2-isopropyl-3-methoxypyrazine in the volatile blend emitted by *E. villosus* plants in the Eastern Cape. These results show that single compounds can be sufficient to attract insect pollinators of *E. villosus* and suggest that evolutionary divergence in cones volatiles between the KwaZulu-Natal and Eastern Cape populations reflect differences in beetle scent preferences between the two regions. It is still uncertain whether these differences in the scent preference of insects in the two regions are the result of innate or conditioned

preferences, and whether the scent preferences, if innate, reflect coevolution or host shifts from other local cycad species.

Introduction

Floral volatiles are important signals for chemical communication between plants and insect pollinators (Pellmyr and Thien, 1986) and often mediate a wide range of plant-insect interactions. For example, floral volatiles may attract pollinators, repel antagonists, and stimulate feeding and oviposition behaviour (review by Raguso, 2008). Floral scents may vary in the number, composition, and relative amounts of the different constituents and in their temporal and spatial emission patterns (Raguso, 2001; Raguso, 2004; Knudsen et al., 2006) and insect pollinators may detect these variations and respond to them accordingly (e.g. Komano-Nomora and Yamaoka, 2009).

In obligate pollination mutualisms where floral volatiles play a major role in pollinator attraction and both plant and pollinator depend on each other for survival, floral volatiles are often considered to mediate specific interactions (Grison-Pigé et al., 2002; Dufaÿ et al., 2003; Svensson et al., 2006; Chen et al., 2009). Such interactions may be mediated by specific compounds (e.g. 4-methylanisole in *Ficus semicordata-Ceratosolen gravely* system, Chen et al., 2009) or a blend of compounds (e.g. 2- phenylethyl alcohol and 2-phenylaceto-nitrile in the *Breynia vitis-idaea–Epicephala* system, Svensson et al., 2010) that attract the pollinators. In protogynous and/ or dioecious plants, pollinators must be attracted to both male and female flowers for pollination to occur (Seymour and Shultz-Motel, 1997; Füssel et al., 2007). In this scenario, the plant-pollinator interaction might involve attraction to male and female flowers as well as repellence from males to enhance pollinator movement between the male and female flowers. One mechanism to achieve

this would be if both male and female flowers emit different chemical compounds which perform attractant and repellent function independently or both male and female flowers emitted similar volatiles which act both as attractants and repellents in different quantities at different times (Tollsten and Knudsen, 1992; Füssel et al., 2007; review in Ashman, 2009).

Floral volatiles are assumed to have originally evolved as deterrents for insects feeding on plant reproductive structures but as insects and plants co-evolved, some compounds were later selected as attractants when the interaction conferred a net benefit to the plant because the herbivores also functioned as pollinators (Pellmyr and Thien, 1986). Selection may therefore have resulted in the presence of volatiles that function as attractants for the benefit of the plant or repellents against herbivores. Terry et al. (2007a) showed that volatiles from the sporophylls of the Australian cycad *Macrozamia lucida* attracted the thrips pollinator *Cycadothrips chadwicki* early in the day, repelled them at midday, and attracted them again in the late afternoon to early evening. These results corresponded to daily field observations which showed that insects left male cones at midday when volatile emissions were high, and were attracted later in the day when cone emissions were low (e.g. Terry et al., 2004a) and suggest that insect pollinators leave the male cones because of increased volatile emissions which may have a repellent function. Further olfactometer experiments using specific compounds emitted by *M. lucida* showed that *C. chadwicki* was attracted to *β*-myrcene at low concentrations but was repelled by high concentrations of the same compound (Terry et al., 2007a and b). These observations indicated that the same cone volatiles can function as both insect attractants and repellents depending on the concentration.Terry et al. (2007a and b).

It is not known if volatile compounds from other cycad taxa function as attractants for pollinators observed in *Macrozamia* occurs in other species. Volatile emissions during pollen dehiscence in male cones and receptivity in female cones of other cycad taxa often coincides with periods of insect activity on

cones suggesting that they attract insect pollinators (Pellmyr et al., 1991; Azuma and Kono, 2006; Proches and Johnson, 2009; Suinyuy et al., 2010). Apart from the *Macrozamia* system studied by Terry et al. (2007a and b), evidence that cone volatiles attract insect pollinators have been obtained in only one other study which demonstrated that pollen shedding male cones of *Zamia furfuracea* attracted *Rhopalotria mollis* beetles (Coleoptera: Curculionoidea) at close range (Tang, 1993) but the attractant is not known and remains to be investigated.

Results from gas chromatography-mass spectrometry (GC-MS) analyses of cone odours from *E. villosus* (Chapter 3) showed that volatile profiles varied across 10 different populations from the north-east to the south-west of the distribution range. The main compounds emitted by cones of *E. villosus* from the southern distribution range were eucalyptol and 2-isopropyl-3-methoxypyrazine whereas plants from the northern range were characterised by emissions of (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene (Chapter 3). The beetle pollinators Erotylidae sp. nov., *M. goodei* and *Porthetes* sp., as well as the seed parasite *A. zamiae* (Donaldson, 1997), were recorded from all 10 populations (Chapter 3). The occurrence of at least four insect species in *E. villosus*, together with a distributional cline in volatile blends, raises important questions about which specific compounds attract which insect species, and whether there is variation in insect response in the different populations. There was thus a need to test the functions of these volatile compounds in relation to the *E. villosus* -insect interactions. Such studies require detailed knowledge of chemistry of cone volatiles, the physiological responses of associated insects (e.g. through gas chromatography –electroantennogram (GC-EAD) studies) and field and laboratory studies of behavioural responses.

When dealing with a complex blend of volatiles, it is important to first identify potentially physiologically active compounds. Gas chromatography-electroantennogram detection (GC-EAD) is an

important tool for identifying which compounds trigger an electrophysiological response. The physiologically active compounds detected in the GC-EAD are relevant chemicals that are of biological importance (Schiestl and Marion-Poll, 2002). Active compounds can then be tested for behavioural activity in olfactometer choice tests and field bioassays (Ayasse et al., 2000; Schiestl and Marion-Poll, 2002; Stensmyr et al., 2002).

The aim of this study was to first to determine if cone volatiles attract insects as expected for the 'pollinator attraction' function for cone volatiles and to identify the active compounds. Also of importance was to determine responses of insect species to various volatile compounds across the distribution range of *E. villosus* given the differences in volatile emissions detected in Chapter 3.

Materials and methods

Study area and plant material

Field bioassays were conducted in the Eastern Cape (EC) at the Ocean View Guest Farm (OVGF) and Umtiza Nature Reserve (UNR) near East London and in KwaZulu-Natal (KZN), in the Kranzkloof Nature Reserve (KKNR) and at the botanical garden of the University of KwaZulu Natal (UKZN) at the Pietermaritzburg (PMB) campus between 2009 and 2010 (Figure 4.1).

Insect species

For olfactometer tests, only female *Porthetes* beetles were used so as not to introduce additional variables associated with different sexes. The insects used in testing attractiveness of cones in the

olfactometer were collected from KKNR and from garden plants of known origins within the range of *E. villosus* in the EC growing at the Kirstenbosch Botanic Garden (KBG), Cape Town. The insects used in testing attractiveness of chemical compounds in the olfactometer were collected from KKNR and from garden plants in the UKZN botanic garden. For electrophysiological tests, insects were collected from KKNR in the KZN and OVGF in the EC directly from male cones by knocking the cone over a beating sheet.

Behavioural tests

Olfactometer tests with cycad cones

Olfactometer experiments were undertaken in the laboratory between 2007 and 2009 using male cones from Eastern Cape plants growing at the Kirstenbosch Botanic Garden, and from KZN plants growing at the KKNR and UKZN. Cones at different developmental stages (Chapter 2) were used to determine whether the known pollinator, *Porthetes* sp., responded to *E. villosus* cone volatiles from pre-dehiscent, dehiscent and post dehiscent male cones. The cones which were collected from the wild were cut at the cone peduncle and placed in water in the laboratory. Cones remain fresh, without substantial loss of moisture for several days (e.g. Tang, 1987b).

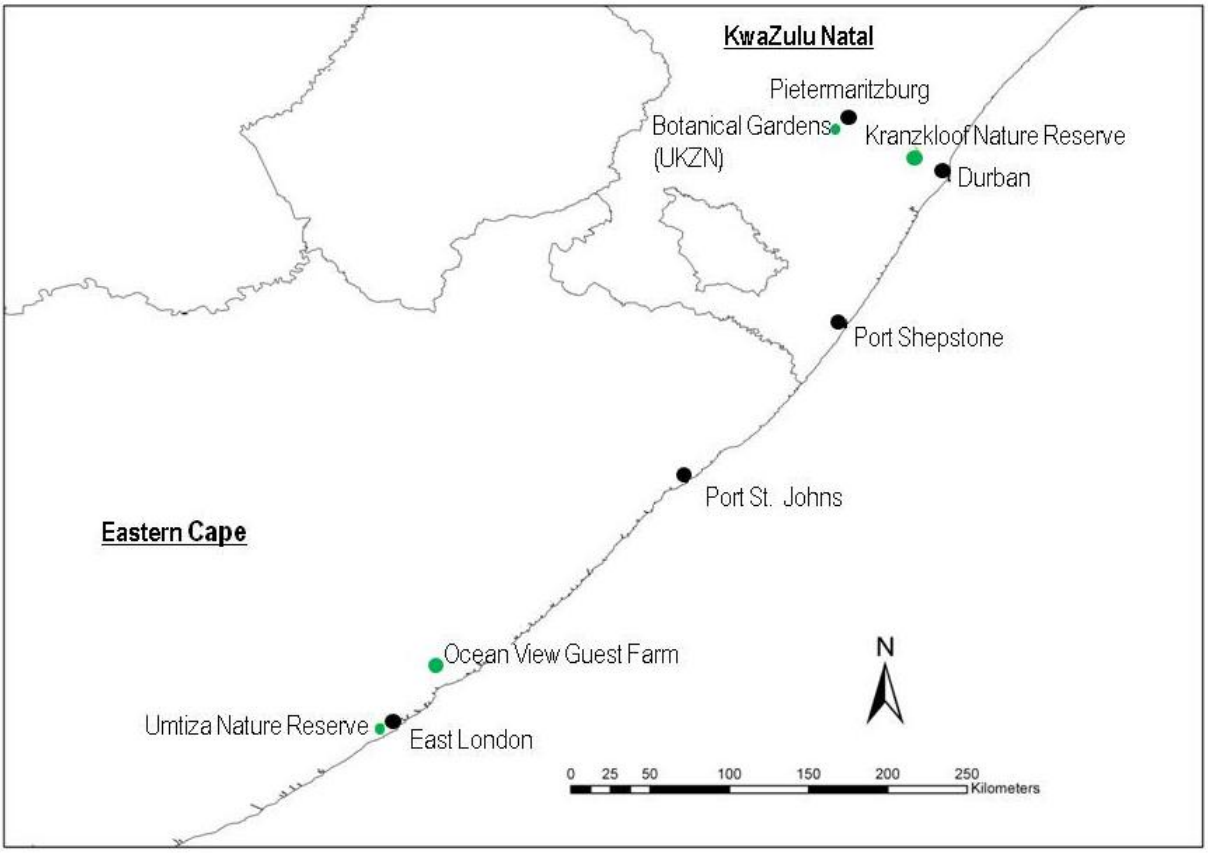

Figure 4.1: Map indicating sites (marked in green) where field bioassays were conducted

Olfactometer assays were conducted using a two arm olfactometer modified from the four arm design of Vet et al. (1983) to create two discrete odour fields (Figure 4.2). Each arm was connected to three 50 ml glass vials and a flow meter. The first vial immediately after the flow meter contained activated charcoal to scrub the ambient air entering the olfactometer, the second vial contained the odour source and the third vial was used to catch beetles moving into the tube. Female weevils were introduced to the centre of the olfactometer at the junction of the two odour fields. A consistent design was followed in which one arm of the olfactometer provided the male cone odour and the second arm provided a control air stream consisting of room air. The flow rate for air entering each arm was set at 200 ml/min. Female *Porthetes* sp. were introduced into the olfactometer in groups of between 15 and 50 individuals. The base of the olfactometer was inscribed with two concentric circles, 1 cm apart, originating at the hole where beetles were introduced into the olfactometer. This created three zones associated with movement towards one of the arms and the zones were designated as R1, R2, R3, and L1, L2, L3 respectively. The choice of the beetle was determined by a) when it entered R2 or L2 and stayed there; b) when it entered R3 or L3 and stayed there; and c) when it finally entered the right or left tubing or crawled into the bottle. Venting of each odour field continued for an hour and the number of beetles collected in the different arms of the olfactometer was counted. After each experiment, the olfactometer was cleaned with 70% EtOH while the glassware was rinsed with acetone and baked at 250 ˚C for two hours. On running the next experiment, the control arm of the olfactometer was switched from left to right or vice versa to avoid bias.

The olfactometer experiments were conducted in the laboratory at room temperature (25 °C) and under uniform lighting to avoid interference with the behaviour of test insects. The experiments were conducted in the morning (08h00 - 11h00), and late afternoon to early evening (16h00 – 19h00).The experiments were replicated in the morning (10x) and evening (6x) for the EC plants and there were 15

replicates for both morning and evening for KZN plants. For each replicate, fresh cone samples and a new batch of insects were used.

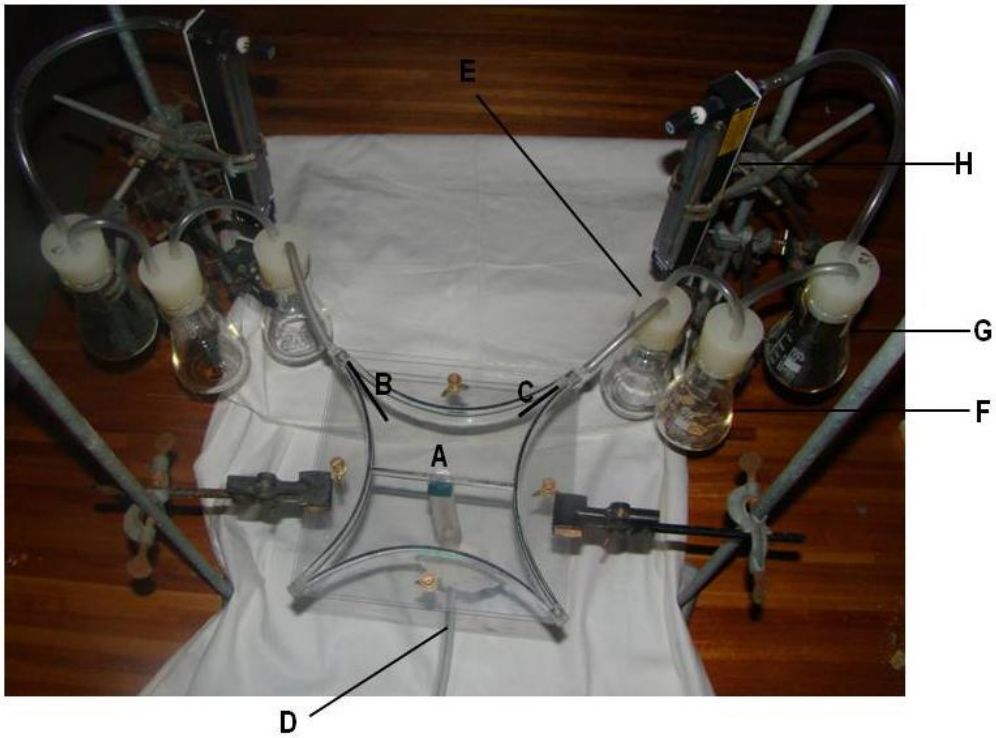

Figure 4.2: A two-arm olfactometer (modified from a four arm version) for choice test experiment. **A**: Olfactometer from the top, **B**: control arm, **C**: treatment arm, **D**: tube connecting pump and insect holder to olfactometer, **E**: vial to catch beetles; **F**: odour source; **G**: activated charcoal to filter incoming air; **H**: flow meter

Gas chromatography-Electroantennogram detection (GC-EAD)

To identify biologically significant compounds emitted by cones of *E. villosus*, cycad insects were tested for electrophysiological responses to volatile compounds by combined gas chromatography-electroantennogram detection and flame ionization detector (GC-EAD/FID) (Schiestl and Marion-Poll, 2002). Experiments were conducted with samples from KZN plants in 2009 and samples from EC plants were tested in 2010. The GC-EAD/FID system consists of a gas chromatography (Varian 3800 Palo Alto, California, USA) and EAD-interface (Syntech, Hilversum, Netherlands), combined with an EAG-amplifier. The GC was equipped with a Carbowax column (DB-wax) of 30m x 0.32 mm internal diameter x 0.25 µm film thickness (Alltech, Deerfield, Illinois, USA), a flame ionisation detector (FID) and a splitless injector. The injector was operated in a splitless mode, and helium was used as a carrier gas at a flow rate of 1 mL/min. The temperature at the start of the run was 40 °C, held for three minutes. The GC oven was ramped up to 240 °C at 10 °C/min and held there for 12 minutes. A Y-tube connection installed at the end of the column splits the stream of volatile compounds into two equal parts, one going to the FID and one to the EAD where a prepared antenna had been inserted. To prevent the antenna from drying out, the airflow through the delivery tube was humidified by passing it through a bottle of water before it reached the antenna of the insect.

For each GC-EAD experiment, odour traps containing cone volatiles of *E. villosus* from EC and KZN (see Chapter 2) were placed in a Varian 1079 injector by means of a Chromatoprobe fitting and thermally desorbed. Each insect preparation was tested for antennal activity before each GC-EAD run by puffing pure air over the antenna. Four insect taxa associated with *E. villosus* cones were tested for their electrophysiological responses to cone volatiles*, i.e. A. zamiae*, Erotylidae sp. nov., *M. goodei*, and *Porthetes* sp. collected from KKNR in the KZN and OVGF in the EC.

Insect antennae were excised from the head using micro-scissors and a few segments on the tip of the antenna were cut off. The excised antenna was mounted between two glass micropipettes using a micromanipulator. The micropipettes had an inner diameter wide enough to allow insertion of one end of an excised antenna. The micropipettes were filled with a ringer solution (0.1 N KCl) to prevent drying out of the antenna and to allow electrical conductivity and each micropipette was connected to an electrode.

Test with individual compounds

To determine whether the level of (3*E*)-1,3-octadiene, 2-isopropyl-3-methoxypyrazine and eucalyptol used in the behavioural tests were ecologically relevant, different volumes 10, 25, and 50 µL of each of the three chemicals were added to matching volumes of paraffin oil to each make up 1000 µL of dilution. From each dilution, 10 µL were applied onto filter paper strips (0.5 cm x 3.0 cm, Whatman No. 1) and sampled for 2 minutes using the same conditions as for sampling cone volatiles. When the samples were analysed, the emission rates of the standards from the 50 µL dilutions were within the ranges of volatile compounds emitted by *E. villosus* cones in natural habitat (total integrated GC areas of the individual compounds and cone volatiles from chromatoprobe trap both ranged between 1.99×10^4 and 4.32×10^4 ng/peak areas). This dilution was therefore used for the olfactometer and bucket trap experiments.

The choice of these compounds was based on the fact that (3*E*)-1,3-octadiene is physiologically active (this study). Although 2-isopropyl-3-methoxypyrazine did not elicit any response on the insect antennae, it has a pungent odour, even at low concentration which is responsible for the strong smell associated with *E. villosus* cones in the EC (Chapter 3) and is thought to attract *E.villosus* insect pollinators (Rattray, 1913). The monoterpene eucalyptol is emitted by plants from all *E. villosus* populations in EC and

KZN, but in higher emission rates in the EC plants (Chapter 3) and it is considered an insect attractant of many plants (Pansarin et al., 2006).

Olfactometer experiments

After electrophysiological analysis, tests were performed to determine the behavioural responses of cycad insects to electrophysiologically active compounds. Individual compounds emitted by KZN plants which elicited electrophysiological responses in the GC-EAD study and other dominant compounds emitted by cycad cones from EC were tested for their effects on insect behaviour. Compounds used were (3*E*)-1,3-octadiene, which is dominant in the volatile blend emitted in KZN populations, and 2-isopropyl-3-methoxypyrazine and eucalyptol, which are dominant in the volatile blend emitted in EC populations. Another compound, (3*E*,5*Z*)-1,3,5-octatriene, elicited electrophysiological responses to Erotylidae sp. nov. and *Porthetes* sp.*,* but could not be tested for behavioural responses because it is not commercially available.

The protocol for this experiment was the same as that of the olfactometer experiment with cycad cones (see above). Dilutions of 50 µl individual compounds and 950 µl paraffin oil (Alpha pharma, South Africa) were prepared and 10 µl of each dilution applied onto filter paper strips (0.5 cm x 3.0 cm, Whatman No. 1) and put into a 50 ml glass vial as the odour source. The same amount of pure paraffin oil was used as a control. The experiments were repeated 27, 18 and 20 times using (3*E*)-1,3-octadiene, 2-isopropyl-3-methoxypyrazine and eucalyptol respectively and a batch of between 15 and 35 beetles were used for each trial.

Field bioassays with bucket traps

To confirm the effects of the electrophysiologically active compounds on cycad insect behaviour, attraction bioassays were carried out in the field using bucket funnel traps™ obtained from Insect science™, South Africa (Figure 4.3). The traps were made up of a cage lid, cage, dome lid, funnel, bucket, and an amber glass bottle for the bait (scent compound). The trap when assembled had a height of 215 mm, diameter of 170 mm, and a weight of 240 gm. In the field, the traps were attached to a pole or plant using a cable tie.

Figure 4.3: Trap made of one colour (yellow bucket, dome lid and funnel) for field bioassay.

Because behaviour of several insect species is influenced by colour, preliminary trials were conducted to determine whether the colour of the traps would affect the behaviour of cycad insects. Four colour combinations, based on the colour of the bucket, lid and dome were tested: yellow bucket, dome lid and funnel; green bucket, dome lid and funnel; green bucket, yellow dome lid and funnel; and yellow

bucket, green dome lid and funnel. Each trap colour had five replicates. Male cones at pollen dehiscent stage collected from the botanical garden of UKZN, PMB campus were placed in the traps and set up randomly in an area with *E. villosus* plants. Another set of traps of the same colours were set out without cones as controls. The traps were monitored every 24 hours (between 09h00 and 10h00) for five days and the number and identity of insects monitored. The comparison of control and treatment traps showed that none of the controls attracted any insects whereas all the treatments attracted insects irrespective of the colour combination. The variation in the number of insects attracted by the cones assessed using restricted maximum likelihood (REML) and generalized linear mixed models (GLMM) indicated that bucket colour had no significant effect in the number of insects attracted to traps ($P > 0.05$).

To test whether individual compounds attracted insects to traps, three compounds were used as baits in bucket traps set up in Umtiza Nature Reserve (UNR), Ocean View Guest Farm (OVGF) in the Eastern Cape (EC), Krantzkloof Nature Reserve (KKNR) and University of KwaZulu Natal (UKZN) in KwaZulu Natal (KZN). For each treatment, 50 µl of either (3*E*)-1,3-octadiene, 2-methoxy-3-isopropylpyrazine, or eucalyptol was added to 950 µl of liquid paraffin (Alpha pharma, South Africa). The combined bait solution was put into a corked bottle and a cotton thread was inserted through the cork to facilitate gradual release of volatile compound. Undiluted paraffin oil, without the test compounds, served as a control. Each bait bottle or control was placed into an individual bucket trap and secured with a piece of wire. The bucket traps were placed about 15 m apart in an area with *E. villosus* individuals. After every 24 hour cycle (between 09h00 and 10h00 the next day) the trap was monitored and the number and identity of insects were recorded. In the UNR and OVGF sites, each treatment and control had five replicates and each trap was monitored for five days. At the KKNR site, treatment and control sampled between days one and four and between days seven and nine each had five replicates while each of those

sampled on day five and six had seven replicates. At the PMB campus of the UKZN, treatments and controls sampled between days one and five each had five replicates while those on days six, seven and eight each had nine, 11 and eight replicates respectively.

Data analysis

Statistical analyses for the olfactometer experiments were undertaken using PASW Statistics 18 (SPSS, Chicago, USA). The sources of variation in the behaviour of insects to the treatments (cone volatiles or individual compounds) in the olfactometer experiments were assessed using a generalized linear model. This allows one to test for differences between treatments and to control for the variation within the trials as several insects were used at the same time. Each observation for a batch of insects represented the proportion of insects that entered the olfactometer (i.e. proportion of responses), and the proportion of beetles that responsed to the treatment arm of the olfactometer. Models used a binomial error distribution and logit link function, and significance of effects was assessed by using likelihood ratio statistics.

Statistical analyses for bucket trap data were undertaken using Genstat version 12.1 (GENSTAT 12.1, 2009). Data were analysed using restricted maximum likelihood (REML) and generalized linear mixed models (GLMM). Restricted maximum likelihood estimation is a method which allows the estimation of variance components in unbalanced data sets (Patterson and Thompson, 1971). These models used a negative binomial error distribution with a logarithm link function and the aggregation factor was set to one. The first analysis tested the differences in the total number of insects attracted by different chemicals in the bucket traps in the different study populations. The region, chemical, and beetle species were fitted as fixed

factors while the population effect, trial and trap number nested within population were fitted as random factors. The subsequent series of analyses tested the differences in the number of individual beetle species per chemical in the different regions. The region and chemical were fitted as fixed factors while the population effect and trials nested within population were fitted as random factors. Further Wald χ^2 statistics generated by REML were used to determine significant fixed factors and their interactions. The Wald χ^2 statistics were quoted with their relevant degrees of freedom and the probability values for the fixed factors. Back transformed means are given with the average standard error of differences between the means.

Results

Olfactometer experiments with cycad cones

In 138 trials with cones from EC and KZN at pre-dehiscent, dehiscent and post dehiscent stages tested in the morning and evening, *Porthetes* sp. always responded by entering the chamber and choosing either the scented or control arm of the olfactometer. The proportion of *Porthetes* sp. responding in the trials at the different developmental stages did not differ between EC and KZN (Figure 4.4A: site effect - χ^2_1 = 0.261, P = 0.609, site x cone stage interactions - χ^2_2 = 1.48, P = 0.476). A small proportion of beetles responded in the trials at pre-dehiscent and post dehiscent stages in the evening (Figure 4.4A) with significant differences between cone stages (χ^2_2 = 18.02, P < 0.0001), time (χ^2_1 = 171.19, P < 0.0001) and site x cone stage x time interactions (χ^2_5 = 185.06, P < 0.0001). The beetles that responded in the trials showed contrasting responses to cone volatiles from EC and KZN (Figure 4.4B, site effect, χ^2_1 = 32.326, P < 0.0001). The beetles showed a strong preference for the scented arm of the olfactometer at all developmental stages (χ^2_2 = 34.84, P < 0.0001) and time of the day (χ^2_1 = 22.47, P < 0.0001, Figure 4.4B).

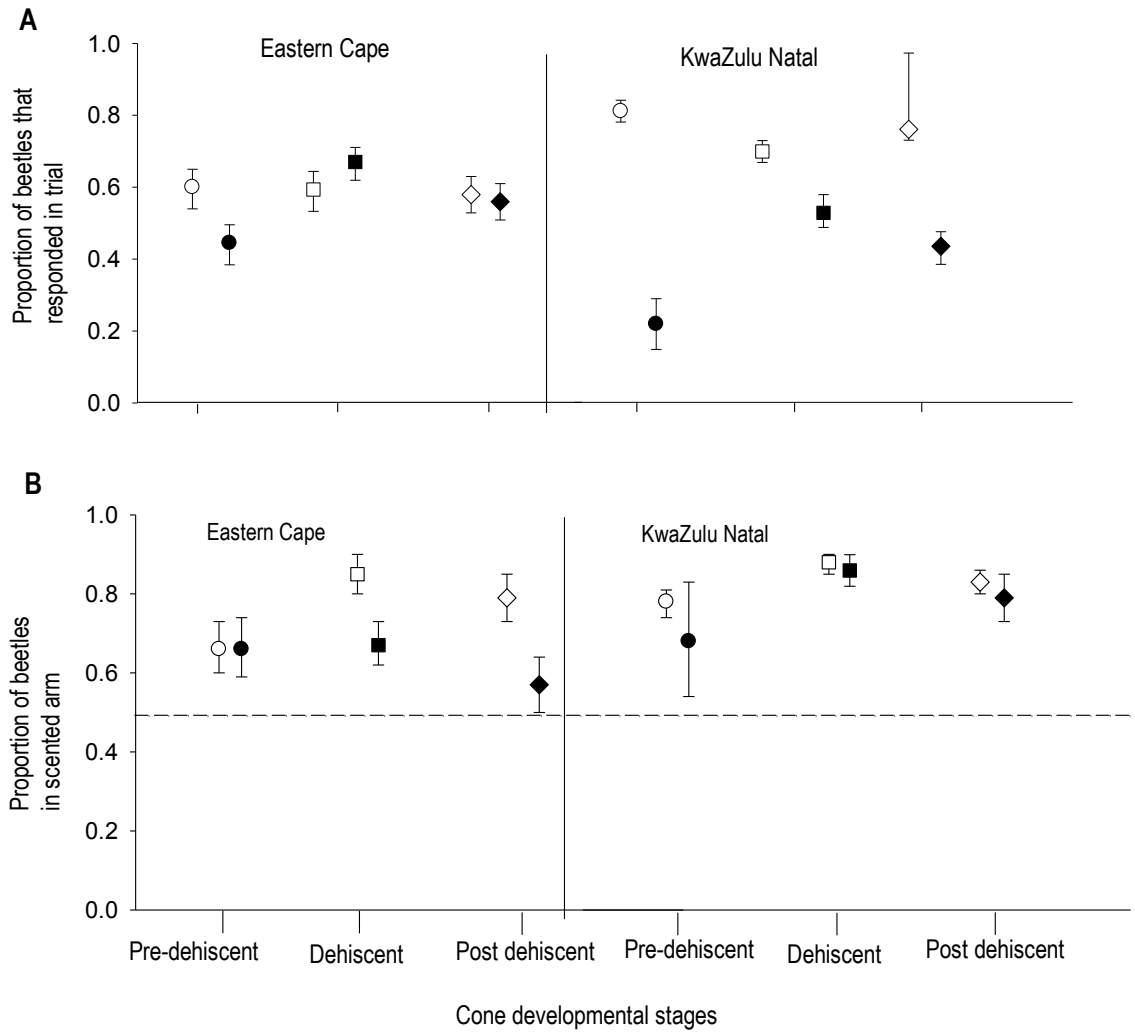

Figure 4.4: Mean (± 95 % CI) behaviour of *Porthetes* sp. during olfactometer choice trials involving *E. villosus* cone volatiles from different sites at different life stages and times of day. **A**) Proportions of beetles that responded in the trials when they entered any arm of the olfactometer from a holding tank. **B**) Proportions of responding beetles that chose the scented arm of the olfactometer, rather than the unscented arm. Means with confidence intervals that do not overlap the 0.5 equal choice line represent significant preference for the scented arm. Beetles in each region are only tested to cones from that region. Open shapes: Morning; Black solid shapes: evening.

The proportion of beetles that preferred the scented arm of the olfactometer at various cone stages differed significantly at different sites ($\chi^2_{1,125}$ = 32.33, P < 0.0001) and times of the day (χ^2_1 = 18.42, P < 0.0001), site x cone stage x time interaction (χ^2_2 = 22.475, P < 0.0001), but not in the site x cone stage interaction (χ^2_2 = 2.606, P = 0.272).

Electrophysiological response to cone volatiles

The gas chromatography-electroantennographic detection/flame ionisation studies show that two compounds, (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene, which are emitted by *E. villosus* in KZN elicited electrophysiological responses in Erotylidae sp.nov. and *Porthetes* sp. (Figures 4.5A and B) collected from KKNR in the KZN. The two insects responded differently to the two compounds. In Erotylidae sp. nov., the antennae were more sensitive to (3*E*)-1,3-octadiene than to (3*E*,5*Z*)-1,3,5-octatriene (Figure 4.5A) whereas the antenna of *Porthetes sp.* responded more strongly to (3*E*,5*Z*)-1,3,5-octatriene than to (3*E*)-1,3-octadiene (figure 4.5B). There was no response from *Antliarhinus zamiae* or *Metacucujus goodei* from the KKNR and all the insects from the KZN region did not respond to volatile compounds from *E. villosus* in the EC. There was also no response detected to any of the compounds especially 2-isopropyl-3-methoxypyrazine and ecalyptol from *E. villosus* in the EC and the beetles from the EC region did not respond to the volatiles compounds (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene from KZN plants.

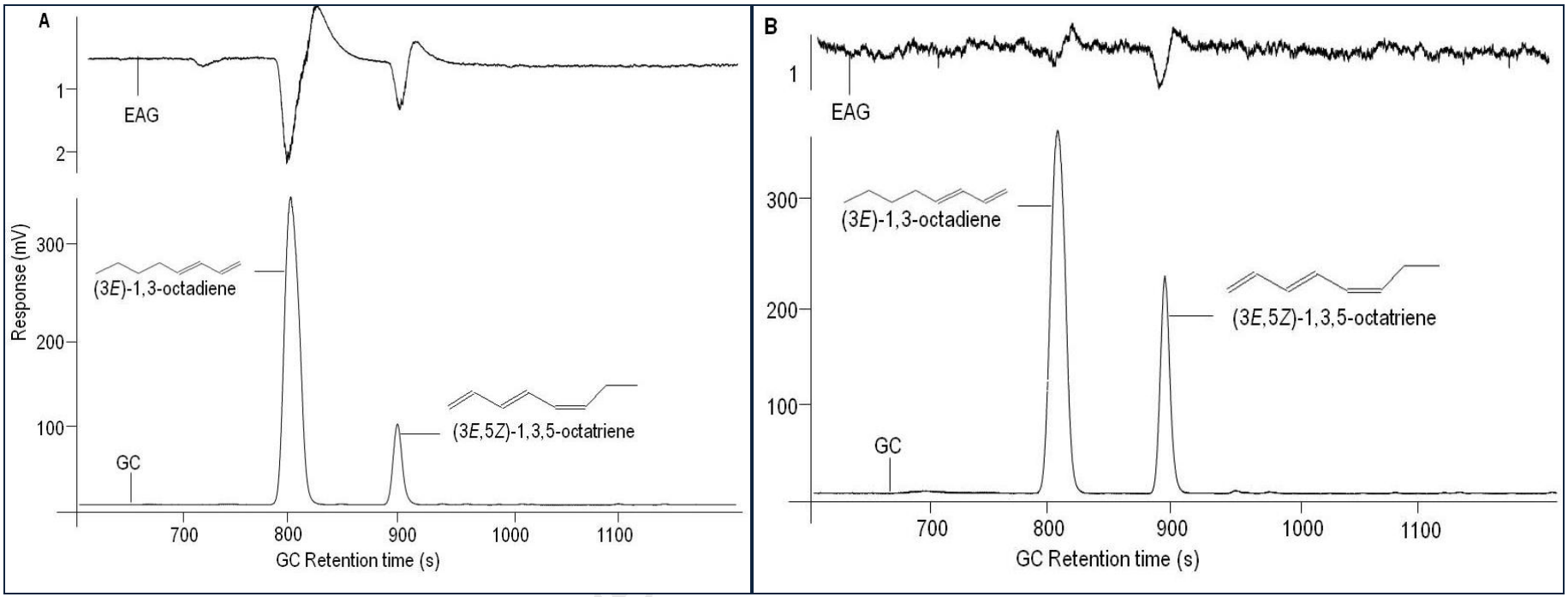

Figure 4.5: Insects' physiological responses to *E. villosus* volatiles. **A**) Erotylidae sp. nov. and **B**) *Porthetes* sp. physiologically respond in a gas chromatography-electroantennographic detection (GC-EAD) to the volatile compounds (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene emitted by male cones of *E. villosus*. Insects and cone volatiles are from E. villosus in the KZN populations.

Chemical compounds in the olfactometer experiments

The overall response of *Porthetes* sp. collected from the KZN populations differed strongly between scent compounds (χ^2_2 = 152.23, P < 0.0001). In all the 65 trials involving (3*E*)-1,3-octadiene, 2-isopropyl-3-methoxypyrazine and eucalyptol, *Porthetes* sp. always made a choice and entered one of the olfactometer arms. *Porthetes* sp. responded more consistently during trials with (3*E*)-1,3-octadiene than with 2-isopropyl-3-methoxypyrazine and eucalyptol (Figure 4.6A). Pairwise comparisons of estimated marginal means based on the trials proportion showed that the response of *Porthetes* sp. to (3*E*)-1,3-octadiene was significantly different from the response to 2-isopropyl-3-methoxypyrazine and eucalyptol (P < 0.0001, Sequential Sidak post hoc procedure). There were no differences between the response of beetles to 2-isopropyl-3-methoxypyrazine and eucalyptol (P = 0.836, Figure 4.6A). When beetles entered the olfactometer, they exhibited different responses to the individual compounds (χ^2_2 = 41.18, P < 0.0001). When offered (3*E*)-1,3-octadiene in the different trials, the majority of the *Porthetes* sp. individuals chose the olfactometer arm containing the scent compound (Figure 4.5B). Pairwise comparisons of estimated marginal means based on the trials proportion showed that the response of beetles to (3*E*)-1,3-octadiene was significantly different from the responses to 2-isopropyl-3-methoxypyrazine and eucalyptol (P < 0.0001, Sequential Sidak post hoc procedure). Although a small proportion of *Porthetes* sp. responded to the olfactometer arm containing 2-isopropyl-3-methoxypyrazine and eucalyptol (Figure 4.6B), the response of beetles to 2-isopropyl-3-methoxypyrazine was significantly different from response to eucalyptol (P = 0.002, Sequential Sidak post hoc procedure). The low response of beetles to 2-isopropyl-3-methoxypyrazine and eucalyptol might be due to the fact that the insects for the experiments were from KZN plants which do not emit 2-isopropyl-3-methoxypyrazine.

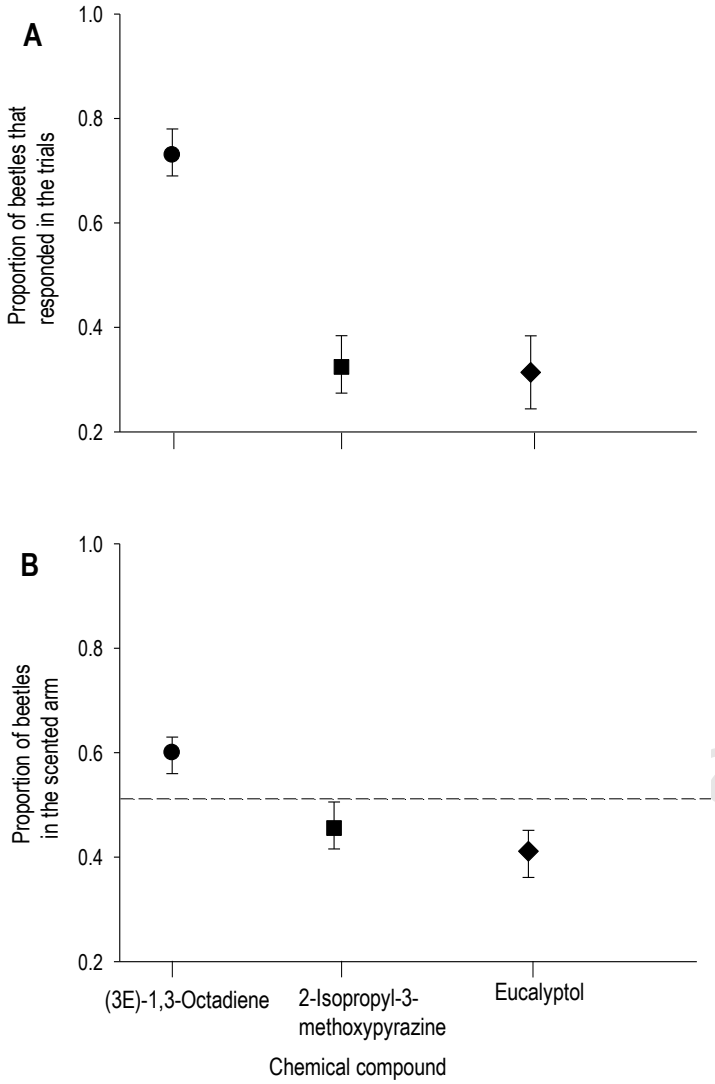

Figure 4.6: Mean (± 95 % CI) behaviour of *Porthetes* sp during choice trials involving the individual volatile compounds in the scent of *E. villosus* male cones, (3*E*)-1,3-octadiene, 2-isopropyl-3-methoxypyrazine, and eucalyptol. **A**) Proportions of beetles that responded in the trials when they entered any arm of the olfactometer from a holding tank. **B**) Proportions of responding beetles that chose the scented arm of the olfactometer, rather than the unscented arm. Beetles are from *E. villosus* populations in the KZN where (3E)-1,3-octadiene is the major volatile compound. Means with confidence intervals that do not overlap the 0.5 equal choice line represent significant preference.

Bucket traps in the field

The comparison of control and treatment traps showed that none of the controls in EC or KZN attracted any insects whereas all the treatment traps attracted insects to varying degrees. Four beetle species, i.e. *A. zamiae*, Erotylidae sp. nov., *M. goodei*, and *Porthetes* sp., were attracted to compounds in the traps. Analyses of region and treatment effects showed that despite the attraction of different beetles to the traps, there was no overall significant effect of region ($P = 0.4$, Table 4.2). The Wald statistic was highly significant for other main effects and interactions, i.e. chemical, beetle species, chemical x region, region x species, chemical x species and region x chemical x species (Table 4.2). The number of beetles attracted by 2-isopropyl-3-methoxypyrazine was significantly greater for *Porthetes* sp. ($P \leq 0.05$) than it was for *M. goodei,* Erotylidae sp. nov., and *A. zamiae* in the EC populations (Figure 4.7). Similarly, the number of beetles attracted by (3*E*)-1,3-octadiene was significantly greater for *Porthetes* sp. ($P \leq 0.05$) than *M. goodei*, Erotylidae sp. nov. and *A. zamiae*, in the KZN populations (Figure 4.7). The significantly greater number of *Porthetes* sp. ($P \leq 0.05$) attracted to 2-isopropyl-3-methoxypyrazine and (3*E*)-1,3-octadiene in different populations in the field (Figure 4.7) highlight the importance of both compounds in the pollination of *E. villosus*.

Further REML analysis of the combined effect of region and chemical compound as factors determining the difference in total number of individual beetle species showed that region had no significant effects on insect abundance ($P = 0.165$, Table 4.2). In contrast, chemical compound was a significant factor for explaining the difference in abundance of trapped *A. zamiae*, Erotylidae sp. nov., and *Porthetes* sp. ($P < 0.0001$, Table 4.2) but not *M. goodei* ($P = 0.284$, Table 4.2). Also, the interaction between chemical and region was significant for all four beetle species attracted to the different chemicals (Table 4.2).

Table 4.2: Wald χ^2 statistics derived from GLMM analyses which tested for differences in the numbers of insects attracted to all the compounds (3*E*)-1,3-Octadiene, Eucalyptol and 2-Isopropyl-3-methoxypyrazine in the bucket trap experiments in the EC and KZN regions.

Fixed term	df	Wald statistic	F pr
Region	1	1.14	0.401
Chemical	2	151	<0.001
Beetle Species	3	416.72	<0.001
Region × Chemical	2	107.57	<0.001
Region × Species	3	24.26	<0.001
Chemical × Species	6	139.68	<0.001
Region × Chemical × Species	6	73.51	<0.001
Porthetes sp.			
Region	1	2.68	0.165
Chemical	2	84.84	<0.001
Region × Chemical	2	82.83	<0.001
Metacucujus goodei			
Region	1	1.2	0.409
Chemical	2	2.52	0.284
Region × Chemical	2	12.67	0.002
Erotylidae sp. nov.			
Region	1	1.57	0.244
Chemical	2	39.64	<0.001
Region × Chemical	2	44.28	<0.001
Antliarhinus zamiae			
Region	1	1.25	0.27
Chemical	2	23.32	<0.001
Region × Chemical	2	10.16	0.007

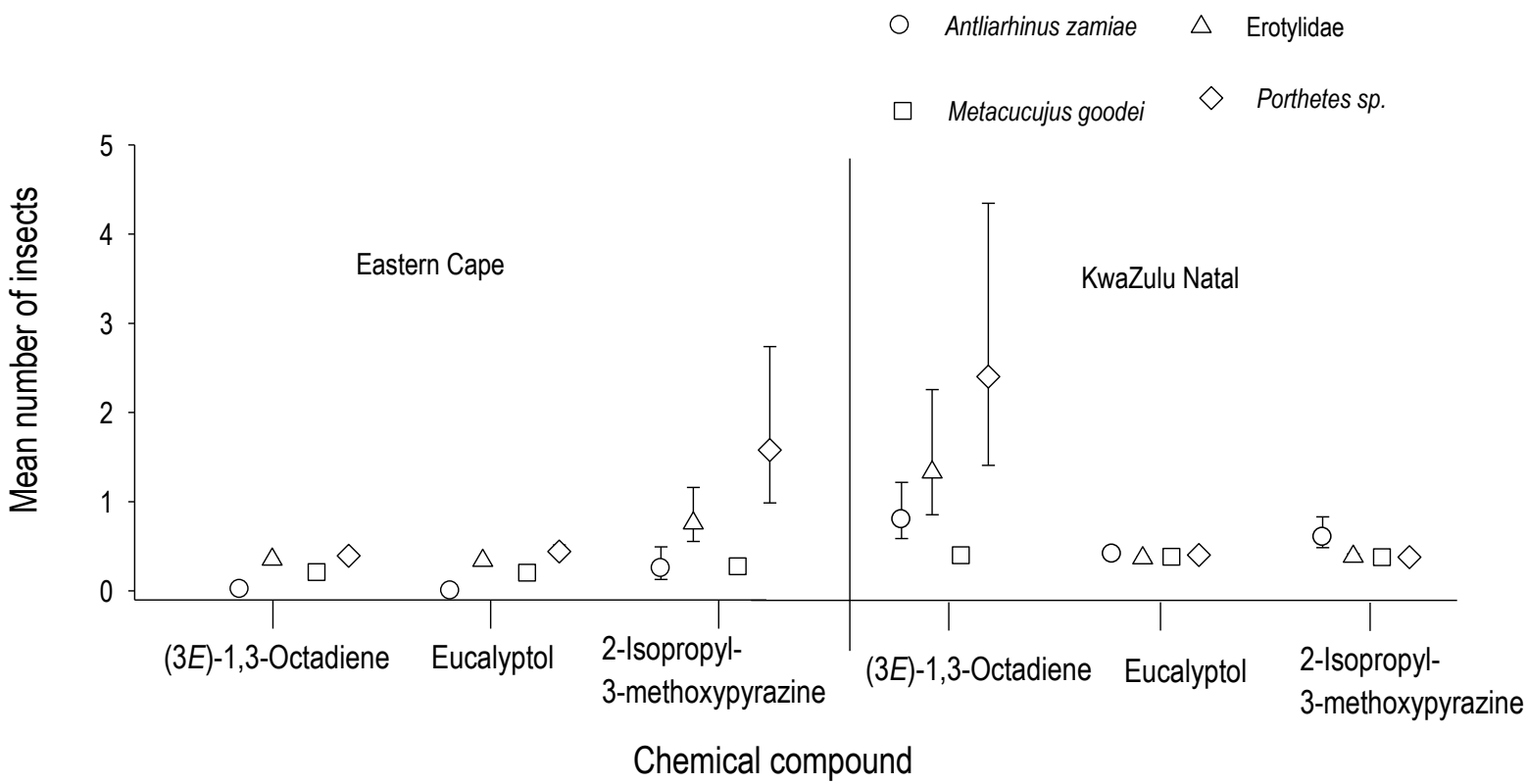

Figure 4.7: Number (mean ± S.E) of insect species attracted to (3*E*)-1,3-Octadiene, Eucalyptol or 2-Isopropyl-3-methoxypyrazine in trapping experiments in different populations. The means and standard errors are back-transformed from the log scale.

Discussion

The purpose of this chapter was to determine whether there is an attraction function of *E. villosus* cone volatiles as observed in *Macrozamia* cycads (Terry et al., 2007a and b). The study was to further identify the active volatile compounds using GC-EAD (Schiestl and Marion-Poll, 2002) and determined the behavioural responses of insects to the chemical compounds that occur in *E. villosus*. The results showed that beetles responded positively to *E. villosus* cone volatiles in the olfactometer (Figures 4.4 and 4.6) and in the bucket traps (Figure 4.7) some of which were physiologically detected by the antennae of Erotylidae sp. nov. and *Porthetes* sp. using coupled GC-EAD (Figures 4.5A and B). The results from physiological and behavioural responses of the different beetle species in the laboratory (olfactometer) and in the field (bucket traps) varied between the compounds and provide strong evidence that volatile compounds function as attractants for pollinators.

Beetles were consistently attracted to the male cone sporophylls of *E. villosus* at all life stages and during different times of the day in olfactometer experiments (Figures 4.4A and B). The volatile compounds emitted by male cones at all developmental stages (Chapter 2) enhanced the attraction of beetles which is strongly evident in the olfactometer experiments (Figures 4.6A and B) and bucket traps (Figure 4.7). The attraction of beetles by cone volatiles was proposed by Rattray (1913) after he observed beetles on the pollen shedding cones of *E. villosus* when they were emitting odour compounds. This result, which confirms Rattray's (1913) observations, is consistent with Tang's (1993) findings that pollen shedding male cones of *Zamia furfuracea* in a sealed jar with perforated lid attracted beetle pollinators *Rhopalotria mollis* at close range and with Terry et al's. (2007a, and b) demonstration that dehiscing male cone sporophylls of *M. lucida* and *M. machinii* attracted *Cycadothrips chadwickii* pollinators in olfactometer experiments. Generally beetles have been observed on cones of cycads sometime before pollen shed when they become aromatic

(e.g. male cones of *Z. furfuracea,* Stevenson et al., 1998) and during periods of pollen shed when they emit odour compounds (e.g. *Stangeria*, Proches and Johnson, 2009; *E. altensteinii,* Suinyuy et al., 2010) indicating that volatile compounds are involved in pollinator attraction. Similarly, in other plants where volatile compound emissions play a major role in pollinator attraction, arrival of insects always corresponds with emission of volatile compounds (e.g. *Cyclocephala hardyi* on *Victoria amazonica*, Prance and Arias, 1975) indicating that it is the volatile compounds that attract them.

The volatile compounds emitted by cones of *E. villosus* consisted of fatty acid derivatives (alcohols, aliphatic acids, aldehydes, esters, ketones, and unsaturated hydrocarbons), benzenoids, terpenoids (monoterpenes and sesquiterpenes) and nitrogen-containing compounds (Chapters 2 and 3). The volatile compounds were characterised by the unsaturated hydrocarbons (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene in the KZN plants and the monoterpene eucalyptol and nitrogen-containing compounds 2-isopropyl-3-methoxypyrazine in the EC plants (Chapters 2 and 3). Contrary to the general arguments that variation in odour profiles indicates attraction of different insect species (Schiestl and Ayasse, 2002), olfactometer experiments showed that three dominant compounds, 2-isopropyl-3-methoxypyrazine from the EC plants, (3*E*)-1,3-octadiene from the KZN plants and eucalyptol from both EC and KZN plants attracted *Porthetes* sp. (Figures 4.6A and B). Although (3*E*)-1,3-octadiene attracted higher numbers of insects than eucalyptol and 2-isopropyl-3-methoxypyrazine (Figure 4.6), it was difficult to assess the most important attractant because insects used in the olfactometer experiment were from the KZN populations only and individual compounds tested represented volatile compounds emitted by plants from the EC and KZN populations. The cone volatiles may vary considerably among populations depending on the local preference of the pollinators (e.g. Schlumpberger and Raguso, 2008) especially as *E. villosus* is visited and pollinated by different insects (Donaldson, 1997). However, field surveys show that the same insect

pollinator species occur across the studied populations of *E. villosus* (Chapter 3) and different individual compounds emitted in different populations attracted the same insect pollinator species (Figure 4.7).

Further confirmation of the attraction of insect pollinators to these compounds was obtained from bucket traps in the field, which showed that *A. zamiae*, Erotylidae sp. nov., *M. goodei* and *Porthetes* sp. were attracted to 2-isopropyl-3-methoxypyraxine, (3*E*)-1,3-octadiene and eucalyptol in the EC and KZN populations of *E. villosus* (Figure 4.7). There was a clear pattern in the number of different insect species responding to the different individual compounds. *Porthetes* sp. responded more to 2-isopropyl-3-methoxypyrazine in the EC populations and to (3*E*)-1,3-octadiene in the KZN populations (Figure 4.6). Erotylidae sp. nov. showed a similar pattern but was lower than *Porthetes* sp. (Figure 4.6). Across all the populations, *A. zamiae* showed a similar response to all the compounds whereas *M. goodei* was the least attracted insect species to all the compounds. The abundance of different insect species attracted to traps containing 2-isopropyl-3-methoxypyrazine in the EC populations and (3*E*)-1,3-octadiene in the KZN populations confirms that these compounds are the main attractants (Figure 4.7). It further suggests that the most abundant beetle species attracted to these compounds are the most important insects. Donaldson (1997) showed that *Porthetes* sp. was the most important pollinator of *E. villosus* while *A. zamiae*, *Erotylidae* sp. nov. and *M. goodei* played minor roles.

The volatile compounds emitted by cones of *E. villosus* (Chapters 2 and 3) are well known compounds that are emitted by cones of other cycads (e.g. Pellmyr et al., 1991; Terry et al., 2004a and b; Azuma and Kono, 2006; Proches and Johnson, 2009; Suinyuy et al., 2010) where they may perform different functions. Although experimental work and other studies showed that insects are attracted to aromatic dehiscing male cones of other cycads (e.g.Tang, 1993; Stevensson et al., 1998; Terry et al., 2004a; Suinyuy et al., 2010), attempts to show attractants have been demonstrated only in *Macrozamia*

cycads (e.g. Terry et al., 2007a and b). It was shown that *β*-myrcene which is a minor compound in the volatile composition of *E. villosus* and a dominant compound in the emissions of the Australian cycads *M. machinii* and *M. lucida* (Terry et al., 2004a and b) function as attractants of *C. chadwickii* pollinators (Terry et al., 2007a and b). This study is similar to that of Terry et al. (2007a and b) in that the attractants are dominant compounds which suggest that dominant compounds might be major attractants of cycad insects. The dominant compound (3*E*)-1,3-octadiene in volatile profile of *E. villosus* in the KZN populations has been detected in cone volatile emissions of *E. altensteinii*, *Z. furfuracea* (Pellmyr et al., 1991), and *E. natalensis* (Suinyuy et al., 2010) but has only been suspected to function as an insect attractant. Its dominance in the volatile emissions of many *Encephalartos* species (Chapter 5) suggests it might also be of biological importance and requires further investigation. Another dominant compound of *E. villosus*, 2-isopropyl-3-methoxypyrazine in the EC populations dominates the volatile profile of *Cycas thouarsii* (Kaiser, 2006) and is emitted by male cones of *E. natalensis* (Suinyuy et al., 2010) but it is not known whether they perform the attractant function in these cycads as observed in *E. villosus* in the EC.

Before the behavioural test for the attraction properties of the different compounds, coupled GC-EAD was used to detect the physiologically active compounds. Detecting the physiological active compounds proved to be a constraint as this technique required a lot of hands-on experience to do properly and the beetles' antennae have a hard exoskeleton leading to poor antennal preparations and responses. When there was a response in the GC-EAD, it was very clear as observed in the detection of physiologically active compounds (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene by Erotylidae sp. nov. and *Porthetes* sp. pollinators (Figures 4.5A and B). The response of the antenna of Erotylidae sp. nov. and *Porthetes* sp. to (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene respectively indicates that these compounds are of biological importance as observed in the behavioural response of insects to (3*E*)-1,3-octadiene (Figures 4.6

and 4.7). Schiestl and Marion-Poll (2002) suggested that in a GC-EAD, only relevant compounds with biological significance will be detected by the insects.

When GC-EAD responses from insects were negative, there was uncertainty about the meaning of the result. The lack of response in the GC-EAD may not necessarily mean that the compound has no biological activity. For example, the GC-EAD studies did not detect any physiological response to 2-isopropyl-3-methoxypyrazine but the bucket traps showed that there is a behavioural response (Figure 4.7). This may be due to the fact that sometimes olfactory receptors in insects do not perform optimally when stimulated with odour compounds eluted from GC because the peaks may be long (10-20 seconds) and asymmetrical (Schiestl and Marion-Poll, 2002). Such a constraint may be overcome by using only electroantennograpm detection (EAD) without the GC by providing odour plumes over the insect antennae every two to three seconds (Schiestl and Marion-Poll, 2002) because olfactory receptors in insects respond best to fast-changing stimuli (Marion-Poll and Thiéry, 1996). Further absence of GC-EAD response to some minor compounds may be because they occur in lower concentrations that are below detection levels. Response of such compounds may also be determined using only EAD with different concentrations of the volatile compounds. Electroantennogram detection should therefore be used in addition to GC-EAD in future studies to investigate physiological responses of insects to cycad volatile compounds.

Although (3*E*,5*Z*)-1,3,5-octatriene was shown to be electrophysiologically active, its behavioural activity was not tested because of the lack of a chemical standard. It is also one of the dominant compounds in the volatile emissions of *E. natalensis* (Suinyuy et al., 2010) and Roman Kaiser (pers. comm.) has indicated that this compound tends to be commonly encountered in floral scent but its role is not known. The only known established role is that it functions as a sperm attractant in the marine brown

algae *Fucus serratus* (Kajiwara et al., 1980; Boland, 1995). Further experiments are therefore required to determine the biological function of (3*E*,5*Z*)-1,3,5-octatriene in plants.

It has been hypothesized that floral volatiles originally evolved as part of chemical defense systems that deterred insects from feeding on reproductive structures of early angiosperms (Pellmyr and Thien, 1986). But as insects co-evolved and became attracted by some compounds, volatiles began to function as attractants for pollinators (Pellmyr and Thien, 1986). The volatile compounds emitted by cones of *E. villosus* (Chapters 2 and 3) are common in some ancient as well as modern insect-pollinated angiosperms where they are associated with repellent functions in plant-insect interaction (Pellmyr et al., 1991). This suggests that *E. villosus* cone volatiles may have maintained the primary function of repellence against herbivores observed in ancient angiosperms. This is reflected in the Australian cycad *M. lucida* in which the attractive phase occurred when dehiscing male cones emitted the major compound *β*-myrcene in relatively low concentrations while the repellent phase occurred when *β*-myrcene was emitted in high concentrations (Terry et al., 2007a and b). Male and female cones of *E. villosus* emitted odours at different stages (Chapters 2 and 3) which consisted of similar compounds but that varied in relative amount. Concentration and emission rates may vary but this was not measured. This therefore implies that (3*E*)-1,3-octadiene, eucalyptol and 2-isopropyl-3-methoxypyrazine emitted by male and female cones of *E. villosus* are attractive to the pollinating insects. The possibility exists that these compounds may also function as repellents at different concentrations. For example the flowers of the orchid *Ophrys sphegodes* emit a blend of alkene compounds to attract the solitary bee *Andrena nigroaenea* (Schiestl et al., 1999) and an ester farnesyl hexanoate as a repellent (Schiestl and Ayasse, 2001). The detection of (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene by Erotylidae sp. nov. and *Porthetes* sp. in the GC-EAD shows that both species have a physiological response to these compounds. In the field, Erotylid beetles responded only weakly to

bucket traps containing (3*E*)-1,3-octadiene indicating that the physiological response may not always trigger an attraction towards the odour. However, repellence was not tested and there was no strong evidence to show that these same compounds acted as repellents. Although this was more difficult to test, repellency cannot be completely ruled out because the responses of beetles to some compounds suggest they may act as repellents. For example the behavioural activity of eucalyptol and 2-isopropyl-3-methoxypyrazine in the olfactometer experiments revealed that very few *Porthetes* sp were attracted as compared to those attracted by (3*E*)-1,3-octadiene (Figures 4.5). Behavioural experiments using bucket traps indicate that eucalyptol and (3*E*)-1,3-octadiene attracted few insects as compared to 2-isopropyl-3-methoxypyrazine in the EC populations of *E. villosus* (Figure 4.7). Also, eucalyptol and 2-isopropyl-3-methoxypyrazine attracted a few beetles in the KZN populations of *E. villosus* compared to those attracted to (3*E*)-1,3-octadiene (Figure 4.7). The low numbers of beetles attracted to these compounds at the different sites may therefore be an indication of repellence. However future studies are required to investigate whether these volatile compounds at different concentrations are attractancts and repellents as observed with *β*-myrcene in the Australian cycad *M. lucida* (Terry et al., 2007a and b).

The compounds tested here for behavioural activity are also common in the volatile composition of some plant taxa where they may also function as attractants and/ or repellents. For example, 2-isopropyl-3-methoxypyrazine previously reported in the floral scent of *Hyacinthus orientalis* (Brunke et al., 1994), *Rothmannia annae* (Kaiser, 2004); *Cycas thouarsii* (Kaiser, 2006) and *E. natalensis* (Suinyuy et al., 2010) may function as a deterrent, repellent or warning compound in high concentration that signal toxicity to many herbivorous insects but are insect attractants in relatively low concentrations (Guilford et al., 1987; Harborne, 1987; Rothschild and Moore, 1987; Kaye et al., 1989; Moore et al., 1990; Woolfson and Rothschild, 1990; Aldrich et al., 1996, 1997). The unsaturated hydrocarbon (3*E*)-1,3-octadiene occurs in

the floral volatiles of the angiosperm *Sauromatum guttatum* and is suggested to be an insect attractant (Skubatz et al., 1996; Hadacek and Weber, 2002). Eucalyptol is also emitted by members of various plant families and performs various functions. It is an attractant of the banana weevil *Cosmopolites sordidus* (Ndieqie at al., 1986), a mosquito feeding and ovipositional repellent (Klocke et al., 1986), and foraging alert pheromone in bumble bees (Granero et al., 2005). Because of its toxicity, it is used as an insecticide against houseflies, blowflies (Sukontason et al., 2004) and the sand fly *Lutzomyia longipalpis* (Maciel et al., 2010). Although these compounds both function as attractants as well as repellents in these angiosperms, the current study only confirms that they attract pollinators in *E. villosus*.

In conclusion, this study represents an important breakthrough in the pollination system of *E. villosus*. It is the first time that biologically active compounds have been identified in *Encephalartos* and reveals a system which is different to that found in other cycads particularly the Australian *Macrozamia* (e.g. push-pull, Terry et al., 2007a and b). It reveals a strong 'pull' factor but does not provide any conclusive evidence for a 'push' factor. The consistency in the number of beetles attracted to these compounds justifies them as the main attractants of *E. villosus* pollinators. Although few beetles were attracted to some compounds in different populations, it is not enough justification to describe them as repellents of beetles in these populations. But the response of beetles to volatiles of *E. villosus* suggests that further investigation is required to establish the 'push' factor which may account for the movement of pollen between male and female plants.

Chapter 5. Variation in the chemical composition of cone volatile emissions within the African cycad genus *Encephalartos*

Abstract

Volatiles play a key role in attraction of pollinators to cycad cones, but the extent to which volatile chemistry varies among cycad species is still poorly documented. Volatile composition of male and female cones of nineteen *Encephalartos* species were analysed using headspace technique and gas chromatograpy-mass spectrometry (GC-MS). A total of 152 compounds were identified among the species included in this study, the most common of which were the monoterpenes *α*-pinene, eucalyptol, and camphene, nitrogen compounds 2-isopropyl-3-methoxypyrazine, 2-methoxy-3-methylpyrazine, and unsaturated hydrocarbons (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene. Male and female cones emitted similar volatile compounds which varied in relative amounts. In a multivariate analysis of volatile profiles using nonmetric multidimensional scaling (NMDS), a number of species clusters were identified according to shared emission of unsaturated hydrocarbons, pyrazines, benzenoids and aldehydes, alkanes and terpenoids. In comparison, terpenoids are common in *Zamia* and dominant in *Macrozamia* species while benzenoids, esters, and alcohols are dominant in *Cycas* and in *Stangeria*. It is likely that volatile variation among *Encephalartos* species reflects both phylogeny and adaptations to specific beetle pollinators.

Introduction

When different plant species are pollinated by the same group of pollinators, and especially by closely related pollinator taxa, they may be expected to have floral volatiles with similar chemical constituents (e.g. Knudsen and Tollsten, 1995). This expectation is based on data showing that floral

volatiles influence the composition and behaviour of pollinators (Dodson et al., 1969; Pellmyr, 1986a; Dobson et al., 1997; Raguso, 2008) and act as important cues in plant-pollinator interactions (Dobson, 2006; Knudsen et al., 2006; Raguso, 2008). However, floral odours can be complex blends that can influence pollinators in different ways (Raguso, 2008) and it is not always clear which compounds act as the main triggers for pollinator behaviour or how these compounds vary between species or populations (Raguso, 2004, 2008).

There are examples in which plant species that are pollinated by the same class of pollinators vary in their floral volatile compounds (e.g. Miyake et al., 1998) and interpopulation variation in volatiles can occur even within the same species (e.g. Anderson et al., 2010; Chapter 3). Such variation in floral volatiles may be due to differences in environmental conditions such as climate and soil chemistry, which can have significant effects on floral scent (Jakobson and Olson, 1994; Majetic et al., 2008, 2009).

Geographic variation in the cone volatiles of *Encephalartos villosus* (Chapter 3) raised important questions about how pollination may be mediated by volatile compounds across different environments. Results from previous chapters showed that in some parts of its range, *E. villosus* emitted the same biologically active compounds as other cycads in the area, e.g. (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene in KwaZulu Natal that is also emitted by *E. natalensis* (Suinyuy et al., 2010). This suggests that there could be local convergence in floral volatiles among unrelated species that share similar pollinators. In contrast, in other parts of its range *E. villosus* emitted compounds that have not yet been isolated from other cycads in the same area, e.g. 2-isopropyl-3-methoxypyrazine in the Eastern Cape (Chapter 3). It is thus not yet clear whether cycads in a given area show convergence or divergence in floral odours.

Southern Africa has a wide range of biomes (Rutherford et al., 2006) and a complex mosaic of vegetation types and is an ideal area in which to examine variation in volatile compounds across different

environments. The 37 species of *Encephalartos* that occur in South Africa (Donaldson, 2003; Hill et al., 2007) are distributed across a range of different biomes and vegetation types. Although many *Encephalartos* species are restricted to a particular vegetation type (e.g. grassland, forest or savannah), and some are endemic to small areas comprising one vegetation type (Dyer, 1965; Donaldson, 1993b; Jones, 1993; Goode, 2001), the majority of species were historically distributed across several vegetation types on different soils. This means that it is possible to determine whether the pattern of geographical differences observed in *E. villosus* is more widespread within the genus *Encephalartos.*

Comparisons of volatile compounds across species should take phylogenetic relatedness into consideration (e.g. Azuma et al., 1997; Williams and Whitten, 1999; Levin et al., 2003). Such an analysis is difficult for *Encephalartos* because the phylogeny for the genus is poorly resolved due to low genetic variation within the genus (Treutlein et al., 2005). Nevertheless, genetic analysis has revealed several well defined clades (Treutlein et al., 2005) and these authors even raised the question about whether these clades represent separate subspecies rather than well differentiated species groups. As a result, it should be possible to compare variation in volatile compounds within and between clades despite the absence of a complete phylogeny. One of the best supported clades is the group that includes *E. villosus* and comprises *E. aplanatus, E. caffer, E. cerinus, E. ngoyanus* and *E. umbeluziensis* that all occur within or close to the current distribution range of *E. villosus.* Although these species are phylogenetically related and geographically close to one another, they have diversified into forest, savannah and grassland vegetation types and therefore provide an opportunity to analyse several factors that may influence volatile profiles.

A second well supported clade is the so-called 'woolly-coned' cycads comprising *E. cycadifolius, E. friderici-guilielmi, E. ghellinckii, E. humilis, E. lanatus* and *E. laevifolius.* This group includes relatively widespread species (e.g. *E. friderici-guilielmi*) and localised endemics (*E. cycadifolius*) within grassland

vegetation types so that it should be possible to determine whether differences occur even within a group that is phylogenetically related and restricted to a single broad vegetation type.

A key part of an analysis of variation in cone volatiles is a comparison of species with closely related pollinators relative to species with different pollinators. Pollination studies on three species of South African *Encephalartos* have shown that there are at least two pollination syndromes within *Encephalartos,* one involving the weevil genus *Porthetes* (Coleoptera: Curculionoidea) (Donaldson, 1997; Suinyuy et al., 2009) and one involving species of cucujid beetles (Coleoptera) belonging to the genus *Metacucujus* (Boganiidae) or the family Erotylidae (Cucujoidea) (Donaldson et al., 1995; Donaldson, 1997; Suinyuy et al., 2009). In some cycad species, e.g. *E. friderici-guilielmi,* both syndromes are present whereas in species such as *E. cycadifolius* only one syndrome has been found. Although the pollination biology of other *Encephalartos* species is not known, the distribution of insects on cones of *Encephalartos* is relatively well studied (Donaldson, 1991; Oberprieler, 1995; Downie et al., 2008; J. Donaldson unpublished data) and it is possible to examine whether variation in cone volatiles is associated with different pollination syndromes.

The first aim of this study was to document variation in volatile compounds from both male and female cones across a relatively large number of *Encephalartos* species that represent different life histories, phylogenetic clades, pollination syndromes and geographical distributions. A second aim was to investigate whether the observed variation in volatile composition was correlated with geographical distribution, phylogenetic clades or pollination syndromes.

Materials and methods

Odour samples were collected from male and/ or female cones of 19 *Encephalartos* species that were either shedding pollen or receptive to pollen between 2007 and 2010. Based on observations from Chapter 3, the Eastern Cape (EC) and KwaZulu Natal (KZN) populations of *E. villosus* were treated separately so that the 19 *Encephalartos* taxa included two samples from *E. villosus*. In total, cones from 246 male plants were sampled, comprising 144 cultivated plants and 102 wild plants. A total of 100 female plants were sampled, comprising 54 in cultivation and 46 in the wild. Details of localities where the plants were sampled, sex and number of plants sampled, biogeographic origin of the plants and their associated vegetation types, phylogenetic affinities and pollination syndromes are listed in table 5.1.

Cone odour collection and chemical analysis

Headspace sampling, as described in Chapter 2, was used to collect odour samples from male and female cones during pollen release and receptivity respectively. Odour samples were collected between 13h00 and 17h00 and analysed using a coupled Varian 3800 gas chromatograph (Varian Palo Alto, California, USA) and Varian 1200 mass spectrometer (GC-MS).

Non-metric multidimensional scaling (MDS), based on Bray-Curtis similarities of square root transformed data, implemented in Primer 6 (Clarke and Gorley, 2006) was used to visualise patterns of variation among samples. The significance of differences in odour samples between sexes and species, vegetation types, phylogenetic clades and pollination syndromes were assessed using a one-way analysis of similarities (ANOSIM with 10000 permutations). To analyse for variation in male and female cones, only species with samples from both sexes were considered. To determine whether volatile variation among

species correlated with pollinator types, phylogenetic relationships and associated vegetation, *Encephalartos* species were categorised into broad pollination syndromes, phylogenetic clades and vegetation types (Table 5.1). A similarity percentage (one-way SIMPER) was used in Primer 6 to identify the compounds that best explained similarities within and between species groupings.

Table 5.1: List of *Encephalartos* taxa from which odour samples were collected, giving the number of male and female plants sampled, location, geographic origin, phylogenetic affinities (sensu Treutlein et al., 2005), vegetation type and pollination syndrome for each taxon.

Species	Male	Female	Sampling locality	Geographic origin	Phylogenetic clade	Vegetation type	Pollination syndrome
E. villosus	7	4	Wild	Kranzkloof NR	*villosus* clade	Forest	*Porthetes* + Cucujid
E. villosus	3	1	UKZN, PMB Botanic Garden	Unknown	*villosus* clade	Forest	*Porthetes* + Cucujid
E. villosus	3		Cycad Centre, PMB	Unknown	*villosus* clade	Forest	*Porthetes* + Cucujid
E. villosus	4		Wild	Nkandla FR	*villosus* clade	Forest	*Porthetes* + Cucujid
E. villosus	5		Wild	Vernon Crookes NR	*villosus* clade	Forest	*Porthetes* + Cucujid
E. villosus	6		Wild	Oribi Gorge NR	*villosus* clade	Forest	*Porthetes* + Cucujid
E. villosus	4		Wild	Umtamvuna NR	*villosus* clade	Forest	*Porthetes* + Cucujid
E. villosus	5	5	Wild	Mount Suillivan area	*villosus* clade	Forest	*Porthetes* + Cucujid
E. villosus	5		Wild	Mpande area	*villosus* clade	Forest	*Porthetes* + Cucujid
E. villosus	6		Wild	Dwesa NR	*villosus* clade	Forest	*Porthetes* + Cucujid
E. villosus	6	4	Wild	Ocean View Farm	*villosus* clade	Forest	*Porthetes* + Cucujid
E. villosus	4		Wild	Umtiza NR	*villosus* clade	Forest	*Porthetes* + Cucujid
E. villosus	5	4	KBG	Unknown	*villosus* clade	Forest	*Porthetes* + Cucujid
E. aplanatus	7		Wild	Siteki, Swaziland	*villosus* clade	Forest	*Porthetes*
E. aplanatus	3		Kirstenbosch NBG	Siteki, Swaziland	*villosus* clade	Forest	*Porthetes* + Cucujid
E. caffer	6		Kirstenbosch NBG	Grahamstown	*villosus* clade	Grassland	*Porthetes* + Cucujid
E. ngoyanus	6	3	Kirstenbosch NBG	Ngoye Forest	*villosus* clade	Grassland	*Porthetes* + Cucujid
E. umbeluziensis	13	4	Hlane Royal NP	Mlawula NR, Swaziland	*villosus* clade	Savanna	*Porthetes* + Cucujid
E. umbeluziensis	4		UKZN, PMB Botanic Garden	Mlawula NR, Swaziland	*villosus* clade	Savanna	*Porthetes* + Cucujid
E. friderici-guilielmi	10	13	Wild	Middledrift farm, Cathcart	*friderici* clade	Grassland	*Porthetes* + Cucujid

Table 5.1 continued

Species	Male	Female	Sampling locality	Geographic origin	Phylogenetic clade	Vegetation biome	Pollination syndrome
E. cycadifolius	9	9	Wild	Winterberg mountains	*friderici* clade	Grassland	Cucujid
E. ghellinckii	9	6	Kirstenbosch NBG	Drakensberg area, Tabankulu	*friderici* clade	Grassland	Cucujid
E. humilis	3		Wild	Oribi veld, Nelspruit	*friderici* clade	Grassland	Cucujid
E. humilis	6	11	Kirstenbosch NBG	Kaapsehoop Mountain	*friderici* clade	Grassland	Cucujid
E. laevifolius	8		Kirstenbosch NBG	Unknown	*friderici* clade	Grassland	*Porthetes* + Cucujid
E. princeps	16	3	Kirstenbosch NBG	Unknown	*senticosus* clade	Thicket	*Porthetes* + Cucujid
E. horridus	11		Kirstenbosch NBG	Centlivres, Port Elizabeth	*latifrons* clade	Thicket	*Porthetes* + Cucujid
E. latifrons	8	6	Kirstenbosch NBG	Trappes Valley, Eastern Cape	*latifrons* clade	Thicket	*Porthetes* + Cucujid
E. trispinosus	5		Kirstenbosch NBG	Alice Farm, Alice	*latifrons clade*	Thicket	*Porthetes* + Cucujid
E. lehmannii	5		Kirstenbosch NBG	Willomore	*latifrons clade*	Thicket	*Porthetes* + Cucujid
E. altensteinii	6		Wild	Kenton-on-Sea	*natalensis* clade	Thicket	*Porthetes* + Cucujid
E. altensteinii	4	6	Kirstenbosch NBG	Mount Coke	*natalensis* clade	Thicket	*Porthetes* + Cucujid
E. senticosus	8		UKZN, PMB Botanic Garden	Jozini	*senticosus* clade	Savanna	*Porthetes* + Cucujid
E. natalensis	10	5	Wild	Hilton College Area	*natalensis* clade	Savanna + Grassland	*Porthetes* + Cucujid
E. natalensis	5	6	Wild	Pietermaritzburg NBG forest	*natalensis* clade	Savanna + Grassland	*Porthetes* + Cucujid
E. natalensis	9	10	UKZN, PMB Botanic Garden	Unknown	*natalensis* clade	Savanna + Grassland	*Porthetes* + Cucujid
E. natalensis	5		Cycad Centre	Unknown	*natalensis* clade	Savanna + Grassland	*Porthetes* + Cucujid
E. ferox	6		UKZN, PMB Botanic Garden	Sodwana Bay Area	*ferox* clade	Wooded grassland	*Porthetes*

UKZN: University of KwaZulu Natal, PMB: Pietermaritzburg, NP: National park, NR: Nature reserve, FR: Forest reserve,

Results

General overview and compound class patterns

The chemical composition of cone odours of the 19 sampled *Encephalartos* taxa (including two *E. villosus* chemotypes) is listed in Appendix 1. The compounds are identified by common names and CAS (Chemical Abstract Service) registry numbers. They are listed according to the estimated Kovats Retention Index (KRI) and in chemical classes. A total of 152 compounds were detected and identified, which included 74 terpenoids (56 monoterpenes and 18 sesquiterpenes), 52 fatty acid derivatives or aliphatics (14 alcohols, 10 aldehydes, nine ketones, six unsaturated hydrocarbons, five aliphatic acids, four alkanes and four esters), 22 benzenoids and four nitrogen-containing compounds. All four nitrogen-containing compounds consisted of pyrazines. The most frequently occurring compounds were benzaldehyde and limonene in 18 species each, heptanal, benzyl alcohol and phenol in 12 species, *β*-caryophyllene in 11 species and (3*E*)-1,3-octadiene and methyl benzoate in nine species. Most of the compounds were detected in small relative amounts (between trace amounts and < 10 %) as only 27 compounds (three alkanes, two unsaturated hydrocarbons, one aliphatic acid, three aldehydes, one ketone, two alcohols, three benzenoids, nine monoterpenes , one sesquiterpene and two nitrogen-containing compounds) reached a relative amount of ≥ 10 %.

The 19 *Encephalartos* taxa can be separated into six groups based on the dominance of the compounds and compound classes. Two of these groups are monospecific – one comprising *E. ferox* and characterised by a dominance of alkanes (99%; Table 5.2) (Appendix 1), and, the second comprising *E. umbeluziensis* characterised by the dominance of sesquiterpenes (97 %) (Table 5.2). The alkanes were absent from all other species tested whereas the sesquiterpene found in *E. umbeluziensis* (dihydroedulan I)

occurred in small relative amounts in other species (Appendix 1). The largest group (eight taxa) was characterised by the prevalence of unsaturated hydrocarbons and comprised *E. villosus* from KZN, *E. natalensis* and *E. senticosus* (between 55 and 75 %), *E. aplanatus*, *E. ngoyanus*, *E. trispinosus* and *E. altensteinii* (between 90 and 100 %) and *E. laevifolius* (37 %) (Table 5.2). The volatile composition was characterised by high emissions of (3*E*)-1,3-octadiene and/ or (3*E*,5*Z*)-1,3,5-octatriene (Appendix 1). Other unsaturated hydrocarbons that occurred in small relative amounts (< 4 %) included (*E*,*E*,*E*)-2,4,6-octatriene and 1,2-Dimethyl-1,4-cyclohexadiene emitted by *E. villosus* from KZN, *E. ngoyanus*, *E. senticosus* and *E. natalensis* (Appendix 1). The next largest grouping (four taxa) comprised species where monoterpenes were the characteristic compounds and included *E. villosus* from EC (60 – 75 %), *E. caffer* (> 80 %), *E. humilis* (> 90 %) and *E. latifrons* (ca. 85 %) (Table 5.2). The specific monoterpenes differed between taxa with high relative amounts of eucalyptol and *β*-pinene in the EC populations of *E. villosus*, *α*-pinene and camphene in the EC populations of *E. villosus*, *E. ghellinckii* and *E. humilis*, *cis*-*β*-ocimene in *E. caffer* and linalool in the emissions of *E. latifrons* (Appendix 1). Aldehydes and benzenoids characterised the emissions of a small group comprising *E. friderici-guilielmi*, *E. cycadifolius*, and *E. horridus* (Table 5.2). The volatile emissions consisted of high relative amounts of hexanal emitted by *E. cycadifolius* and *E. friderici-guilielmi* samples and heptanal and benzaldehyde in all three species (Appendix 1).The final group was characterised by nitrogen-containing compounds, specifically 2-methoxy-3-methylpyrazine, and included *E. princeps* and *E. lehmanii* (70 - 80 %) (Table 5.2). Although 2-isopropyl-3-methoxypyrazine was a biologically important compound in the emissions of *E. villosus* from the EC (Chapter 3), it contributed ca. 10 % of the total emission of this taxon and therefore did not influence the grouping for EC populations of *E. villosus* as compared to eucalyptol that was emitted in high relative amount (Appendix 1).

Table 5.2: Cone volatile composition of 19 *Encephalartos* species and 346 samples summarised according to relative amount (%) of compound classes. The number of samples for each species is given in parentheses. K = KwaZulu Natal, E = Eastern Cape, M = Male, F = Female, Unsat. HC = unsaturated hydrocarbons, tr = trace amounts (< 0.01 %)

Compound class Species & sample size	Alkanes	Unsat. HC	Aliphatic acids	Aldehydes	Ketones	Alcohols	Esters	Benzenoids	Monoterpenes	Sesquiterpenes	Nitrogen-containing compounds
E. villosus^{EM} (32)	-	-	-	7.30	0.37	0.70	-	15.09	66.76	tr	9.77
E. villosus^{EF} (9)	-	-	-	6.53	-	3.17	-	13.43	74.08	-	2.79
E. villosus^{KM} (33)	-	72.46	-	3.73	0.72	2.16	0.08	3.10	16.14	0.36	1.28
E. villosus^{KF} (5)	-	65.83	-	4.95	-	15.46	0.90	1.85	10.96	0.04	-
E. aplanatus^{M} (10)	-	99.7	-	0.15	tr	0.04	-	0.07	0.03	tr	-
E. cffer^{M} (6)	-	-	-	-	0.07	3.83	-	0.39	86.53	3.02	6.16
E. ngoyanus^{M} (6)	-	99.55	-	0.16	0.26	-	-	0.03	0.01	-	-
E. ngoyanus^{F} (3)	-	98.86	-	0.21	0.02	-	-	0.71	0.19	0.02	-
E. umbeluziensis^{M} (17)	-	-	-	0.07	-	0.04	-	0.14	2.21	97.55	-
E. umeluziensis^{F} (4)	-	-	-	-	-	-	-	3.48	6.52	90.00	-
E. friderici-guilielmi^{M} (10)	-	-	14.35	42.1	-	6.95	-	14.76	19.49	2.35	-
E. friderici-guilielmi^{F} (12)	-	-	3.73	56.65	-	10.38	-	15.60	13.04	0.60	-
E. cycadifolius^{M} (9)	-	-	2.13	15.56	-	18.69	-	21.85	34.61	7.15	-
E. cycadifolius^{F} (9)	-	-	3.21	28.93	-	6.23	-	36.71	18.84	6.09	-
E. ghellinckii^{M} (9)	-	0.80	-	11.45	21.09	5.87	0.11	15.45	45.08	0.16	-
E. ghellinckii^{F} (9)	-	-	1.5	57.24	1.94	4.10	0.12	20.64	13.64	0.82	-

Table 5.2 continued

Compound class Species & sample size	Alkanes	Unsat. HC	Aliphatic acids	Aldehydes	Ketones	Alcohols	Esters	Benzenoids	Monoterpenes	Sesquiterpenes	Nitrogen-containing compounds
E. humilis^{M} (9)	-	-	-	2.58	-	-	-	5.69	91.72	tr	-
E. humilis^{F} (12)	-	-	-	0.24	-	0.01	-	4.84	94.62	0.30	-
E. laevifolius^{M} (8)	-	37.22	-	9.51	1.00	3.17	-	22.34	26.77	-	-
E. princeps^{M} (16)	-	5.02	1.96	0.06	-	0.78	0.39	14.75	5.35	0.04	71.64
E. princeps^{F} (3)	-	36.42	-	-	-	-	-	37.13	-	-	26.45
E. horridus^{M} (11)	-	-	-	22.98	-	-	-	58.45	18.56	-	-
E. latifrons^{M} (8)	-	-	-	5.60	2.72	1.45	-	3.99	84.03	2.20	-
E. latifrons^{F} (6)	-	-	-	14.83	1.93	-	-	57.22	22.51	2.55	0.96
E. trispinosus^{M} (5)	-	93.8	-	0.92	1.88	-	-	0.32	0.13	2.95	-
E. lehmannii^{M} (5)	-	-	-	2.43	-	6.41	3.62	8.75	-	-	78.79
E. altensteinii^{M} (11)	-	99.14	-	0.06	0.04	-	-	0.09	0.10	-	0.57
E. altensteinii^{F} (6)	-	99.99	-	-	-	-	-	-	0.01	-	-
E. senticosus^{M} (8)	-	76.34	0.01	2.71	-	0.17	-	3.02	17.76	-	-
E. natalensis^{M} (22)	-	67.39	-	1.36	6.93	1.02	-	6.10	17.09	tr	0.12
E. natalensis^{F} (21)	-	55.59	-	2.12	0.01	-	-	22.88	19.19	0.22	-
E. ferox^{M} (6)	98.58	-	0.13	-	-	-	-	0.32	tr	0.96	-

Species-specific patterns of volatile compounds

The number of compounds varied markedly between male and female plants for species with both sexes. In female cones, from as little as three compounds occurred in *E. altensteinii* to 31 in *E. natalensis* and in male cones, from 12 in *E. altensteinii* to 59 in *E. villosus* from KZN (Appendix 1). Despite overall differences in the number of compounds, a Bray-Curtis NMDS analysis of odour compounds showed no clear separation between male and female cones. Consequently, a two-way cross design (with replicates) showed a marginal but significant difference in the relative amounts of compounds making up odours from male and female cones of the same species (NMDS stress value 0.2; ANOSIM, R (sex) = 0.253, $P < 0.01$). The marginal separation can be accounted for by the fact that each species pair emitted similar volatile compounds. For each species pair, pairwise significant differences were found only between male and female *E. ghellinckii* ($R = 0.73$, $P = 0.05$) and between male and female *E. latifrons* ($R = 0.933$, $P = 0.03$). When comparing each species pair (male and female *E. ghellinckii*) using one-way SIMPER, tetradecanal, camphene, 2-nonanone, *α*-pinene and benzaldehyde accounted for 46.91 % of the difference between the sexes. Linalool, benzaldehyde *cis*-*β*-ocimene, heptanal, benzyl alcohol and *trans*-*β*-ocimene accounted for 59.6 % of the difference between male and female *E. latifrons*. The variation in volatile composition was quantitative and not qualitative. The marginal separation between male and female cones volatiles indicates that sample of species for which only one sex is analysed is a fair reflection of the species as a whole.

Analysis of all samples (with either one or both sexes present) showed that the number of compounds varied markedly between species. The number of compounds ranged from eight in *E. lehmannii* to 73 in *E. villosus* from KZN. There were marked differences in chemical composition between the cone odours of different *Encephalartos* species (Appendix 1; Figure 5.1) and even between closely

related species (NMDS stress value 0.21; one-way ANOSIM, factor species: Global R = 0.698, $P < 0.01$). Most pairwise species comparisons were significantly different and the variations were accounted for by the dominant compounds which contributed to the *Encephalartos* groupings discussed above. The scent profiles of *E. ferox* and *E. umbeluziensis* were most distinct and well separated from the other species (range of R for pair-wise contrast between species = 0.779 – 1; $P < 0.01$) and therefore did not cluster with any other species (Figure 5.1). There was no distinction in the volatile profiles of *E. villosus* (KZN), *E. aplanatus*, *E. ngoyanus*, *E. senticosus*, *E. trispinosus*, *E. altensteinii*, and some *E. natalensis* cones, which were characterised by high relative amounts of (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene together with small amounts of (*E*,*E*,*E*)-2,4,6-octatriene and 1,2-dimethyl-1,4-cyclohexadiene. Samples of *E. cycadifolius* and *E. friderici-guilielmi* were characterised by benzaldehyde and heptanal whereas *E. ghellinckii* and *E. humilis* samples were characterised by camphene and α-pinene (Figure 5.1). Similarly, some samples of *E. princeps* and *E. lehmannii* formed a cluster and were characterised by 2-methoxy-3-methylpyrazine.

Analysis of odour profiles using NMDS with phylogenetic clade as a factor revealed a marginal but significant separation between clades (one-way ANOSIM, clade, $R = 0.307$, $P < 0.01$) (Figure 5.2). The scent profile of *E. ferox,* which is not associated with any of the other clades analysed here (Treutlein et al., 2005), was distinct and well separated from the other clades ($R = 0.660 – 0.979$; $P < 0.01$). Taxa within the *E. friderici-guilielmi* clade all clustered together with the exception of *E. laevifolius*, in which cone odours contained hydrocarbons and were more similar to species in the *E. natalensis* clade (Figure 5.2).

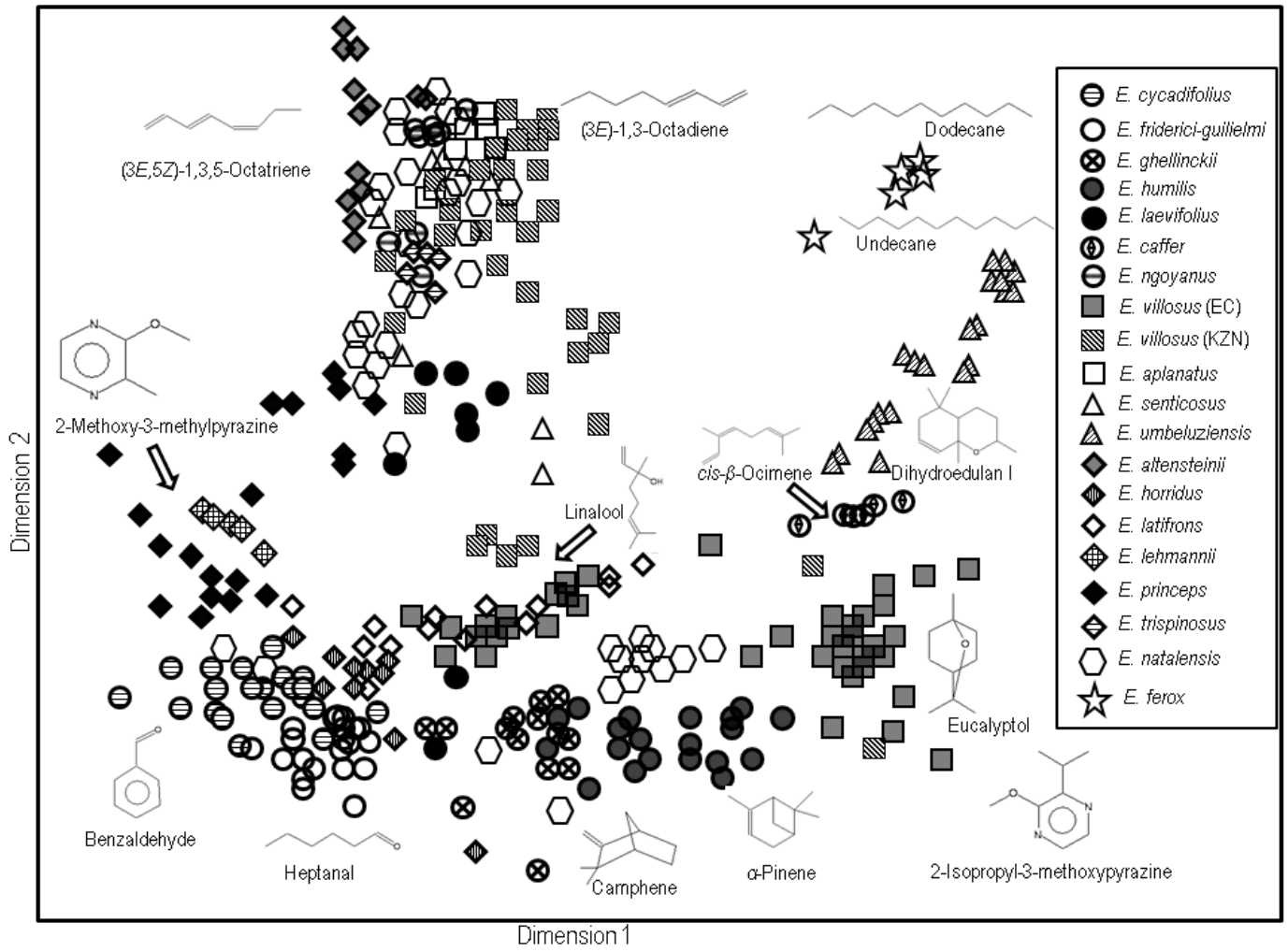

Figure 5.1: Ordination based on non-metric multidimensional scaling (NMDS) using Bray-Curtis similarity of male and female cone volatile composition comprising 152 compounds from 19 *Encephalartos* species across the distribution range in South Africa. (2D stress value = 0.21; ANOSIM, factor species, Global R = 0.698, $P < 0.01$). The structures and names of the 14 main compounds dominating the volatile profile of cone odour of different *Encephalartos* species are presented in the figure

Despite the apparent closeness of taxa within the *E. friderici-guilielmi* complex, significant differences between species within the clade were found (one-way ANOSIM, factor species, $R = 0.814$, $P < 0.01$). In another clade, *E. lehmannii*, *E. latifrons* and *E. horridus* clustered together but *E. trispinosus* from the same clade had odour profiles more closely aligned with the *E. natalensis* clade due to the dominance of hydrocarbons. Taxa within the *E. villosus* clade did not form a coherent cluster and were significantly different from one another (one-way ANOSIM, factor species, $R = 0.752$, $P < 0.01$). Within this clade, two taxa (*E. umbeluziensis* and *E. caffer*) each had distinct profiles whereas *E. villosus* (EC) had strong similarities with *E. latifrons* from a different clade, and *E. villosus* (KZN) and *E. ngoyanus* had similar odour profiles to the *E. natalensis* clade. Comparison of odour profiles between taxa from the Eastern Cape (9 taxa) and KwaZulu Natal (8 taxa) showed that there was an overrepresentation of hydrocarbons in the odour profiles of KZN taxa (50%) compared to EC taxa (22%) and an overrepresentation of 2-methoxy-3-methylpyrazine in EC taxa (44%) compared to KZN taxa (12.5%). These patterns were consistent with those found in *E. villosus* but they were not statistically significant ($\chi^2 = 2.73$, $P>0.05$). Further analyses of volatile composition using vegetation types as a factor showed a small but statistically significant separation between taxa from different vegetation types (one-way ANOSIM, factor vegetation, $R = 0.344$, $P < 0.01$) (Figure 5.3). There were significant differences in odour profiles between all vegetation types with the greatest separation between *Encephalartos* species from the wooded grassland vegetation type and species from all other vegetation types ($R = 0.667 – 0.886$; $P < 0.01$) and the least separation between species from forest and savanna plus grassland vegetation types ($R = 0.15$; $P < 0.01$).

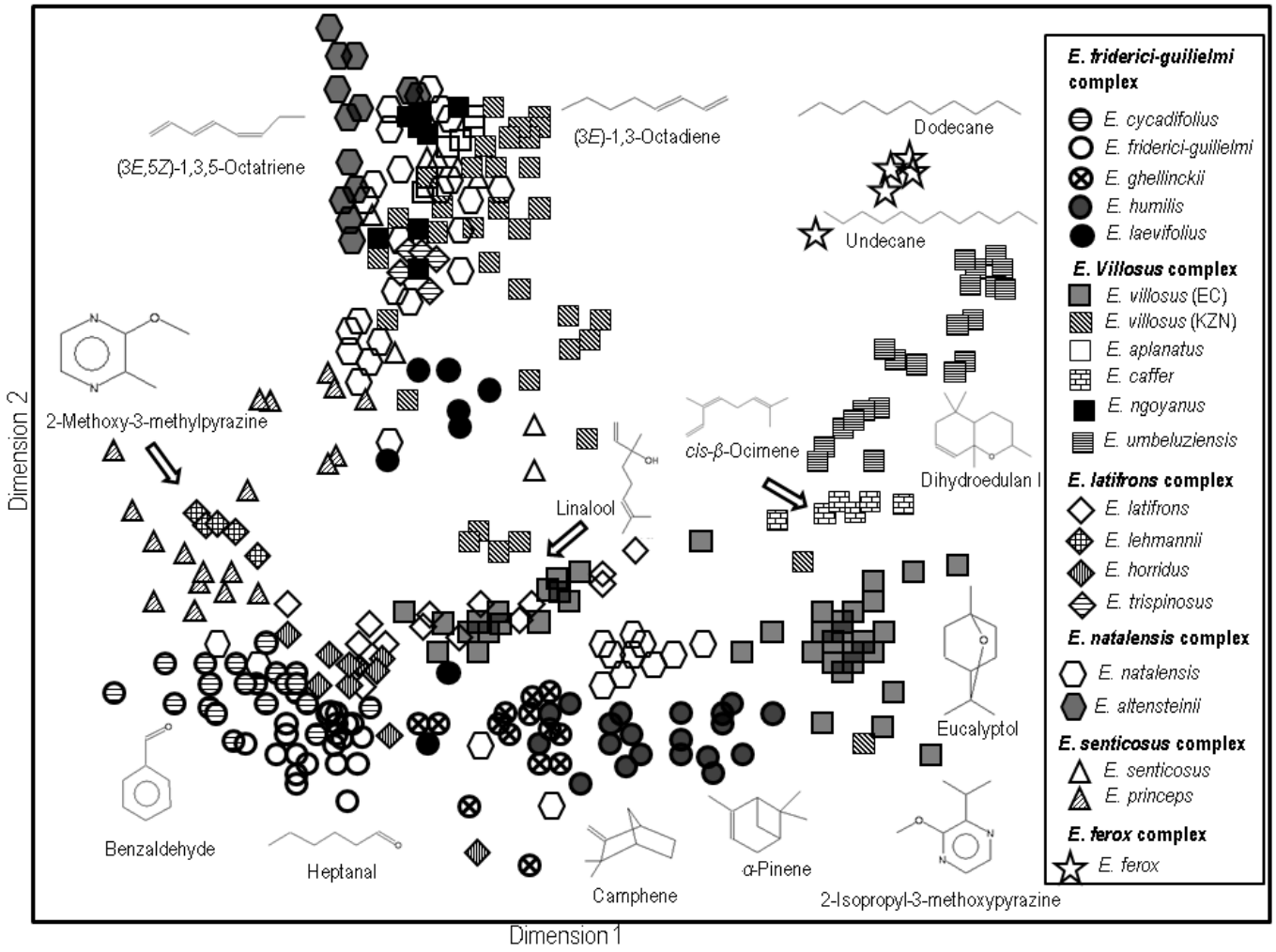

Figure 5.2: Ordination based on non-metric multidimensional scaling (NMDS) using Bray-Curtis similarity of male and female cone volatile composition comprising 152 compounds from 19 *Encephalartos* species in different phylogenetic clades. (2D stress value = 0.21; one-way ANOSIM, factor phylogenetic clade, $R = 0.307$, $P < 0.01$). The structures and names of the 14 main compounds dominating the volatile profile of cone odour of different *Encepahalartos* are presented in the figure

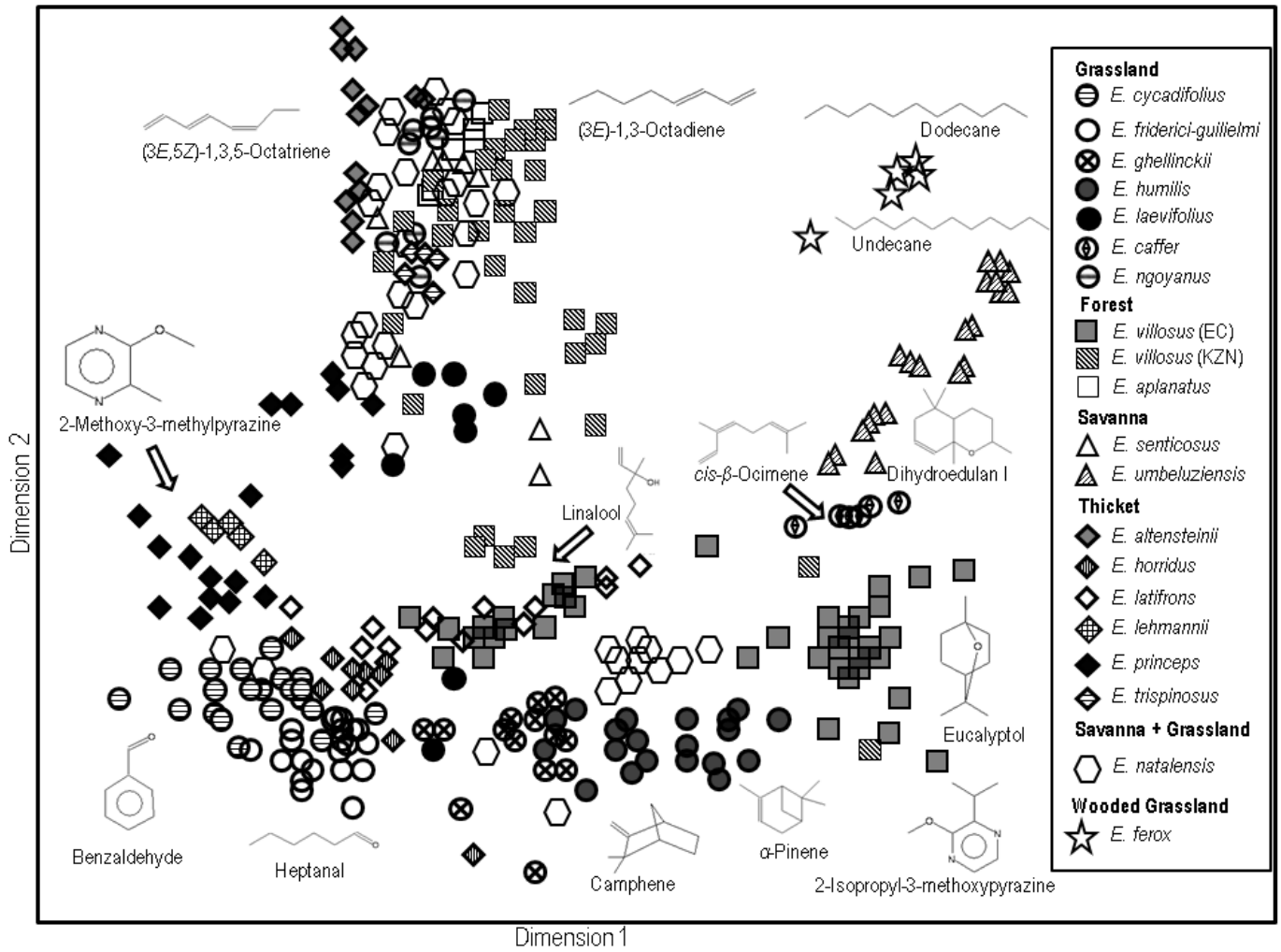

Figure 5.3: Ordination based on non-metric multidimensional scaling (NMDS) using Bray-Curtis similarity of male and female cone volatile composition comprising 152 compounds from 19 *Encephalartos* species occurring in different vegetation types in South Africa. (2D stress value = 0.21; ANOSIM, factor vegetation type, Global R = 0.344, $P < 0.01$). The structures and names of the 14 main compounds dominating the volatile profile of cone odour of different *Encephalartos* are presented in the figure

The NMDS analysis of odour composition based on similarities in pollination syndromes showed that *Encephalartos* species that have been classified as having only a cucujid pollination syndrome (*E. cycadifolius*, *E. ghellinckii* and *E. humilis*) all grouped together (Figure 5.4). The two species associated only with a *Porthetes* pollination syndrome, i.e. *E. ferox* and *E. aplanatus,* were distinct from each other but were also distinct from the cucujid pollinated species (R = 0.837, P < 0.01) (Figure 5.4). The volatile profiles of *Encephalartos* species with *Porthetes* plus cucujid pollination syndromes were wide spread in the odour space and were not significantly different from *Encephalartos* with either *Porthetes* or cucujid pollination syndromes (Figure 5.4) (NMDS stress value 0.21; one-way ANOSIM, factor pollination syndrome, R = 0.111, P < 0.01). Pairwise comparisons also failed to reveal any significant differences between species with both pollination syndromes and those with only one of the syndromes (range of R for pair-wise comparison between *Porthetes* plus cucujid and other pollination syndromes = 0.052 - 0.138, P > 0.05). Further comparison using one-way SIMPER, showed that 52.8% of the difference between species with both pollination syndromes was accounted for by variation in the relative amounts of (3*E*)-1,3-octadiene, (3*E*,5*Z*)-1,3,5)-octatriene, camphene, benzaldehyde and *α*-pinene . Camphene, benzaldehye and *α*-pinene were characteristic of *Encephalartos* species with a cucujid pollination syndrome while (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5)-octatriene were found in many of the species with a *Porthetes* syndrome.

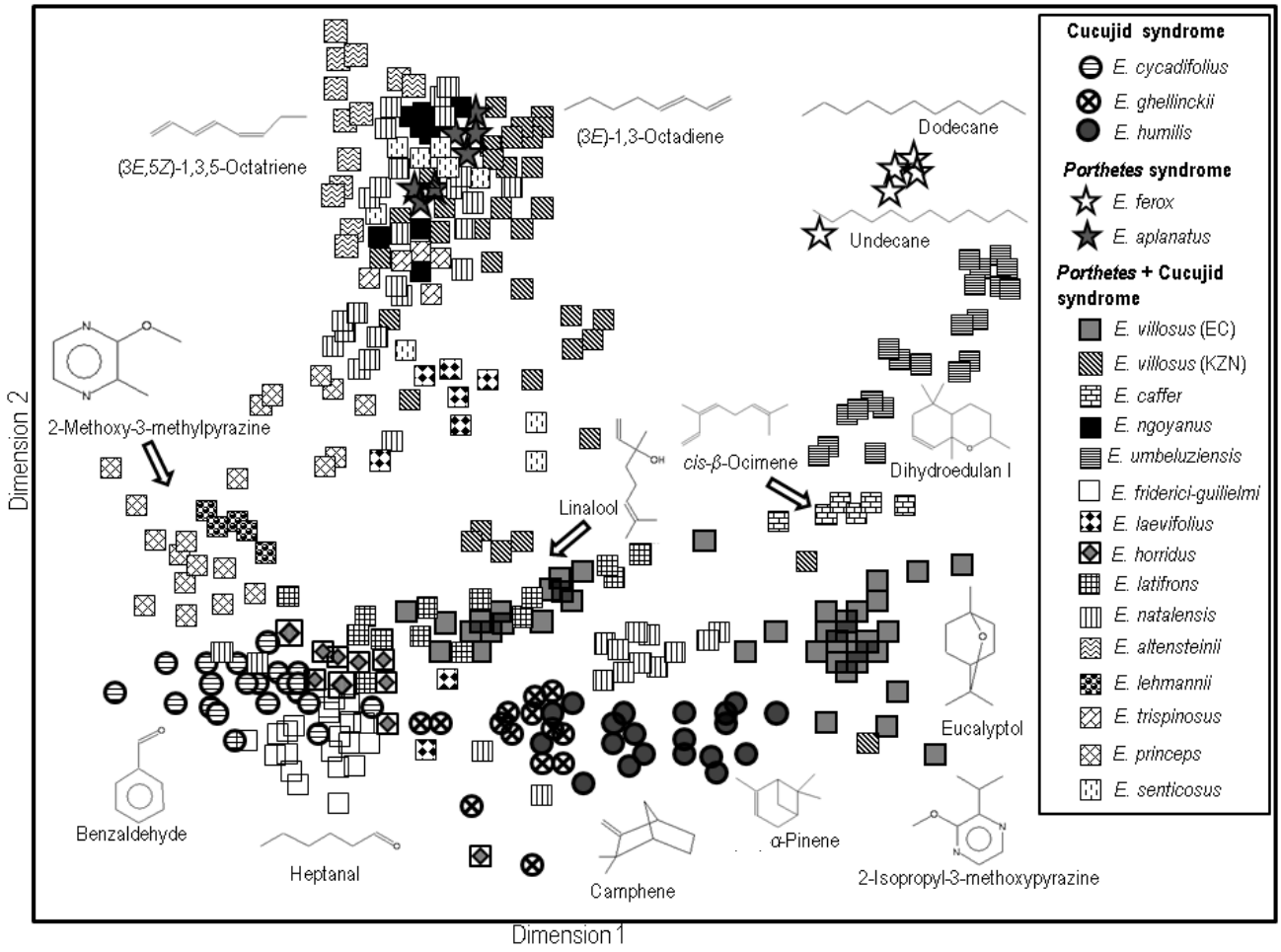

Figure 5.4: Ordination based on non-metric multidimensional scaling (NMDS) using Bray-Curtis similarity of male and female cone volatile composition comprising 152 compounds from 19 *Encephalartos* species associated with different pollination syndromes (2D stress value = 0.21; one-way ANOSIM, factor pollination syndrome, $R = 0.111$, $P < 0.01$). The structures and names of the 14 main compounds dominating the volatile profile of cone odour ofdifferent *Encephalartos* are presented in the figure

Discussion

The purpose of this chapter was to identify the volatile compounds emitted by both male and female cones of a wide range of *Encephalartos* species and to determine whether observed similarities in cone volatiles could be explained by phylogenetic relatedness, pollination syndromes, or other factors such as geographical distribution and occurrence in similar vegetation types. This is the first study that provides such a comprehensive breakdown of cone odour composition in a relatively large number of *Encephalartos* species (ca. 30% of all taxa, 51% of South African taxa). Previous studies have analysed scent mostly in a few single species of *Encephalartos* (e.g. *E. altensteinii*, Pellmyr et al., 1991; Tang, 1993; *E. natalensis*, Suinyuy et al., 2010) or comparing a small number of *Encephalartos* species with species from other cycad genera (e.g. Pellmyr et al., 1991; Tang, 1993). The results showed that *Encephalartos* species emitted a wide variety of compounds comprising unsaturated hydrocarbons, monoterpenes, aldehydes and benzenoids, nitrogen-containing compounds, sesquiterpenes, alkanes, aliphatic alcohols, aliphatic acids, ketones and esters (Table 5.2). An important question is whether these compounds have been gained and lost independently in the process of evolution (e.g. Barkman, 2001; Levin et al., 2001), or whether there are consistent patterns that correlate with phylogeny, pollination syndrome (Nogueira et al., 2001) and ecology so that particular compounds are restricted to particular lineages, pollination modes (Knudsen et al., 1993; Kaiser and Tollsten, 1995) or vegetation types (Randlkofer et al., 2010).

Altogether 152 compounds were identified from the 19 taxa included in this study. Of these, 27 compounds occurred in high relative amounts (≥ 10 %) and in most species there were one or two dominant compounds (relative amount >30%). One important observation from this study was the overall similarity in cone odours from male and female cones. The NMDS analyses showed that, based on relative

amounts of different compounds, the cone odours of male and female cones of the same species were generally similar to one another even if the overall concentrations differed.

The results show that each of the 19 *Encephalartos* taxa had its own characteristic volatile profile (Appendix 1). Nevertheless, it was possible to identify groups of taxa with similar volatile compounds (Figure 5.1) and NMDS analysis revealed that the 19 *Encephalartos* species can be grouped into six distinct groups based on the relative abundance of 15 dominant compounds grouped into seven compound classes (Figure 5.1). The two most distinct groups were each monospecific comprising *E. ferox,* characterised by emissions of alkanes, and *E. umbeluziensis* characterised by emission of sesquiterpenes (dihydroedulan I). The largest group (eight taxa) was characterised by cone odours dominated by unsaturated hydrocarbons (mostly (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene) (Appendix 1) and the second largest group (six taxa) was characterised by emissions of different monoterpenes (e.g. eucalyptol, camphene, linalool, *α*-pinene and *cis*-*β*-ocimene) (Figure 5.1). The remaining taxa fell into two groups, one (three taxa) with odours characterised by benzenoid (benzaldehyde) and aldehyde (heptanal) compounds, and the second (two taxa) characterised by nitrogen-containing compounds (2-methoxy-3-methylpyrazine).

The above grouping of *Encephalartos* taxa based on similarities in cone odours did not concur with the phylogenetic clades identified by Treutlein et al. (2005). Nevertheless, the NMDS analysis showed weak but statistically significant support for the hypothesis that species within a clade would be more similar to one another than to species from a different clade. Only *E. ferox*, which has a unique odour profile dominated by alkanes, was entirely consistent with this hypothesis. The greatest phylogenetic similarity amongst larger clades was observed in the *E. friderici-guilielmi* clade where four of the five species clustered together. This is not surprising given that the *E. friderici-guilielmi* clade had strong bootstrap support (98%) in phylogenetic analyses (Treutlein et al., 2005) and appears to have separated

from other clades early in the evolution of the genus *Encephalartos.* Moreover, the group has several consistent morphological features and the species all occur in grasslands. Within this clade, only *E. laevifolius* had a very different odour composition due to the relatively high levels of (3*E*)-1,3-octadiene, which is absent in other species within the clade. This compound is common in species from KwaZulu-Natal and it is interesting to note that historically *E. laevifolius* was widespread with populations in KZN that occurred close to other species of *Encephalartos* which also have cone odours containing (3*E*)-1,3-octadiene.

The one clade in which phylogenetic relatedness had no effect on similarity in cone odours was the clade containing *E. villosus* as well as *E. aplanatus, E. caffer, E. ngoyanus*, and *E. umbeluziensis*. This clade also had strong bootstrap support (99%) in phylogenetic analyses (Treutlein et al., 2005) but the data from Chapters 2 and 3 showed that even within *E. villosus* there was considerable variation in cone odours between populations. There was a strong similarity in the odour profiles of *E. villosus* (KZN), *E. aplanatus* and *E. ngoyanus* due to the dominance of unsaturated hydrocarbons ((3*E*)-1,3-octadiene and/ or (3*E*,5*Z*)-1,3,5-octatriene). In contrast, the odour composition of *E. villosus* (EC) and *E. caffer* were dominated by monoterpenes and the cone odour of *E. umbeluziensis* was dominated by sesquiterpenes. The distinction in odour composition was such that odours from *E. villosus* (EC), *E. caffer,* and *E. umbeluziensis* had no trace of unsaturated hydrocarbons whereas odours from *E. aplanatus* and *E. ngoyanus* had only trace amounts of monoterpenes. All the species except *E. umbeluziensis* had negligible amounts of sesquiterpenes. This clade therefore shows considerably more diversification in odour composition than was observed in any other clade.

The largest grouping within *Encephalartos* comprised species where cone odours were dominated by the unsaturated hydrocarbons (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene. There is no clear

phylogenetic link between these taxa (include 4 clades identified by Treutlein et al. (2005)) but there is a strong geographical link in that most of the species emitting these compounds have current or historical distributions that included populations in KwaZulu Natal (66%). The geographical pattern in cone odour observed in *E. villosus* (Chapter 3), in which plants from KZN populations emitted (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene also fits this overall pattern.

Despite the apparent prevalence of unsaturated hydrocarbons in the cone odours of KZN cycads, the data did not support the hypothesis that species from the same vegetation type would be most similar. Species with high relative amounts of unsaturated hydrocarbons occur in forest, savanna and grassland habitats. Analysis of other species within *Encephalartos* showed similarities among grassland species and a distinct profile for the single species from wooded grasslands (*E. ferox*). Within the taxa studied, *E. ferox* is also phylogenetically distinct so it is impossible to separate the phylogenetic effects from factors such as vegetation type. The grassland species are also mostly from the *E. friderici-guilielmi* clade so it is difficult to separate similarities due to phylogenetic conservatism from possible effects associated with grassland habitat. However, it is noteworthy that *E. laevifolius* differs from other species within the *E. friderici-guilielmi* clade even though it also occurs in grasslands indicating that divergence in odour composition is possible within the same lineage occurring in the same habitat. Similarly, major differences in odour composition between *E. caffer* and *E. ngoyanus* (part of the *E. villosus* clade) and all the other grassland species within the *E. friderici-guilielmi* clade suggests that there is no convergence in odour composition among taxa occurring in grasslands.

In highly specialised plant-pollinator interactions mediated by floral scent, the odour profile is sometimes dominated by one or few compounds (e.g. Knudsen and Mori, 1996; Grison-Pigé et al., 2002; Terry et al., 2007a and b) and these dominant compounds may account for variation among species (e.g.

Okamoto et al., 2007). Similarities in volatile compounds between species from different phylogenetic lineages may indicate that they have evolved pollination systems that involve the emission of similar volatile profiles (e.g. Knudsen and Tollsten, 1993; Andersson et al., 2002) or that they share the same pollination syndrome. The dominance of a few compounds is consistent with what was observed in the cone odours of most *Encephalartos* taxa and I tested whether similarities in odour compounds could be associated with particular pollinator syndromes. The NMDS analysis of *Encephalartos* taxa using pollinator syndrome as a factor showed that only the three species with a cucujid pollination syndrome (and no *Porthetes* syndrome) clustered together (Figure 5.4). All of these species belong to the *E. friderici-guilielmi* clade and occur in grassland vegetation types so there are other factors that may explain similarities in odour composition. Within this small group, *E. humilis* and *E. ghellinckii* have the most similar odour profiles and provide some support for the hypothesis that similarities in odour are associated with similarities in pollinator syndromes. However, the more general pattern seems to be a diversification of odour composition even among related species sharing the same pollinator syndrome. For example, cone odours from *E. cycadifolius* differ from the other taxa with only a cucujid pollinator syndrome because they are characterised by benzaldehyde and heptanal, which are shared with the closely related *E. friderici-guilielmi*. At the same time, *E. friderici-guilielmi* and *E. laevifolius* have completely different cone odours from each other even though they belong to the same clade, occur in the same vegetation type and have similar pollination syndromes.

Variation in volatile compounds between species within the same clade, with the same pollinator syndrome and the same vegetation types implies that specific components have been gained and lost independently in the process of evolution (Barkman, 2001; Levin et al., 2001). It is possible that these changes in odour composition are associated with the evolution of particular pollinator interactions and reflect a situation where volatile compounds function as reproductive isolation barriers (e.g. Mant et al.,

2005). Many *Encephalartos* species are now geographically isolated (Goode, 2001) so it is not clear whether isolation barriers have played a role in their evolution. One possible example is *E. caffer,* which occurs close to *E. villosus* and *E. ngoyanus* from the same clade in parts of its range but has evolved an interaction with a separate species of *Porthetes* (Oberprieler, 1996). The odour of *E. caffer* is characterised by *cis*-*β*-ocimene that distinguishes it from *E. villosus* (EC) populations whereas *E. villosus* (KZN) and *E. ngoyanus* are characterised by emissions of (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene. In a contrary example from the same clade, *E. villosus* (EC), *E. villosus* (KZN), *E. aplanatus* and *E. umbeluziensis* have three distinct odour profiles but share the same pollinator species (Downie et al., 2008). This implies that diversification in odour composition is not always associated with diversification in pollinators as would be expected in the evolution of reproductive isolation mechanisms.

The majority of the compounds recorded from *Encephalartos* taxa were emitted in small relative amounts ranging from trace amounts to just above 9 % (Appendix 1). Such compounds may play important roles in plant-pollinator interactions and they have been suggested to contribute to species isolation in co-occurring plants (Okamoto et al., 2007). Terpenoids are the most numerous compounds in the volatile profiles of *Encephalartos*, but only nine monoterpenes and a sesquiterpene occurred in high relative amounts (≥ 10 %). The majority of terpenoids occurred in small relative amounts and their possible influence on pollinator behaviour is unknown. Some may be biologically active although some may simply be artefacts arising from the scent sampling process, e.g. (*E*,*E*)-2,6-dimethyl-3,5,7-octatrien-2-ol, (*E*,*E*)-2,6-dimethyl-1,3,5,7-octatetraene (from *E. caffer*) and 2,6-dimethyl-3,5,7-octatrien-2-ol (from *E. latifrons*) may result from oxidation and rearrangement when large amounts of *cis*-*β*-ocimene are trapped on the carbon filters (Brunke, 1994; Tholl et al., 2006).

Many of the volatile compounds found in *Encephalartos* species occur in other cycad genera or other plants although their function in these species is often not known. For example (3*E*)-1,3-octadiene occurs in a large number of *Encephalartos* species as well as other cycad taxa (e.g. *Z. furfuracea*, Pellmyr et al., 1991) and some angiosperms (Hadacek and Weber, 2002; Knudsen et al., 2006). Results from Chapter 4 showed that (3*E*)-1,3-octadiene elicits a physiological response from pollinator insects of *E. villosus* and is an effective trap bait for pollinator insects from this species in the field. However, the activity of (3*E*)-1,3-octadiene in other *Encephalartos* species requires investigation. The other biologically active compound that attracted pollinators in the field was 2-isopropyl-3-methoxypyrazine from *E. villosus* (EC). This compound was also present in *E. natalensis* (Suinyuy et al., 2010) and *E. caffer* (Appendix 1) and has been recorded in other cycad genera (e.g. *Cycas thouarsii*, Kaiser, 2006).

As observed in *Encephalartos,* terpenoids are common in *Zamia* and dominant in *Macrozamia* species (Pellmyr et al., 1991; Tang, 1993; Terry et al., 2004a and b, 2008) while benzenoids, esters, and alcohols are dominant in *Cycas* (Pellmyr et al., 1991; Tang, 1993; Azuma and Kono, 2006), and in *Stangeria* (Proches and Johnson, 2009). Some of the volatile constituents in these cycads are active compounds that perform different functions and are widespread in other plants (Kajiwara et al., 1980; Boland, 1995; Knudsen et al., 2006). For example in some *Macrozamia* species, *β*-myrcene acts as an attractant and repellent of the pollinator *Cycadothrips chadwickii* at different concentrations (Terry et al., 2007a and b). It also occurs in several other plants and acts an attractant of the pine wood nematode, *Bursaphelenchus xylophilus* (Ishakawa et al., 1986). Likewise, linalool is widespread in the volatile profile of hundreds of plants (Raguso and Pichersky, 1999; Knudsen et al., 2006) and is an attractant of bees and other insects (e.g. Raguso and Pichersky, 1995; Dudareva et al., 1996; Borg-Karlson et al., 2003). It is also dominant in cone odours of *Macrozamia machinii* (Terry et al., 2004a and b).

Male and female cones emitted similar volatile compounds, but male cones volatile compounds occurred in higher relative amounts than female cone volatiles. Because cycads are dioecious, they require a mechanism to enhance pollinator movement between male and female cones for pollination to occur. Tang (1987a) hypothesized that female cones mimic male cones by emitting similar volatile compounds which are weaker. Cone mimicry is possible for *Encephalartos* species in this study because both male and female cones emit similar volatile compounds. Ashman (2009) in her review asserts that in intersexual mimicry, male and female flowers of the same species should use similar cues (advertisement) so as to maintain constancy of pollinators. Intersexual mimicry seem to occur in *Zamia pumila* (Breckon and Oritz, 1983), *Cycas rumphii* (Pellmyr et al., 1991), *Macrozamia machinii, M. macleayii* and *M. lucida* (Terry et al., 2004a), *C. revoluta* (Azuma and Kono, 2006), and *Stangeria eriopus* (Proches and Johnson, 2009).

One common factor between studies of cone odours in *Cycas*, *Macrozamia*, *Zamia* and *Encephalartos* is that there is interspecific variation in the composition of volatile odours. In Australian *Macrozamia*, variation in volatile composition among the different species seems to be best explained by pollination syndrome. The genus is subdivided in two sections based on morphological characters (Forster, 2004) and species in both section *macrozamia* and *parazamia* emit *β*-myrcene as a major compound when they are associated with thrips *(Cycadothrips*) pollinators (Forster et al., 1994; Terry, 2001; Terry et al., 2004a and b). In contrast other species within these sections emit other compounds when associated with beetle pollinators, e.g. *Macrozamia machinii* (section *parazamia*) emits linalool (Terry et al., 2004and b) while *M. moorei* (section *macrozamia*) emits *α*-terpinene and *P*-cymene (Pellmyr et al., 1991; Tang, 1993; Terry et al., 2004b) and is associated with *Ulomioides australis* (Forster et al., 1994; Terry et al., 2004b). Similarly, studies of volatile emission in *Zamia furfuracea* and *Z. pumila* suggest that pollination syndrome accounts for the variation in volatile composition among the species. *Zamia furfuracea* emits (3*E*)-1,3-octadiene and linalool (Pellmyr et al., 1991; Tang, 1993) and

is associated with *Rhopalotria mollis* (Norstog and Fawcett, 1989; Stevenson et al., 1998) while *Z. pumila* which is associated with *R. slossoni* and *Pharaxonotha zamiae* (Tang, 1987a; Fawcett and Norstog, 1993) is characterised by methyl salicylate (Pellmyr et al., 1991; Tang, 1993). Interspecific variation in volatile composition has also been recorded among *Cycas* species. Pellmyr et al. (1991) identified 2-pentanol, 2-heptanol, 2-pentanone, methyl-3-methylbutanoate and 2-pentyl-acetate as the dominant compounds in *C. rumphii* whereas estragole was detected as the main compound in *C. revoluta* (Azuma and Kono, 2006). However, it is not possible to determine whether these compounds are associated with particular pollinators.

High interspecific variation in floral scent has been observed in other plant taxa (e.g. Thien et al., 1975; Pellmyr et al., 1987; Knudsen and Tollsten, 1991; Barkman et al., 1997; Dobson et al., 1997; Levin et al., 2001; Raguso et al., 2006) and has been variously attributed to phylogenetic relationships (e.g. Azuma et al., 1997; Nogueira et al., 2001; Grison et al., 1999; Raguso et al., 2006), pollination syndrome (e.g. Füssel et al., 1997) or both (Dobson et al., 1997; Raguso et al., 2003). Some studies argue that there is a more complex ecological signal (e.g. Levin et al., 2001; Jürgens et al., 2002) as neither phylogeny nor pollination syndrome can adequately account for variation in volatile compounds among species. Further studies are required to identify the factors that explain variation in the composition of *Encephalartos* cone odours. Overall, the study provides some evidence that phylogenetic history explains some of the variation in cone odours but there has been considerable diversification of cone odours in some lineages. The limited analysis of the link with pollinators, using pollinator syndromes, failed to show a distinct pattern but further analysis using more detailed information on the composition and phylogeny of pollinators may show how pollinators have interacted with specific odour compounds.

Chapter 6. The role of cone volatiles and heating in the pollination of *Encephalartos*

Abstract

This chapter summarises the results presented in the previous chapters and places the full scope of the work within the context of current knowledge about cycad pollination systems and chemically mediated pollinator interactions. Findings about the roles of volatile compounds and heat production in the pollination of *Encephalartos* are compared to those for cycads in general. The general significance of variation in cone volatile emissions among different populations of *E. villosus* and among *Encephalartos* species is explored. Based on these findings for *E. villosus*, it appears that pollination mutualisms in *Encephalartos* are mediated by cone volatiles, and that there can be high levels of intraspecific variation in the compounds that are used as cues by beetle pollinators. Further work will need to be done to dissect the roles of coevolution versus unilateral evolution in driving divergence among cycad populations and species.

Introduction

Ever since the role of insects in cycad pollination was first proposed (Pearson, 1906; Rattray, 1913; Marloth, 1914) and later confirmed in *Zamia* (Norstog et. al., 1986; Tang 1987a), *Encephalartos* (Donaldson et al., 1995; Donaldson, 1997; Suinyuy et al., 2009) and other cycad genera (e.g. Mound and Terry, 2001; Terry, 2001; Terry et. al., 2005; Proches and Johnson, 2009), there have been questions about the role that cone volatiles and thermogenesis play in mediating this interaction (Pearson, 1906; Rattray, 1913; Tang, 1993; Terry et al., 2007a and b). In other highly specialised plant-insect pollinator interactions, the emission of volatile compounds in association with heat production

has been shown to influence pollinator behaviour (Pellmyr and Thien, 1986; Seymour and Shultz-Motel, 1997; Thien et al., 2000), indicating that floral odour and heat production are adaptations for insect pollination (Meeuse, 1975). Despite the early reports suggesting that pollen-shedding cones of cycads heat up (Poisson, 1878; Jacot-Guillarmod, 1958) and emit volatile compounds which probably attract insects (Pearson, 1906; Rattray, 1913), their roles have been investigated in only a few cycad species (e.g. Tang, 1993; Terry et al., 2007a and b).

The limited number of studies of cycad pollination have nevertheless revealed some remarkable insights. First, there is a limited diversity of cycad pollinators that colonise the cones of different cycads. Most of the pollinator species belong to the beetle superfamilies Curculionoidea and Cucujoidea. Phylogenetic studies of these groups indicate that the beetle families associated with cycads on different continents are not closely related (Oberprieler, 1995; Leschen, 2003). Oberprieler (2004) suggested that the pollinator assemblage indicates convergent evolution and include species that may have shifted from angiosperm hosts. Also, some pollinators are restricted to a single cycad genus. For example cycad thrips, *Cycadothrips* spp., have so far only been recorded from cones of *Macrozamia* (Terry, 2001; Mound and Terry, 2001; Terry et al., 2005) and the only recorded Lepidopteran putative pollinator, *Anatrachyntis,* visit the cones of *Cycas micronesica* (Terry et al., 2009; Marler, 2010). One common observation with all the cycad-pollinator interactions is that the pollinators used the male cones as brood sites. There are marked differences in cone volatile composition between cycad genera and between species (e.g. Pellmyr et al., 1991; Tang, 1993; Terry et al., 2004a and b) and different compounds have been found to be associated with different groups of pollinators. For example *Cycadothrips* respond to *β*-myrcene in *Macrozamia lucida* and *M. macleayii* while weevil pollinators *Tranes* sp. respond to linalool in *M. machinii* (Terry et al., 2007a and b). Contrary to the typical role of volatiles in attracting pollinators, cone volatiles in *Macrozamia* form part of a more

complex push-pull interaction in which *Cycadothrips* are attracted by *β*-myrcene at low concentration and are repelled by high concentrations of the same compound at (Terry et al., 2007a and b).

These studies have raised further questions about the nature of cycad pollinator interactions, specifically how these interactions have evolved and diversified, and what role cone volatile emissions and heating have played in shaping the relationship between cycads and their insect pollinators. In the series of studies reported in this thesis I examined the role of cone volatiles and, to a lesser extent, the role of thermogenesis in the pollination of *Encephalartos* with particular emphasis on *E. villosus.* The thesis addressed three main questions:

- Do cone volatile emissions play a role in the pollination ecology of *Encephalartos*?
- Is there evidence of push-pull interactions in *Encephalartos*?
- Do patterns of volatile emissions in *Encephalartos* indicate phylogenetic conservatism within the group or do they reflect adaptation to particular pollination syndromes or habitats?

In this chapter I synthesize the findings of the earlier chapters that addressed these three questions, place the results in the broader context of theory about chemically mediated plant-insect interactions, and identify questions for further study.

Do cone volatile emissions play a role in the pollination of *Encephalartos villosus*?

Pearson (1906) and Rattray (1913) noticed that pollen shedding cones of *Encephalartos* emitted distinct odours and suggested that the odour attracted the beetles observed on cycad cones. This observation was confirmed in this study which showed that patterns of cone volatile emissions in *E. villosus* coincided with periods of pollination when insects were active on pollen shedding and receptive

female cones (Chapters 2 and 3). More importantly, the hypothesis that cone odours act as attractants for pollinating insects was confirmed by gas chromatography electroantennographic detection (GC-EAD), olfactometer experiments, and field studies (Chapter 4).

The volatile compounds emitted by male and female cones of *E. villosus* consisted of fatty acid derivatives (alcohols, aliphatic acids, aldehydes, esters, ketones, and unsaturated hydrocarbons), benzenoids, terpenoids (monoterpenes and sesquiterpenes) and nitrogen-containing compounds. They were characterised mainly by eucalyptol, 2-isopropyl-3-methoxypyrazine, (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene (Chapters 2 and 3). Two of the dominant compounds, (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene, elicited physiological responses in the antennae of two pollinator species (Chapter 4). Further olfactometer experiments and traps set in the field confirmed that (3*E*)-1,3-octadiene attracted pollinators, at least at the concentrations tested in this study. Two additional compounds, 2-isopropyl-3-methoxypyrazine and eucalyptol, elicited attraction responses in olfactometer tests and in field traps even though no physiological responses were detected in GC-EAD experiments. The implication is that these compounds can individually attract particular pollinators. The other physiologically active compound, (3*E*,5*Z*)-1,3,5-octatriene, was not tested for behavioural activity because of the lack of commercial availability of a chemical standard.

An important and unexpected result was the occurrence of different geographically-structured chemotypes of *E. villosus* based on the volatile compounds emitted by male and female cones. The volatile profiles of plants from populations south of the Umtamvuna river (in the Eastern Cape, EC) were characterised by eucalyptol and 2-isopropyl-3-methoxypyrazine while plants from north of the Umtamvuna River (KwaZulu-Natal, KZN) were characterised mostly by emissions of (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene (Chapters 2 and 3). Based on other studies where specific pollinator interactions are mediated by floral volatiles, it was expected that such a significant shift in volatile

compounds in *E. villosus* would be associated with a shift in pollinators. However, surveys of insect pollinators revealed that the same species of *Porthetes, Metacucujus*, *Antliarhinus* and Erotylidae occurred across the range of *E. villosus* (Chapters 2 and 3) and were attracted to both 2-isopropyl-3-methoxypyrazine and (3*E*)-1,3-octadiene in bucket traps (Chapter 4). Despite the apparent uniformity in insect pollinators, differences in insect responses to *E. villosus* volatiles in the two regions (Chapter 4) shows that there is geographical variation in volatile preferences within insect species, especially for Erotylidae and *Porthetes* sp. At this stage it is not possible to determine whether these differences represent possible differentiation in volatile preferences between morphologically cryptic insect species. Further genetic work is also required to determine whether the geographical divergence in this system is a result of coevolution between *E. villosus* and its pollinators.

The volatile compounds emitted by male and female cones of *E. villosus* (Chapters 2 and 3) also occur in other *Encephalartos* species (Suinyuy et al., 2010; Chapter 5) and other cycad taxa in different relative amounts or concentrations. Perhaps most significant is that (3*E*)-1,3-octadiene, which was shown here to elicit both physiological and behavioural responses in pollinators of *E. villosus* (Chapter 4), is also emitted as a dominant compound by at least seven other *Encephalartos* species (Chapter 5) and by *Zamia furfuracea* (Pellmyr et al., 1991). Given the results obtained from *E. villosus* there is good reason to expect that (3*E*)-1,3-octadiene will also influence pollinator behaviour in other cycad taxa. In the case of *Encephalartos,* the eight species in which cone odours are dominated by (3*E*)-1,3-octadiene all have a *Porthetes* spp. as one of the pollinator taxa but there are at least four species of *Porthetes* involved (Oberprieler, 1995; Downie et. al., 2008). This suggests that while (3*E*)-1,3-octadiene may be an important attractant for *Porthetes,* host specific interactions between different *Porthetes* spp. and their host plants are not dependent entirely on this compound, despite the finding in Chapter 4 that (3*E*)-1,3-octadiene alone was sufficient to attract the *Porthetes* beetles that pollinate *E. villosus*.

The other biologically active compound in *E. villosus* was 2-isopropyl-3-methoxypyrazine (Chapter 4). This compound which attracted *E. villosus* insect pollinators in bucket traps (Chapter 4) occurred predominantly in the southern populations of *E. villosus* and the only other record within *Encephalartos* was trace amounts detected in cone odours of *E. natalensis* (Suinyuy et al., 2010; Chapter 5). A stand out feature of 2-isopropyl-3-methoxypyrazine is its pungent odour, even at low concentrations, which is responsible for the strong smell associated with certain *E. villosus* cones (Rattray, 1913). In other cycad genera, the only record of 2-isopropyl-3-methoxypyrazine is from *Cycas thouarsii* where it has been recorded as a major compound in cone volatiles (Kaiser, 2006). Nothing is known about the pollination ecology of *C. thouarsii* so it is not possible to speculate on the possible role of 2-isopropyl-3-methoxypyrazine in this species. It should also be noted that *β*-myrcene was detected as a minor compound in the volatile profile of *E. villosus* (Chapters 2 and 3). This is a dominant compound in the emissions of the Australian cycads *Macrozamia lucida* and *M. macleayii* and is the main compound influencing the behaviour of thrips pollinators on *Macrozamia* spp. (Terry et al., 2004a and b). No responses to *β*-myrcene were recorded in this thesis but the role of minor compounds in *Encephalartos* has yet to be fully explored.

Evidence for push-pull interactions in *Encephalartos*

Push-pull interactions involving cycads and insect pollinators are caused by regular daily changes in levels of volatile emissions and heat production (Terry et al., 2004a, 2007a and b) resulting in periods when the cone attracts pollinators alternating with periods when the cone is repellent to pollinators. In many cycads, male cones are the brood sites for pollinators, while female cones are apparently visited by mistake. It would thus benefit the male fitness of plants if insect pollinators were expelled from male cones resulting in greater pollen dispersal. Terry et al. (2007a and b) showed that in

pollen shedding cones of *Macrozamia lucida*, attraction occurred when the major compound *β*-myrcene was emitted in relatively low concentrations and cone temperature was low, whereas the repellent phase occurred when *β*-myrcene was emitted in high concentrations and cone temperatures were at their peak. Notable shifts in pollinator behaviour accompanied the changes in volatile concentrations and cone temperature.

In pollen shedding cones of *E. villosus*, there were no significant differences in the amounts of different volatile compounds emitted throughout the day. The daily pattern of cone volatile emission in *E. villosus* therefore seems contrary to the expected pattern for push-pull interactions. In addition, the presence of insects on male cones of *E. villosus* at all developmental stages (Chapter 2) suggests that volatile compounds act mostly as insect attractants (Chapter 2). This was supported by the results from olfactometer tests in which male cone sporophylls at all developmental stages, as well as specific compounds, attracted insect pollinators (Chapter 4). In their investigation of push-pull interactions in *Macrozamia*, Terry et al. (2007a and b) looked at the absolute concentrations of the volatile compounds and were able to distinguish changes in pollinator behaviour associated with changes in absolute concentrations of the same compound. In contrast, the analyses in this study were restricted to comparisons of the relative amounts of volatile compounds in *E. villosus*. As a result, it is not possible to exclude the possibility that higher or lower concentrations of the active compounds may have had an effect on pollinator behaviour. Despite this limitation, the available evidence shows that cone volatiles have a significant and measurable effect on pollinator attraction whereas there is no evidence that volatiles alone have a repellent (push) function in pollinator interactions of *E. villosus*.

In contrast to cone volatiles, there was a distinct daily pattern in cone thermogenesis with low cone temperatures in the morning, high cone temperatures in the afternoon, and peak cone temperatures in the evening (Chapter 2). Such a daily pattern of cone thermogenesis is consistent with

what would be expected in a push-pull interaction. Confirmation of such an interaction would require evidence that changes in cone temperature associated with thermogenesis cause daily movement of insect pollinators between male and female cones. Field observations of dehiscing male cones of *E. villosus* at peak temperatures in the evening indicated that insects were generally less abundant than earlier in the afternoon preceding peak cone temperature (Chapter 2). The implication is that insects might have moved away from male cones in response to higher cone temperatures, although there was no mass exodus of pollinators from male cones as observed with *Cycadothrips* pollination systems in species of *Macrozamia* or even with weevil pollinated species such as *M. machinii* (Terry et al., 2004a). Although heating associated with periods of thermogenesis may influence daily movement of pollinators on cones of *E. villosus* this was not explicitly tested. Appropriate testing would have to overcome methodological constraints such as the difficulty of separating the effects of temperature from the effects of cone volatiles. In addition, insects associated with *E. villosus* were often unresponsive when placed in artificial experimental chambers and it would therefore be necessary to separate authentic behavioural responses to experimental treatments from artefacts of the experimental system. Also, because of the infrequent coning nature of *Encephalartos*, cones were not available to conduct different experimental studies.

Compounds from diverse chemical classes have been identified as pollinator attractants, particularly terpenoids, hydrocarbons, esters, alcohols, aldehydes and fatty acids occurring in the floral scent of angiosperms (kite et al., 1991; Knudsen et al., 1993). The range of pollinator attractants in cycads is poorly known except for work on *Macrozamia* (Terry et al., 2007a and b). Identification of pollinator attractants in *E. villosus* required coupled gas chromatography electroantennogram detection (GC-EAD) experiments and behavioural assays to identify physiologically and behaviourally active compounds.

In the experiments outlined in Chapter 4, I specifically tested the responses of pollinators to (3*E*)-1,3-octadiene, 2-isopropyl-3-methoxypyrazine and eucalyptol. Although all the compounds attracted insects to some degree, the number of insects attracted to the different compounds varied. For example, in KZN populations, *Porthetes sp., Antliarhinus zamiae* and Erotylidae sp. all responded far more strongly to (3*E*)-1,3-octadiene than to either 2-isopropyl-3-methoxypyrazine or eucalyptol. The differences could simply reflect different roles as attractants but it does not exclude the possibility that those compounds which attracted fewer insects may have a repellent effect. Results in Chapter 2 showed that insect pollinators were more abundant on dehiscent male cones when they were emitting volatiles in high relative amounts, but declined after pollen shed when the volatile emission was low and the cone started deteriorating. This implies that volatile compounds are attracting insect pollinators to dehiscing male cones where they mate, oviposit and breed in the sporophyll tissues. After dehiscence when the cones are deteriorating and volatile emission has ceased, the insects leave in search of suitable cones. In this case, volatile compounds of *E. villosus* would function only as attractants and have no repellent effects as the insects would leave only when the cone starts deteriorating and is no longer a suitable brood site.

Although the few insects that were observed leaving the male cones in the evening could be responding to high cone temperatures, the movement did not seem to signify a strong daily repellent effect. In contrast, the change in the insect abundance at different developmental stages represented a much stronger pattern that is more consistent with a life cycle push-pull interaction pattern in which active attraction of insects to volatile compounds occurs at one stage in the male cone development cycle followed by passive dispersal due to the declining quality of the cone later on (Donaldson, 2007). *Rhopalotria mollis* pollinators of *Zamia furfuracea* (Norstog and Fawcett, 1989) and *Tranes* weevils pollinators of *Lepidozamia peroffskyana* (Hall et al., 2004) showed similar behaviour to *E. villosus* pollinators and also leave the male cones as they deteriorate.

Despite the change in pollinator abundance recorded at different developmental stages in *E. villosus*, there are still uncertainties related to the role of cone volatiles as a 'push' factor in *Encephalartos*. Because pollinators must be attracted to both male and female *E. villosus* cones for pollination to occur, it was expected that insects would respond to similar volatile odours in male and female cones. However, the uniformity in the relative amounts of cone volatile compounds at all the developmental stages in male and female cones of *E. villosus* may mask the potential for a 'push' factor based on changes in absolute concentrations. Future work should measure the absolute concentrations of the compounds in order to determine if their function changes at different concentrations (e.g. Terry et al., 2007a and b). In addition, one of the physiologically active compounds, (3*E*,5*Z*)-1,3,5-octatriene, was not tested for behavioural activity because of lack of chemical standard and future work should determine the role of this compound once it is synthesized.

Evidence for adaptive shifts in volatile emissions within *Encephalartos*

Although early reports suggested that odour was emitted from pollen shedding cones of *E. villosus* (Pearson, 1906; Rattray, 1913), *E. altensteinii* and *Stangeria* (Rattray, 1913), cycad cone volatiles have been investigated in only a small number of cycad taxa namely; three species of *Cycas* (Pellmyr et al., 1991; Tang, 1993; Azuma and Kono, 2006; Terry et al., in press), two of *Encephalartos* (Pellmyr et al., 1991; Tang, 1993; Suinyuy et al., 2010), seven *Macrozamia* (Pellmyr et al., 1991; Tang, 1993; Terry et al., 2004a and b, 2008), the monotypic *Stangeria eriopus* (Proches and Johnson, 2009) and two species of *Zamia* (Pellmyr et al., 1991; Tang, 1993). The work presented here for 19 species of *Encephalartos* therefore represents a substantial increase in the available data on cycad volatiles and makes it possible to analyse patterns in volatile emissions across a broad cross section of species.

The GC-MS analyses for *Encephalartos* spp. showed that the volatile profiles consisted of blends of compounds belonging to several chemical classes, typically fatty acid derivatives, benzenoids, terpernoids and sometimes nitrogen-containing compounds that may vary in the number, composition, and relative amounts of the different constituents. The particular constituents and pattern of odour emission comprise the signals that may influence the composition and behaviour of pollinators (Dodson et al., 1969; Pellmyr, 1986a; Dobson et al., 1997; Raguso, 2008) and would therefore be expected to reflect any adaptive changes associated with pollination ecology.

The sampling of *E. villosus* volatiles from populations across the distribution range (Chapter 3) revealed at least two chemotypes with a shift occurring between populations south and north of the Umtamvuna river. Because the distribution range of *E. villosus* overlaps with other species, the observed variation in the cone volatiles raised questions about how pollination may be mediated by volatile compounds across different environments especially when other species of *Encephalartos* occurred nearby. It raised questions as to whether scent chemistry is divergent, resulting in interactions with different pollinators, or convergent perhaps due to adaptation to the same pollinator.

The geographical distribution of chemotypes in *E. villosus* (Chapters 2 and 3) shows some convergence with patterns of volatile emissions in other *Encephalartos* taxa (Chapter 5) that occur in the same region. Analysis of cone volatiles from all 19 species of *Encephalartos* revealed that eight species contain (3*E*)-1,3-octadiene as a dominant compound in cone volatiles (Chapter 5) and five of these species occur in KwaZulu Natal. This suggests that there could be local convergence in floral volatiles among unrelated species that share similar pollinators. It is true that all of these species appear to have the same pollinator syndrome in that species of *Porthetes* as well as cucujoid beetles have been recorded from cones of all of these species in KZN (Chapter 5). However, the pattern is not statistically significant because at least three species that also contain (3*E*)-1,3-octadiene do not occur

in the same geographical area. In addition, convergence in cone volatiles was not recorded in other areas such as the Eastern Cape, where populations of *E. villosus* occur close to other species where there is little or no convergence in cone volatiles. In these populations, *E. villosus* emits 2-isopropyl-3-methoxypyrazine, which has not yet been isolated from other cycads in the same area even though it was also shown to be an attractant for pollinators (Chapter 4). Instead, cone odours from species of *Encephalartos* in the EC consisted of a diversity of compounds made up of (3*E*)-1,3-octadiene, monoterpenes, benzaldehydes, aldehydes and pyrazine compounds in high relative amounts, even though eight of the species in this region share the same pollinator syndrome (Chapter 5).

It is notable that *Porthetes* spp. are associated only with *Encephalartos* so they do represent a cycad specific pollination syndrome. Nevertheless, there is a high degree of host specificity within the genus. Surveys have revealed at least 19 species (Oberprieler 1995; Downie et al., 2008) of *Porthetes,* of which 11 have been recorded from a single host plant and a further four have only two host plants (Downie et al., 2008; J.S. Donaldson unpublished data). This suggests that divergence in odours between *Encephalartos* species may be an adaptation to different species of pollinators (Knudsen and Tollsten, 1993; Tollsten and Bergström, 1993; Tollsten and Øvstedal, 1994) even though they form part of the same broad pollinator syndrome.

An important question in examining adaptation to specific pollinators is the degree of phylogenetic conservatism in odour composition within *Encephalartos.* The results of Chapter 5 showed that closely related species do sometimes have similar odour profiles, such as *E. altensteinii* and *E. natalensis,* which emit (3*E*)-1,3-octadiene (Pellmyr et al., 1991; Suinyuy et al., 2010; Chapter 5). However, there was compelling evidence for shifts in odour profiles within clades of *Encephalartos.* Within the *E. friderici-guilielmi* clade (Treutlein et al., 2005) cone odours mostly comprised benzenoids, aldehydes and monoterpenes and the species clustered together in NMDS analyses (Chapter 5).

Despite this clustering, there was divergence within the clade between species with different pollinator syndromes such as between *E. ghellinckii* and *E. humilis,* which emit monoterpenes and are associated with a cucujid pollination syndrome, and *E. friderici-guilielmi* which emits more aldehydes and is associated with a *Porthetes* plus cucujid pollination syndrome (Chapter 5). Within this clade, *E. laevifolius* had a completely different odour profile that was more similar to species in other clades.

The strongest evidence for shifts in odour composition within a clade was found in the *E. villosus* clade. Populations of *E. villosus* from KZN, had similar volatile profiles to *E. ngoyanus* and *E. aplanatus*, but these differed substantially from EC populations of *E. villosus, E. caffer* and *E. umbeluziensis* (Chapter 5). Variation in volatile compounds between species as well as the type of variation has been recorded in other plants (e.g. Tollsten and Bergström, 1993; Tollsten & Øvstedal, 1994; Azuma et al., 2001) and is typically interpreted as an adaptation to different pollinators (Knudsen and Tollsten, 1993; Tollsten and Bergström, 1993; Tollsten and Øvstedal, 1994). However, despite substantial differences in volatile compounds, all populations of *E. villosus* (both EC and KZN), as well as *E. aplanatus* and *E. umbeluziensis* appear to be pollinated by the same species of *Porthetes* based on morphological and molecular data (Downie et al., 2008). This shows that divergence in volatile compounds can occur without a shift in pollinators (e.g. Ellis and Johnson, 2009; Chapter 3). The attraction of the same beetles by different compounds in closely related *Encephalartos* taxa raises questions about convergence of function in different volatile compounds and is an area that requires further investigation. It also raises questions about the population structure of pollinator species where the pollinator is responding to different compounds in different populations.

Random genetic drift and gene flow across species boundaries may be important sources of variation in volatile composition between populations of *E. villosus*. Generally, *E. villosus* plants are widely distributed and occur in small isolated populations. Disjunctions between the widespread

populations limit gene flow throw pollen and seed dispersal. Plant species that have limited pollen and seed dispersal ability tend to have more genetic differentiation among populations than species with more potential for gene movement (Hamrick and Godt, 1996). The volatile variation observed in *E. villosus* could therefore be as a result of random genetic drift in the widespread isolated populations (Tollsten and Bergström, 1993). The same may be true of pollinator populations, although studies on *Porthetes* associated with *E. friderici-guilielmi* showed little genetic differentiation between populations (Downie and Williams, 2009) and suggested that gene flow is occurring between quite widely distributed populations.

The observed variations in volatile compounds within *Encephalartos* suggests a complex ecological signal that is not fully explained by phylogenetic conservatism within clades or by adaptation to particular pollination syndromes, or convergent adaptation to available pollinators in a particular area. More detailed studies of interactions with specific pollinators and the interaction of pollinators with a variety of compounds may help to better understand the evolution of these interactions and how they are influenced by shifts in volatile compounds.

Significance of this study

This study represents an important advance in our understanding of pollination systems in *Encephalartos,* but the results also have wider significance for our understanding of the role of cone volatiles in cycad pollination systems and how these differ from chemically mediated pollination systems in angiosperms.

It is interesting that the unsaturated hydrocarbons, particularly the alkenes that attract insect pollinators in *Encephalartos,* are also physiologically and behaviourally active compounds in angiosperms with specialised pollination systems (e.g. Ayasse et al., 2000; Schiestl and Ayasse, 2002;

Svensson et al., 2005). Whether the alkenes evolved as pollinator attractants before the divergence of cycads and angiosperms is not known, but the biologically active alkene compounds in *Encephalartos* are present in the volatile profiles of some basal plant families in the angiosperms (e.g. Skubatz et al., 1996; Hadacek and Weber, 2002). A related question is whether the weevil pollinators which are attracted to alkenes in modern cycads have evolved on cycads or whether they are derived from ancestors occurring on angiosperm hosts that may have had similar volatile compounds. Based on a phylogenetic analysis of weevil families, Oberprieler (2004) suggested that the evolution of cycad pollinator interactions has involved shifts from angiosperm hosts to cycads. The occurrence of alkenes in the cone volatiles of modern cycads, which attract pollinators, as well as in the floral volatiles of basal angiosperms provides some evidence that similarities in chemical signalling could have facilitated host shifts in pollinating species.

The results regarding the importance of pyrazine compounds in mediating cycad-pollinator interactions add a further dimension to the link between cycads and angiosperms. Pyrazines in the floral scent profile of some angiosperms (e.g. Mookherjee et al., 1990; Borg-Karlson et al., 1994; Knudsen and Ståhl, 1994) have been interpreted to function as offensive, deterrent, repellent and warning compounds (Guilford et al., 1987; Rothschild and Moore, 1987; Kaye et al., 1989; Moore et al., 1990). In contrast, pyrazine compounds were recorded in the volatile profile of several *Encephalartos* species (Chapter 5) and one of these compounds attracted insect pollinators of *E. villosus* (Chapter 4). The attraction of pollinators is more consistent with observations on palms in which pyrazines acted as attractants for *Baridine* weevil pollinators of *Aphandra natalia* palms (Ervik et al., 1999). In addition to the similarity in attractant compounds in both cycads and palms, weevil pollination in palms is similar to that in cycads where male reproductive structures offer brood sites to pollinators (Anstett, 1999). Since some cycad pollinating weevils are closely related to those of some palms, it has been suggested that

pollinators from palms have shifted to cycads (Oberprieler, 2004). Similarities in attractant compounds between palms and cycads lend some credibility to this hypothesis.

This study extends our knowledge about obligate pollination mutualisms in which the floral odour is dominated by one or few compounds (e.g. Knudsen and Mori, 1996; Grison-Pigé et al., 2002; Terry et al., 2007a and b). In this study it was shown that (3*E*)-1,3-octadiene and 2-isopropyl-3-methoxypyrazine, emitted by male and female cones of *E. villosus* in different parts of its distribution range, can attract pollinating insects in isolation from other compounds. These compounds function as a 'private channel' of communication that elicits the required behavioural response from the pollinating insect (e.g. Schiestl et al., 2003; Schiestl and Peakall, 2005; review by Raguso, 2008; Chen et al., 2009). This is contrary to other obligate plant-pollinator interactions in which a blend of compounds usually attracts the insect pollinator (e.g. Svensson et al., 2010). Pollinator interactions in *Encephalartos* seem to be as reciprocally specialized as figs, suggesting that highly specialized plant-insect interactions are not unique to angiosperms, and also show that odours in which a single dominant compound is behaviourally active can occur even within highly specialised interactions.

Another important result of this study was the similarity in the volatile profile of male and female cones of most *Encephalartos* spp. Although the methods used in this study did not allow comparison of absolute concentrations, the differences in peak heights indicated that female cones emitted similar volatile compounds to male cones but in lower amounts. Therefore male and female plants appear to use the same chemical signals to influence the behaviour of pollinators. However, male cones offer pollen, brood sites and shelter to the insects while female cones offer no rewards. This implies that female cones are pollinated by deceit because their volatile compounds resemble those of male cones and Tang (1987a) hypothesized that female cones mimic male cones by emitting similar volatile compounds which are weaker. The similarity in cone volatiles between male and female cones of *Encephalartos* species appears to support the intersexual mimicry hypothesis in which male and female

flowers use similar cues to attract the same pollinators (Ashman, 2009) but the female produce weaker signals. Intersexual mimicry has been observed in dioecious figs (Grison-Pigé et al., 2001), in the palm *Chaemarops humilis* (Dufaÿ et al., 2004) and in *Silene latifolia* (Waelti et al., 2009). The weaker cues produced by female cones of cycad may just be enough to enhance insect visitation to deliver pollen but not strong to influence mating and oviposition that leads to seed destruction (Seymour et al., 2004). In push-pull systems as advocated by Terry et al (2007a and b), pollen flow is facilitated by repeating cycles of strong attraction and repellence in male cones coupled with weaker attraction in female cones. The pollination system of *E. villosus* is different from the push-pull interaction found in *Macrozamia* (Terry et al., 2007a and b) and it is still not clear as to what influence insect movements between male and female cones which leads to pollen flow.

An additional dimension in the interaction between *Encephalartos* spp. and their pollinators is the presence of other non-pollinating herbivores and seed parasites. It is noteworthy that pollinator taxa, such as Erotylidae, *Porthetes* sp. and *Metacucujus* spp. all use the male cone as a brood site whereas the majority of the non-pollinator herbivores develop in female cone tissues. The insects associated with female cones of *Encephalartos* comprise at least 20 species of beetle in three genera and includes groups that feed on the central axis, sporophylls and seeds. This situation poses an interesting paradox in that male and female cones appear to emit similar volatile compounds, yet there is a clear separation in the way that insects respond to the cones when searching for brood sites. Future experiments comparing the chemical ecology of *Antliarhinus* seed predators and pollinators could yield important insights into how this separation occurs.

In conclusion, the research presented in this thesis has provided several novel insights into the relationship between cone volatiles and pollination in *Encephalartos* that has implications for our understanding of other chemically mediated pollination interactions. The results have shown that cycad-

insect pollination systems are highly specialised and comparable with similarly specialised interactions between angiosperms and insects. Despite some very clear insights, such as the role of single dominant compounds in attracting pollinators, the results also point to a complex set of interactions in which the factors driving changes in volatile composition and the concomitant responses of pollinators and herbivores is difficult to predict.

Future directions

The studies in this thesis raised new questions that warrant investigation. In Chapter 2, it was shown that cone thermogenesis during pollen shed resulted in daily spikes in cone temperature that were consistent with push-pull interactions (e.g. Terry et al., 2004a). However, cone heating did not seem to result in mass movement away from male cones. As a result, the function of cone thermogenesis remains unknown. It has been speculated that increased temperature can increase volatilization of cone compounds or increase insect movement (Tang, 1993; Terry et al., 2004a), or enhancing growth and development of pollinator larvae (Ervik and Barfod, 1999). These hypotheses need to be tested to determine the role of cone thermogenesis.

Geographic variation in volatile compounds has not been recorded before in cycads but most studies have not looked for variation between populations of the same species. The data from *E. villosus* showed substantial differences between populations and limited sampling from other species such as *E. natalensis* and *E. laevifolius* indicated that there were also geographical differences between populations. Such variation suggests a more dynamic relationship between volatile chemistry and pollinator interactions than has previously been thought to occur. It is necessary to investigate this divergence in cone volatile compounds in other taxa in order to understand its significance. Other cycad

taxa with wider distributions should be investigated, e.g. *Cycas media*, *Dioon edule*, *E. barteri*, *E. caffer*, *E. hildebrandtii*, *E. laevifolius*, *E. natalensis*, *E. ngoyanus* and *Macrozamia riedlei*.

Several aspects of chemical ecology of cycad insects are still to be explored. This study shows that volatile compounds attract insect pollinators (Chapter 4), but repellent compounds and oviposition stimulants are not yet known. Most critical is the fact that *Antliarhinus zamiae,* a seed parasite, occurs in many of the *Encephalartos* taxa in South Africa (Donaldson, 1993a). Comparative studies of pollinators and seed predators seem to provide a unique opportunity to examine how different responses to cone volatiles influence the evolution of these interactions.

Finally, cycads have involved an obligate pollination mutualism but it is not clear whether there has been co-evolution between the cycads and their pollinators. It is difficult to investigate co-speciation of weevils and *Encephalartos* because the phylogeny of *Encephalartos* (e.g. Treutlein et al., 2005) is not yet well resolved. However, the phylogeny of the weevil tribe Amorphocerini is well studied (e.g. Downie et al., 2008) and includes the pollinator genus *Porthetes* as well as the cone herbivore *Amorphocerus.* Further studies of how these beetles have diversified in relation to the chemical ecology of their host plants could provide insights into the evolution of the interactions and speciation associated with changes in host chemistry.

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Appendices

Appendix 1: Occurrence and relative amounts (%) of volatile compounds emitted male and female cones of some *Encephalartos* spp[d] during pollen shed and receptivity. Compounds are identified by common names and CAS (Chemical Abstract Service) registry number and listed according to estimated Kovats retention index (KRI) within each compound class. Values are mean percentage of total peak area. The number of samples in which the compounds were identified is given in parentheses.

Plant species and sex			Evill[KM]	Evill[KF]	Evill[EM]	Evill[EF]	Eapl[M]	E cf[M]	Engy[M]	Engy[F]	E umb[M]	Eumb[F]	E frid[M]	Efrid[F]	Ecyc[M]	Ecyc[F]	Eghe[M]	Eghe[F]	Ehum[M]	Ehum[F]	Elaev[M]	Epr[M]	E.pr[F]	Ehor.[M]	Elat[M]	Elat[F]	Etrisp[M]	Eleh[M]	Ealt[M]	Ealt[F]	Esen[M]	Enat[M]	Enat[F]	Efer[M]
Number of samples			33	5	32	9	10	6	6	3	17	4	10	13	9	9	9	5	9	12	8	16	3	11	8	6	5	6	8	6	8	29	21	6
Number of compounds			72	26	61	27	17	11	13	10	26	9	18	16	14	10	37	26	16	27	16	16	6	9	21	22	23	9	12	3	17	43	30	13
Compound	**CAS**	KRI																																
ALIPHATICS																																		
Alkanes																																		
Decane[a]	124-18-5	1070	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.03 (6)
Undecane[b]	1120-21-4	1139	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	38.11 (6)
Dodecane[a]	112-40-3	1249	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	43.40 (6)
Tridecane[b]	629-50-5	1340	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10.29 (6)
Unsaturated hydrocarbons																																		
(3*E*)-1,3-Octadiene[a]	1002-33-1	1062	24.78 (33)	27.74 (5)	-	-	47.15(10)	-	62.28 (6)	93.58 (3)	-	-	-	-	-	-	-	-	-	-	25.66 (6)	5.02 (8)	36.42 (2)	-	-	-	93.57 (5)	-	87.63 (8)	86.82 (6)	41.14 (8)	42.39 (18)	25.39 (11)	-
1,3,7-Octatriene[c]	1002-35-3	1090	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.70 (5)	-	-	-	-	-
(3*E*,5*Z*)-1,3,5-Octatriene[b]	40087-61-4	1148	40.89 (33)	4.77 (5)	-	-	51.61(10)	-	36.80 (6)	5.25 (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.24 (5)	-	10.12 (8)	11.24 (6)	30.08 (8)	15.59 (17)	21.84 (12)	-
(*E*,*E*,*E*)-2,4,6-Octatriene[b]	15192-80-0	1216	3.23 (27)	tr (4)	-	-	0.50 (6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.22 (4)	0.22 (8)	0.01 (2)	-
1,2-Dimethyl-1,4-cyclohexadiene[b]	17351-28-9	1217	1.44 (23)	tr (4)	-	-	0.19 (5)	-	0.36 (6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02 (3)	0.45 (8)	-
7,7-Dimethyl-3,4-octadiene[c]	61129-34-8	1485	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.45 (8)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aliphatic acids																																		
Acetic acid[a]	64-19-7	1467	-	-	0.02 (1)	0.61 (1)	-	-	-	-	-	-	-	-	-	-	-	0.38 (2)	-	-	-	-	-	-	-	-	-	-	-	-	tr (1)	-	-	0.13 (6)
Isovaleric acid[b]	503-74-2	1682	-	-	-	-	-	-	-	-	-	-	5.32 (7)	-	0.92 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Methyl-butanoic acid[b]	116-53-0	1689	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.61 (4)	-	-	-	-	-	-	-	-	-	-	-	-
Hexanoic acid[a]	142-62-1	1852	-	-	0.09 (1)	tr (1)	-	-	-	-	-	-	0.31 (3)	0.93 (5)	-	0.65 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Heptanoic acid[a]	111-14-8	1969	-	-	0.04 (1)	0.23 (1)	-	-	-	-	-	-	0.47 (2)	0.59 (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aldehydes																																		
Hexanal[a]	66-25-1	1125	0.86 (5)	5.70 (5)	-	tr (2)	-	-	-	-	0.07 (5)	-	5.23 (10)	7.07 (12)	3.35 (9)	7.36 (9)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.79(4)	0.39 (8)	0.51 (8)	-
Heptanal[a]	111-71-7	1209	4.88 (13)	3.81 (4)	7.30 (9)	6.48 (2)	0.02 (5)	-	-	-	-	-	12.85 (10)	16.33 (12)	3.96 (5)	5.07 (9)	2.95 (9)	2.63 (4)	2.15 (6)	0.21 (4)	4.86 (6)	-	-	22.81 (7)	5.55 (8)	14.75 (3)	0.77 (5)	2.43 (5)	-	-	-	1.69 (11)	1.14 (7)	-
(*Z*)-2-Heptenal[b]	57266-86-1	1340	tr (3)	-	-	-	tr (2)	-	0.07 (6)	0.13 (2)	-	-	0.30 (7)	-	-	-	0.19 (9)	0.23 (4)	-	-	-	-	-	-	-	-	0.05 (5)	-	0.04 (4)	-	0.19 (3)	0.04 (12)	0.03 (5)	-
(*E*)-2-Octenal[b]	2548-87-0	1446	0.15 (5)	-	-	-	-	-	-	-	-	-	0.78 (8)	2.00 (9)	0.42 (5)	0.44 (9)	0.44 (9)	0.37 (4)	-	-	-	-	-	-	-	-	0.06 (5)	-	-	-	-	-	-	-
(2*E*,4*E*)-Hepta-2-4-dienal[b]	4313-5-3	1513	0.01 (8)	0.08 (1)	-	-	0.13 (6)	-	0.06 (6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr (1)	-	-	-
(*E*)-2-Nonenal[b]	18829-56-6	1545	-	-	-	-	-	-	-	-	-	-	1.07 (8)	0.96 (8)	-	-	0.57 (8)	0.96 (5)	-	0.01 (2)	-	-	-	0.17 (1)	-	-	-	-	-	-	-	-	-	-
2,4-Octadienal[b]	30361-28-5	1610	tr (8)	0.01 (1)	-	-	tr (3)	-	0.03 (6)	0.07 (2)	-	-	-	-	-	-	-	-	-	-	0.06 (4)	-	-	-	-	-	0.04 (5)	-	0.04 (5)	-	-	0.01 (5)	-	-
(*E*)-2-Decenal[b]	3913-81-3	1663	-	-	-	-	-	-	-	-	-	-	-	0.08 (3)	-	-	0.16 (9)	0.38 (5)	0.02 (3)	0.01 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tridecanal[b]	10486-19-8	1834	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.46 (8)	4.31 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tetradecanal[b]	124-25-4	1941	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.87 (7)	33.23 (4)	-	0.01 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ketones																																		
3-Octanone[a]	106-68-3	1274	1.18 (11)	-	0.37 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02 (3)	0.01 (2)	-
3-Hydroxy-2-butanone[b]	513-86-0	1302	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.86 (5)	-	-	-	-	-	-	-
2-Nonanone[a]	821-55-6	1403	-	-	-	-	-	-	0.19 (6)	-	-	-	-	-	-	-	13.80 (9)	0.53 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	5.16 (4)	-	-

Appendix 1 continued

Plant species and sex			EvillKM	EvillKF	EvillEM	EvillEF	EaplM	E cfM	EngyM	EngyF	E umbM	EumbF	E fridM	EfridF	EcycM	EcycF	EgheM	EgheF	EhumM	EhumF	ElaevM	EprM	E.prF	EhorM	ElatM	ElatF	EtrispM	ElehM	EaltM	EallF	EsenM	EnatM	EnatF	EferM
Number of samples			33	5	32	9	10	6	6	3	17	4	10	13	9	9	9	5	9	12	8	16	3	11	8	6	5	6	8	6	8	29	21	6
Number of compounds			72	26	61	27	17	11	13	10	26	9	18	16	14	10	37	26	16	27	16	16	6	9	21	22	23	9	12	3	17	43	30	13
Compound	**CAS**	KRI																																
2,2,6-Trimethyl-6-vinyldihydro-2H-pyran-3(4H)-one[c]	33933-72-1	1489	0.01 (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2,5-hexanedione[b]	110-13-4	1524	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01 (5)	-	-
3,3,6-Trimethyl-1,5-heptadien-4-one[b]	546-49-6	1583	tr (1)	-	-	-	tr (1)	-	0.06 (6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.03 (2)	-	0.05 (5)	-	-	0.01 (9)	-	-
2-Undecanone[b]	112-12-9	1615	-	-	-	-	-	-	0.02 (6)	0.02 (2)	-	-	-	-	-	-	0.11 (7)	0.10 (3)	-	-	0.42 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-
Eucarvone[b]	503-93-5	1741	-	-	-	-	-	0.07 (6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexahydrofarnesyl acetone[c]	502-69-2	2135	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.70 (8)	1.88 (5)	-	-	-	-	-	-	-	-
Alcohols																																		
3-Methyl-1-butanol[b]	123-51-3	1225	-	-	-	-	-	-	-	-	-	-	-	-	10.76 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Hexanol[a]	111-27-3	1357	-	-	-	-	-	-	-	-	-	-	0.19 (2)	1.42 (5)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(*Z*)-3-Hexen-1-ol[b]	928-96-1	1390	0.05 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-Octanol[a]	589-98-0	1386	1.57 (16)	-	0.69 (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02 (3)	-	-
1-Octen-3-ol[a]	3391-86-4	1456	2.01 (30)	5.64 (3)	-	3.15 (4)	0.03 (4)	-	-	-	0.02 (5)	-	-	-	-	-	-	tr (1)	-	-	0.14 (3)	-	-	-	-	-	-	6.41 (5)	-	-	-	0.01 (3)	-	-
2-Nonanol[a]	628-99-9	1523	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.65 (7)	0.33 (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	0.73 (4)	-	-
1-Octanol[a]	111-87-5	1563	-	-	-	-	-	-	-	-	-	-	2.25 (7)	3.88 (8)	0.89 (5)	1.97 (9)	1.09 (9)	0.50 (3)	-	-	-	0.60 (4)	-	-	-	-	-	-	-	-	0.12 (4)	-	-	-
cis-3-Octen-1-Ol[c]	20125-84-2	1569	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr (3)	-	-
(*E*)-2-Octen-1-ol[b]	18409-17-1	1625	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.69 (6)	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Nonanol[a]	143-08-8	1668	-	-	-	-	-	-	-	-	-	-	0.66 (5)	-	-	-	0.40 (4)	0.11 (1)	-	0.01 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lavandulol[b]	498-16-8	1690	-	-	tr (5)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(*E,E*)-2,6-Dimethyl-3,5,7-octatriene-2-ol[c]		1834	-	-	-	-	-	3.75 (6)	-	-	-	-	-	-	-	-	-	-	-	-	0.27 (5)	-	-	-	0.70 (4)	-	-	-	-	-	-	-	-	-
2,6-Dimethyl-3,7-octadiene-2,6-diol[b]	13741-21-4	1946	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.29 (8)	-	-	-	-	-	-	-	-	-
1,7-Octadien-3-ol[c]	30385-19-4	2086	0.09 (5)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Esters																																		
Methyl isovalerate[b]	556-24-1	1083	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.62 (5)	-	-	-	-	-	-
2-Phenethyl hexanoate[c]	6290-37-5	1270	0.14 (2)	4.29 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl levulinate[c]	624-45-3	1586	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.06 (8)	0.03 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dimethyl glutarate[c]	1119-40-0	1723	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.29 (5)	-	-	-	-	-	-	-	-	-	-	-	-
BENZENOIDS																																		
Anisole[a]	100-66-3	1357	tr (1)	0.87 (1)	-	-	-	-	-	-	-	-	-	-	-	-	0.43 (9)	1.28 (5)	3.55 (8)	3.00 (12)	3.83 (4)	0.16 (7)	-	-	-	-	-	-	-	-	-	10.42 (12)	11.34 (5)	-
Durenol[c]	527-35-5	1417	-	-	-	-	-	0.26 (6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzaldehyde[a]	100-52-7	1553	0.84 (33)	1.59 (5)	5.30 (30)	7.15 (9)	0.06 (10)	0.12 (6)	0.02 (6)	0.55 (3)	-	-	5.90 (10)	6.93 (12)	8.66 (9)	1.12 (9)	8.71 (9)	4.43 (5)	1.22 (9)	1.31 (12)	6.04 (8)	6.98 (16)	27.50 (3)	37.00 (10)	3.17 (8)	40.53 (6)	0.27 (5)	2.28 (5)	0.12 (5)	-	1.56 (8)	1.01 (25)	2.99 (21)	0.06 (6)
2-Isopropyl-5-methyl-anisole[c]	1076-56-8	1611	-	-	tr (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl benzoate[a]	93-58-3	1646	-	-	tr (4)	-	-	-	-	-	-	-	0.02 (9)	0.64 (6)	-	-	0.01 (2)	0.30 (5)	0.05 (8)	0.03 (12)	0.03 (2)	-	-	4.07 (7)	-	0.59 (3)	-	6.27 (5)	-	-	-	0.11 (9)	0.15 (6)	-
Estragole[b]	140-67-0	1690	0.08 (1)	-	0.28 (11)	0.31 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01 (4)	1.70 (4)	-	-	-	-	-	-	-	-
1-Ethenyl-4-methoxy-benzene[c]	637-69-4	1703	0.08 (7)	-	tr (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Appendix 1 continued

Plant species and sex			EvilI^{KM}	EvilI^{KF}	EvilI^{EM}	EvilI^{EF}	EapI^{M}	E cf^{M}	Engy^{M}	Engy^{F}	E umb^{M}	Eumb^{F}	E frid^{M}	Efrid^{F}	Ecyc^{M}	Ecyc^{F}	Eghe^{M}	Eghe^{F}	Ehum^{M}	Ehum^{F}	Elaev^{M}	Epr^{M}	E.pr^{F}	Ehor.^{M}	Elat^{M}	Elat^{F}	Etrisp^{M}	Eleh^{M}	Ealt^{M}	Ealt^{F}	Esen^{M}	Enat^{M}	Enat^{F}	Efer^{M}
Number of samples			33	5	32	9	10	6	6	3	17	4	10	13	9	9	9	5	9	12	8	16	3	11	8	6	5	6	8	6	8	29	21	6
Number of compounds			72	26	61	27	17	11	13	10	26	9	18	16	14	10	37	26	16	27	16	16	6	9	21	22	23	9	12	3	17	43	30	13
Compound	**CAS**	KRI																																
Salicylaldehyde[b]	090-02-08	1711	0.08 (7)	-	-	-	-	-	-	-	-	-	-	-	-	-	0.25 (7)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Methylbenzylalcohol[a]	98-85-1	1795	tr (5)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr (1)	-	-
1,4-Dimethoxy-benzene[c]	150-78-7	1760	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.10 (4)	-	-	-	-	-	-	-
Methyl salicylate[a]	119-36-8	1808	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.05 (5)	-	-	-	-	-	-	-	-	-	-	0.02 (3)	-	-	-	tr (6)	tr (3)	-
2,5-Dimethylbenzaldehyde[c]	5779-94-2	1845	tr (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01 (5)	-	-	-	-	-
Guaiacol[b]	90-05-1	1888	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.09 (6)	-	-	0.04 (7)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl alcohol[a]	100-51-6	1896	0.06 (9)	-	-	-	-	-	-	0.03 (2)	tr (6)	0.27 (4)	2.06 (8)	-	-	-	0.60 (9)	0.21 (4)	0.06 (3)	0.02 (3)	-	3.91 (10)	0.54 (2)	14.50 (11)	0.66 (6)	11.19 (6)	0.05 (5)	0.11 (5)	-	-	-	0.70 (14)	2.18 (11)	-
2-Phenylethanol[a]	060-12-8	1933	0.03 (8)	-	1.02 (11)	3.73 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-Methoxy-benzaldehyde[c]	591-31-1	1933	0.34 (8)	-	-	-	-	-	-	-	-	-	-	0.18 (1)	-	-	-	-	-	0.11 (11)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenol[a]	108-95-2	2032	-	-	-	-	tr (5)	-	tr (6)	0.13 (3)	0.02 (8)	3.22 (4)	-	-	-	-	0.08 (7)	0.10 (4)	0.14 (8)	0.06 (10)	0.27 (6)	2.79 (16)	4.98 (2)	2.90 (10)	0.11 (2)	1.71 (6)	0.01 (4)	-	-	-	0.52 (4)	0.23 (24)	0.75 (18)	0.08 (6)
p-Anisaldehyde[a]	123-11-5	2061	0.01 (20)	0.10 (2)	0.63 (21)	0.89 (7)	0.03 (6)	-	-	-	0.12 (17)	-	-	-	-	-	-	-	-	-	0.42 (2)	-	-	-	-	-	-	0.01 (5)	-	-	0.10 (4)	-	-	tr (6)
2-Ethylphenol[a]	90-00-6	2089	2.31 (33)	tr (2)	7.75 (31)	1.01 (6)	-	-	0.01 (6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cresol[a]	106-44-5	2099	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.43 (11)	4.11 (3)	-	-	0.80 (5)	-	-	-	-	-	0.01 (5)	-	-
Eugenol[a]	97-53-0	2191	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.18 (6)
Vanillin[a]	121-33-5	2551	-	-	-	-	-	-	-	-	-	-	0.13 (7)	0.21 (6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TERPENOIDS																																		
Monoterpenes																																		
α-Pinene[a]	7785-70-8	1095	0.06 (11)	2.02 (3)	9.02 (24)	11.73 (5)	-	-	-	-	0.07 (7)	-	-	-	-	-	8.23 (9)	0.42 (4)	13.28 (8)	18.41 (12)	-	-	-	-	-	-	-	-	-	-	-	15.41 (10)	11.44 (5)	-
Camphene[a]	79-92-5	1112	-	0.04 (1)	5.85 (20)	3.57 (4)	-	-	-	-	0.07 (4)	-	-	-	-	-	19.41 (9)	4.49 (3)	66.87 (8)	-	-	-	-	-	-	-	-	-	-	-	-	0.01 (2)	-	-
β-Pinene[a]	127-91-3	1194	0.41 (12)	1.04 (2)	11.18 (24)	8.37 (6)	-	1.83 (6)	-	-	0.88 (16)	6.02 (2)	-	-	-	-	-	-	-	0.21 (7)	1.18 (5)	-	-	-	3.41 (8)	1.00 (2)	-	-	-	-	-	1.66 (13)	1.45 (5)	-
β-Myrcene[a]	123-35-3	1199	0.04 (8)	0.95 (4)	0.06 (8)	tr (4)	-	-	-	-	-	-	-	-	0.73 (1)	-	-	-	-	-	-	3.92 (1)	-	-	-	-	-	-	-	-	0.01 (1)	0.18 (7)	0.19 (2)	-
α-Terpinene[a]	99-86-5	1220	2.91 (5)	-	11.05 (20)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Limonene[a]	138-86-3	1224	-	1.60 (2)	0.08 (8)	3.22 (4)	-	0.19 (7)	-	0.19 (3)	0.09 (5)	-	10.90 (9)	6.09 (8)	5.33 (2)	tr (1)	0.53 (9)	1.30 (5)	2.98 (8)	3.17 (12)	3.46 (4)	1.29 (9)	-	17.03 (9)	3.71 (8)	8.51 (4)	-	-	0.06 (9)	0.01 (2)	0.09 (2)	0.31 (8)	0.20 (3)	-
Eucalyptol[a]	470-82-6	1231	0.65 (17)	0.01 (1)	16.81 (32)	22.30 (8)	-	-	-	-	0.05 (10)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
trans-β-Ocimene[a]	3779-61-1	1267	0.15 (9)	-	0.13 (3)	-	-	3.79 (7)	-	-	0.13 (6)	-	1.35 (2)	0.67 (3)	5.94 (7)	1.26 (9)	-	-	-	-	-	-	-	-	7.40 (7)	-	-	-	-	-	-	-	-	-
γ-Terpinene[a]	99-85-4	1269	0.18 (4)	-	1.75 (19)	-	-	-	-	-	-	-	-	-	-	-	0.12 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cis-β-Ocimene[a]	3338-55-4	1275	0.01 (6)	tr (1)	tr (1)	-	-	79.18 (6)	-	-	0.20 (5)	-	-	-	-	-	-	-	-	-	10.14 (8)	-	-	-	10.13 (8)	0.48 (1)	-	-	0.04 (4)	-	0.77 (5)	0.11 (4)	-	-
p-Cymene[a]	99-87-6	1294	0.99 (4)	-	3.09 (18)	5.62 (3)	-	-	-	-	0.57 (5)	0.23 (2)	-	-	-	-	1.00 (7)	0.02 (1)	-	-	0.19 (8)	0.10 (4)	-	-	-	-	-	-	-	-	0.03 (4)	0.03 (9)	0.01 (1)	-
α-Terpinolene[a]	586-62-9	1304	-	-	0.18 (8)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Allo-ocimene[b]	673-84-7	1391	-	-	tr (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6-camphenone[a]	55659-42-2	1419	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.04 (9)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Perillene[b]	539-52-6	1428	-	-	tr (6)	-	-	-	-	-	-	-	-	-	0.17 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr (3)	-	-

Appendix 1 continued

Plant species and sex			EvillKM	EvillKF	EvillEM	EvillEF	EaplM	E cfM	EngyM	EngyF	E umbM	EumbF	E fridM	EfridF	EcycM	EcycF	EgheM	EgheF	EhumM	EhumF	ElaevM	EprM	E.prF	EhorM	ElatM	ElatF	EtrispM	ElehM	EaltM	EaltF	EsenM	EnatM	EnatF	EferM
Number of samples			33	5	32	9	10	6	6	3	17	4	10	13	9	9	9	5	9	12	8	16	3	11	8	6	5	6	8	6	8	29	21	6
Number of compounds			72	26	61	27	17	11	13	10	26	9	18	16	14	10	37	26	16	27	16	16	6	9	21	22	23	9	12	3	17	43	30	13
Compound	**CAS**	KRI																																
trans-Linalool oxide (Furanoid)[a]	34995-77-2	1453	0.48 (1)	-	0.27 (5)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02 (4)	-	-	0.61 (8)	0.84 (5)	-	-	-	-	-	tr (1)	0.33 (2)	-
cis-Linalool oxide (Furanoid)[a]	5989-33-3	1467	0.06 (8)	-	tr (6)	0.25 (4)	-	-	-	-	0.03 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	0.71 (8)	0.08 (6)	-	-	-	-	-	1.21 (8)	2.10 (12)	-
Nerol oxide[b]	1786-08-9	1488	0.01 (5)	-	0.01 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Irone[b]	79-69-6	1535	-	-	tr (5)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Camphor[a]	464-48-2	1543	-	-	tr (5)	-	0.03 (5)	-	-	-	-	-	-	-	-	-	-	-	0.02 (3)	0.03 (10)	-	-	-	-	-	-	-	-	-	-	-	-	0.02 (2)	-
Linalool[a]	78-70-6	1562	4.12 (33)	1.70 (2)	0.49 (25)	1.64 (6)	-	-	-	-	-	-	-	-	5.71 (8)	10.13 (9)	-	-	0.01 (1)	-	-	-	-	0.71 (1)	56.88 (8)	11.00 (4)	0.08 (5)	-	-	-	0.29 (4)	0.08 (19)	0.05 (8)	tr (6)
cis-*p*-Mentha-2-en-1-ol[b]	29803-82-5	1576	-	-	0.22 (14)	tr (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pinocarvone[a]	30460-92-5	1597	tr (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.04 (7)	0.09 (12)	-	-	-	-	-	-	-	-	-	-	-	tr (7)	tr (4)	-
Camphene hydrate[c]	465-31-6	1613	tr (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	0.03 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4-Terpineol[a]	562-74-3	1613	0.03 (9)	-	0.07 (8)	tr (1)	-	-	-	-	-	-	-	-	-	-	0.25 (7)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-ionol[c]	25312-34-9	1616	-	-	0.04 (4)	-	-	-	-	-	0.02 (10)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
p-Menth-1-en-4-ol[a]	20126-76-5	1618	0.05 (14)	-	tr (7)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Cyclocitral[b]	432-25-7	1647	0.08 (14)	0.69 (1)	tr (2)	-	tr (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01 (4)	-	-	tr (5)	-	-
Myrtenal[a]	564-94-3	1650	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01 (2)	-	0.01 (3)	0.03 (10)	-	-	-	-	-	-	-	-	-	-	-	tr (2)	tr (1)	-
Thujopsene[a]	470-40-6	1653	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.15 (3)	-	-	-	-	-	-	-	-	-	-	-	-	-
cis-Verbenol[a]	18881-04-4	1696	-	-	tr (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Citral[b]	106-26-3	1703	0.25 (13)	0.03 (1)	1.10 (27)	0.69 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Terpineol[a]	10482-56-1	1716	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.82 (6)	0.30 (8)	0.27 (6)	-	-	-	-	-	-	-	-
Isobornylacetate[c]	125-12-2	1721	0.04 (5)	-	0.09 (8)	-	-	-	-	-	-	-	-	-	-	-	0.06 (5)	-	-	-	-	-	-	-	-	0.23 (2)	-	-	-	-	-	-	-	-
Borneol[a]	507-70-0	1725	-	-	-	-	tr (2)	-	-	-	tr (2)	-	-	-	-	-	-	0.02 (2)	0.01 (3)	tr (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pinocarveol[a]	5947-36-4	1730	-	-	-	-	-	0.14 (5)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Cyclogeraniol[c]	6627-74-3	1737	0.13 (5)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citral[b]	106-26-3	1732	0.03 (8)	-	0.04 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
trans-Linaool oxide (Pyranoid)[a]	14009-71-3	1755	-	-	0.01 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.08 (3)	tr (2)	-	-	-	-	-	-	tr (1)	-
Piperitone oxide[c]	5286-38-4	1760	0.02 (2)	-	0.01 (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cis-Linalool oxide (Pyranoid)[a]	5989-33-3	1781	0.02 (8)	-	0.05 (5)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.06 (4)	tr (2)	-	-	-	-	0.14 (5)	-	tr (1)	-
trans-*p*-Menth-2-en-7-ol[c]	19898-87-4	1791	0.01 (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	0.09 (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cis-Geraniol[a]	106-25-2	1803	-	-	0.10 (11)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydro-β-ionone[c]	17283-81-7	1807	tr (8)	-	-	-	-	-	0.01 (5)	-	tr (6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Myrtenol[a]	515-00-4	1815	0.14 (12)	0.02 (1)	-	-	-	-	-	-	-	-	-	-	-	-	0.01 (2)	-	-	tr (1)	-	-	-	-	-	-	-	-	-	-	-	tr (3)	tr (2)	-
Perilla aldehyde[b]	18031-40-8	1817	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.08 (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Grandisol[b]	26532-22-9	1821	0.18 (8)	-	-	-	-	-	-	-	0.03 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydro-α-ionone[c]	31499-72-6	1855	-	-	-	-	-	-	-	-	tr (4)	tr (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Appendix 1 continued

Plant species and sex			EvillKM	EvillKF	EvillEM	EvillEF	EaplM	E cfM	EngyM	EngyF	E umbM	EumbF	E fridM	EfridF	EcycM	EcycF	EgheM	EgheF	EhumM	EhumF	ElaevM	EprM	E.prF	Ehor.M	ElatM	ElatF	EtrispM	ElehM	EaltM	EaltF	EsenM	EnatM	EnatF	EferM
Number of samples			33	5	32	9	10	6	6	3	17	4	10	13	9	9	9	5	9	12	8	16	3	11	8	6	5	6	8	6	8	29	21	6
Number of compounds			72	26	61	27	17	11	13	10	26	9	18	16	14	10	37	26	16	27	16	16	6	9	21	22	23	9	12	3	17	43	30	13
Compound	**CAS**	KRI																																
p-Cymen-8-ol[a]	1197-01-9	1872	tr (1)	-	tr (12)	-	-	-	-	-	-	-	-	-	-	-	0.11 (8)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Ionone[a]	127-41-3	1875	0.23 (17)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
exo-2-Hydroxycineole[b]	92999-78-5	1877	0.02 (5)	-	tr (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cis-Myrtanol[b]	15358-92-6	1893	0.05 (7)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.05 (4)	-	-	-	-	-	-	-
β-ionol	22029-76-1	1939	-	-	-	-	-	-	-	-	0.02 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(*E*)-*β*-Ionone[b]	79-77-6	1989	0.04 (7)	-	-	-	-	-	-	-	0.03 (11)	0.27 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
p-Cymen-3-ol[a]	89-83-8	2225	0.18 (5)	-	0.24 (14)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
p-Cymen-2-ol[a]	499-75-2	2232	-	-	0.02 (8)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sesquiterpenes																																		
(3E,5E)-2,6-Dimethyl-1,3,5,7-octatetraene[c]	460-01-5	1467	-	-	-	-	-	2.96 (6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Longipinene[c]	05989-08-2	1492	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.35 (5)	-	-	-	-	-	-	-
α-Ylangene[b]	14912-44-8	1505	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.04 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydroedulan I[c]	63335-66-0	1538	tr (7)	-	tr (5)	-	-	-	-	-	97.21 (17)	89.58 (4)	-	-	-	-	-	-	-	-	-	-	-	-	0.47 (7)	0.39 (4)	0.04 (5)	-	-	-	-	-	-	-
D-longifolene[b]	475-20-7	1600	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.08 (8)	0.23 (5)	-	0.01 (1)	-	-	-	-	-	-	0.23 (5)	-	-	-	-	-	-	-
α-Bergamotene[b]	17699-05-7	1604	0.03 (5)	-	tr (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.57 97)	2.13 (4)	0.01 (3)	-	-	-	-	-	-	-
β-Caryophyllene[a]	87-44-5	1636	tr (2)	-	0.01 (1)	0.03 (1)	-	-	-	0.02 (2)	tr (2)	-	-	-	0.55 (2)	-	0.02 (3)	-	tr (2)	0.04 (9)	-	-	-	-	0.14 (6)	-	0.02 (4)	-	-	-	-	tr (8)	0.01 (5)	0.88 (6)
Megastigma-4,6(Z),8(E)-triene[c]	51468-85-0	1660	-	-	-	-	-	-	-	-	0.06 (9)	0.15 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Himachalene[c]	3853-83-6	1676	-	-	-	-	-	-	-	-	0.01 (4)	0.27 (2)	-	-	-	-	-	-	-	0.04 (8)	-	-	-	-	-	-	1.53 (5)	-	-	-	-	-	-	-
α-Caryophyllene[a]	6753-98-6	1705	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.02 (2)	-	-	-	-	tr (1)	0.14 (1)	0.05 (6)
α-Amorphene[b]	483-75-0	1719	-	-	tr (1)	0.03 (1)	-	-	-	-	-	-	-	-	-	1.08 (6)	-	-	-	0.13 (11)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Cubebene[b]	13744-15-5	1738	tr (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Himalachene[c]	1461-03-6	1740	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.06 (5)	-	-	-	-	-	-	-
Allo-Aromadendrene[a]	25246-27-9	1751	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.03 (7)	-	-	-	-	-	-	0.57 (5)	-	-	-	-	-	-	-
(*E*,*E*)-*α*-Farnesene[a]	502-61-4	1760	tr (2)	-	tr (2)	-	-	-	-	-	-	-	1.45 (4)	0.34 (3)	2.90 (3)	-	-	-	-	-	-	0.04 (4)	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Curcumene[b]	644-30-4	1796	0.08 (14)	0.01 (1)	-	0.01 (1)	-	-	-	-	0.01 (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Caryophyllene oxide[a]	1139-30-6	2020	-	-	-	-	tr (2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.01 (6)
9,10-Aromadendrene	85048-01-7	2287	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.12(4)	-	-	-	-	-	-	-
NITROGEN-CONTAINING COMPOUNDS																																		
2,5-Dimethylpyrazine[c]	123-32-0	1336	tr (9)	-	tr (8)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Methoxy-3-methylpyrazine[c]	2847-30-5	1392	-	-	tr (3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	63.62 (16)	26.45 (3)	-	-	1.00 (4)	-	78.79 (5)	0.78(5)	-	-	0.09 (5)	-	-
2-Isopropyl-3-methoxypyrazine[a]	25773-40-4	1452	1.16 (4)	-	9.30 (32)	2.46 (9)	-	6.05 (6)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr (2)	-	-
2-Methoxy-3-(1-methylpropyl)-pyrazine[c]	24168-70-5	1519	-	-	0.01 (12)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]Identifications based on mass spectrum, kovats retention index and authentic standard. [b]Identifications based on mass spectrum and kovats retention index. [c]Identification based on mass spectrum only. [d]Abbreviations: tr = trace amounts (< 0.01 %). [M]Male plant; [F]Female plant, *E. villosus* (Evill), *E. aplanatus* (Eapl), *E. caffer* (Ecf), *E. ngoyanus* (Ecf), *E. umbeluziensis* (Eumb), *E friderici-guilielmi* (Efrid), *E. cycadifolius* (Ecyc), *E. ghellinckii* (Eghe), *E. humilis* (Ehum), *E. laevifolius* (Elaev), *E. princeps* (Epr), *E. horridus* (Ehor), *E. latifrons* (Elat), *E. trispinosus* (Etrisp), *E. lehmannii* (Eleh), *E. altensteinii* (Ealt), *E. senticosus* (Esen), *E. natalensis* (Enat), *E. ferox* (Efer)

Appendix 2

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Insect pollination in the African cycad *Encephalartos friderici-guilielmi* Lehm

T.N. Suinyuy [a,d,*], J.S. Donaldson [a,b,d], S.D. Johnson [c]

[a] *KRC, SA National Biodiversity Institute, Private Bag X7, Claremont 7735, Cape Town, South Africa*
[b] *Research Associate, Fairchild Tropical Botanic Garden, Miami, Florida, USA*
[c] *School of Biological and Conservation Sciences, University of KwaZulu-Natal Pietermaritzburg, Private Bag X01, Scottsville 3201, South Africa*
[d] *Department of Botany, University of Cape Town, South Africa*

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Abstract

Studies of the pollination ecology of the South African cycad, *Encephalartos friderici-guilielmi* Lehm, were carried out to determine the role played by insects and to identify the key pollinators. Surveys of insects on the male and female cones at the time of pollination indicated that three beetle species were present in sufficient numbers during pollination to be potential pollinators, i.e. an undescribed Erotylidae sp. (Cucujoidea), *Metacucujus encephalarti* (Cucujoidea), and *Porthetes hispidus* (Curculionidae). Pollen laden specimens of all three species were found on female cones with mean pollen loads ranging between 391 and 1019 pollen grains per beetle. In behavioural experiments, individuals of *P. hispidus* and *M. encephalarti* deposited fluorescent dye on the micropyles of receptive ovules. Enclosure of pollen laden specimens of *P. hispidus*, *M. encephalarti*, or Erotylidae sp. nov. on female cones resulted in seed set that was statistically similar to open controls and significantly higher than insect exclusion treatments. These data support the conclusion that *E. friderici-guilielmi* is insect pollinated and provide further evidence for the role of cucujoid (Erotylidae, Boganiidae) and curculionoid (*Porthetes*) beetles in the pollination of *Encephalartos* spp.

Keywords: Cycad pollination; *Encephalartos friderici-guilielmi*; Erotylidae beetle; *Metacucujus encephalarti*; *Porthetes hispidus*; Zamiaceae

1. Introduction

Early observations made on African cycads suggested that insects played an important role in cycad pollination (Pearson, 1906; Rattray, 1913; Marloth, 1914). These early observations were disregarded after the influential publication of Chamberlain (1935) who concluded that insects were not important and that all cycads were wind pollinated. However, subsequent experimental studies have confirmed that entomophily occurs in at least three African cycad taxa (Donaldson et al., 1995; Donaldson, 1997; Proches and Johnson, 2009) and is widespread in cycads from other regions (Norstog et al., 1986; Tang, 1987; Norstog and Fawcett, 1989; Chadwick, 1993; Terry, 2001; Wilson, 2002; Hall et al., 2004). Some authors argue that entomophily originated early in the evolution of cycads and is probably ubiquitous in modern cycads (Norstog and Nicholls, 1997; Stevenson et al., 1998).

Beetles (Coleoptera) have been identified as the most common pollinators of cycads (Oberprieler, 2004) and most of the pollinator species belong to the superfamilies Curculionoidea and Cucujoidea (Oberprieler, 1995). However, phylogenetic studies of these groups indicate that the beetle families associated with cycads on different continents are not closely related (Oberprieler, 1995; Leschen, 2003) and that these cycad–insect interactions have evolved independently on the different continents. Comparative studies of cycads on different continents are required to determine the nature of these pollination systems and to understand how pollinator mutualisms have evolved and diversified.

Studies within the African genus *Encephalartos* are particularly interesting because this genus has a high species richness of insects associated with cones and includes several

* Corresponding author. Tel.: +27 21 799 8718; fax: +27 21 797 6903. Fax2email: 0865207016.
E-mail address: Suinyuy@sanbi.org (T.N. Suinyuy).

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species-specific interactions (Oberprieler, 1995, 2004; Donaldson, 1997, 1999). A role for insects in the pollination of two *Encephalartos* species has been confirmed by experimental studies (Donaldson et al., 1995; Donaldson, 1997). However, it is not clear whether the high levels of insect species richness on *Encephalartos* is due to co-speciation between cycads and their pollinators (Downie et al., 2008). This type of analysis is not possible without a better understanding of the pollination biology of more species of *Encephalartos*.

An additional reason for studies of cycad pollination is that cycads are among the most threatened groups of plants worldwide (Donaldson, 2003) and the management of cycad populations requires information on pollination ecology. The absence of fertile seeds in some cycad species has been interpreted to be a result of pollinator extinctions (Vovides et al., 1997), but in many cases pollinators still need to be identified in order to plan effective conservation programmes (Terry, 2001).

In this study, we investigated the pollination biology of *Encephalartos friderici-guilielmi* Lehm. This species belongs to a clade of morphologically similar species that has been consistently identified in several phylogenetic studies of *Encephalartos* and comprises *E. cycadifolius*, *E. friderici-guilielmi*, *E. ghellinckii*, *E. humilis*, *E. laevifolius*, and *E. lanatus* (e.g. Treutlein et al., 2005). Within this species complex, there is considerable variation in the number and composition of insect species (mostly beetles) associated with the male and female cones (Donaldson, 1997; Oberprieler, 2004) and that may be involved in pollination. Studies of *E. cycadifolius* showed that two species of cucujoid beetles (Coleoptera: Cucujoidea), an undescribed Erotylidae sp. and *Metacucujus encephalarti* Endrödy-Younga (Boganiidae), were the main pollinators (Donaldson et al., 1995), whereas Endrödy-Younga (1991) concluded that *Metacucujus* beetles were not involved in the pollination of *E. lanatus* even though they were present on cones. Studies of *E. friderici-guilielmi* will provide additional insights into pollination systems in this clade because, in addition to cucujoid beetles, this species also has several weevil species associated with the cones. The weevils tend to be more species specific than other beetles and one weevil species in the genus *Porthetes* has been identified as a pollinator of other *Encephalartos* species (Donaldson, 1997).

Pollination experiments were conducted on *E. friderici-guilielmi* to determine whether insects are involved in pollination and to identify the most important pollinators.

2. Materials and methods

2.1. Study species and site

Encephalartos friderici-guilielmi is endemic to South Africa and occurs in grasslands of the Eastern Cape and KwaZulu-Natal provinces. The summers are hot while, in winter, snow and frost are common. Rainfall ranges from 375 to 500 mm per year with maximum in summer (Jones, 2002). The population size is estimated to comprise between 5000 and 10,000 mature individuals and the species is classified as Near Threatened according to IUCN criteria (Donaldson, 2003). Studies on *E. friderici-guilielmi* were conducted sporadically between 1989 and 2000 and intensively from September 2008 to March 2009 in a population situated south west of Cathcart in the Eastern Cape Province of South Africa.

2.2. Study system

Encephalartos friderici-guilielmi is a medium-size to large cycad with an erect or sprawling trunk up to 4 m tall (Fig. 1a and b). It generates new stems from basal suckers and mature plants may have as many as eight stems. Both male and female plants produce between one and eight dense woolly cones per stem. Male cones range from 20–40 cm in height while the female cones range from 20–35 cm in height and 15–25 cm in diameter. When male cones shed pollen, the cone extends and the sporophylls become separated so that pollen is freely dispersed from the cone.

Mast seeding is a common phenomenon in cycads and occurs in *E. friderici-guilielmi* populations (Donaldson, 1993). Initial studies were carried out during periods of intermittent coning between 1991 and 2000 and during a mass coning event in 2008 when a large proportion of the plants produced cones. The last mass coning event was ca. 15 years ago (N. McMaster pers. comm.) but a few plants have produced cones in the interval between mass coning years.

2.3. Insect visitors to E. friderici-guilielmi cones

To determine which insects visited *E. friderici-guilielmi*, both male and female cones were sampled at three stages of development, i.e. pre-dehiscent, dehiscent, and post dehiscent for male cones and pre-pollen receptive, receptive, and post receptive stages for female cones. Sampling was undertaken in the morning (07:00–11:00), afternoon (13:30–15:30), and evening (18:00–20:30). At the same times, the behaviour and movement of the insects was monitored on wild plants. Cones were dissected and all the insect species present in the cones were recorded.

2.4. Pollen loads and the ability of different insects to deposit pollen onto the micropyle

Based on the survey of insects and insect behaviour, three species were identified for further pollination studies, i.e. *Porthetes hispidus* (Boheman) (Coleoptera: Curculionidae), *Metacucujus encephalarti* (Coleoptera: Boganiidae), and an undescribed species of Erotylidae (Coleoptera: Cucujoidea) (Fig. 1c,d and f). To determine pollen loads, individual insects of all three species were collected in the field as they were foraging, arriving, and leaving male and female cones. The insects were stored in 2 mL Eppendorf tubes containing xylene and Kaiser glycerol gelatin (Merck, Germany). Pollen was washed from the insect bodies and counted using the method of MacGillivray (1987). Pollen loads were measured in 24, 39, and 56 specimens of *P. hispidus*, *M. encephalarti*, and Erotylidae sp nov. respectively.

The ability of pollinators to transfer pollen to the micropyle was tested using the method of Donaldson et al. (1995). Pollen

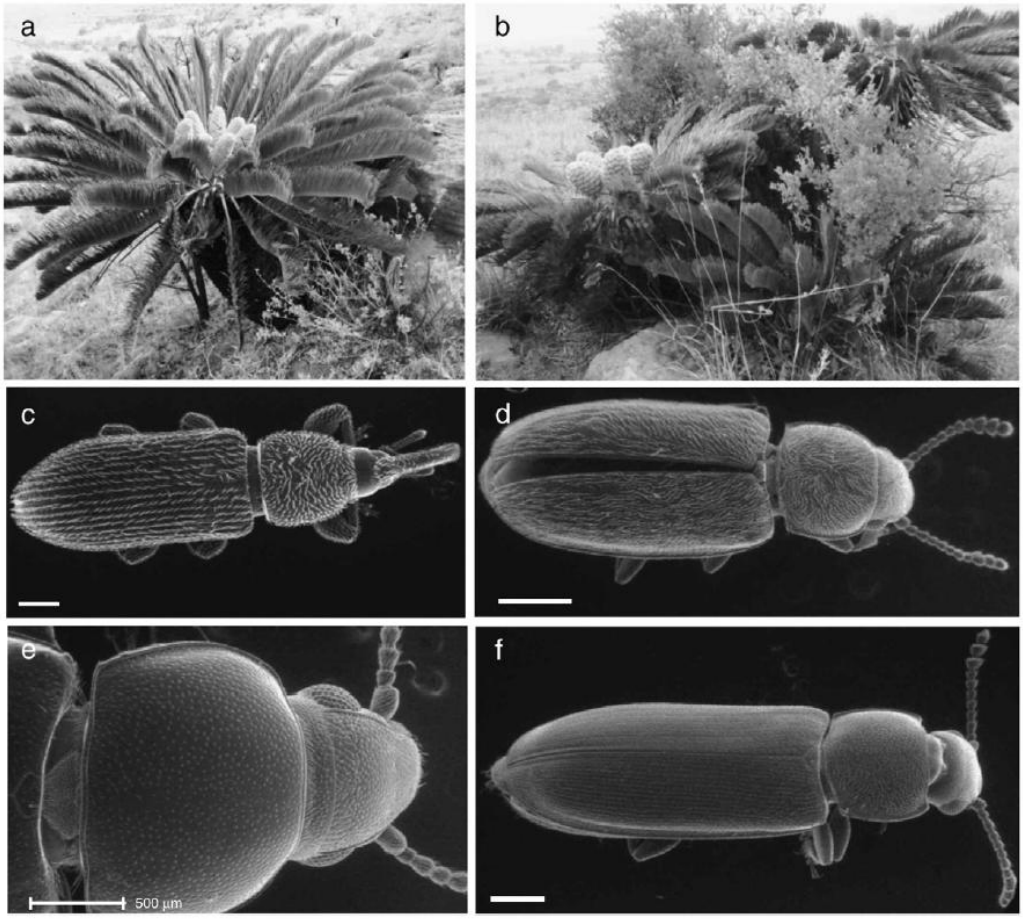

Fig. 1. (a–f) Male *Encephalartos friderici-guilielmi* with cones at pollen shed. (b) Female *Encephalartos friderici-guilielmi* with cones at pollen receptive stage. (c) *Porthetes hispidus*. (d) *Metacucujus encephalarti*. (e) Head of an undescribed Erotylid beetle. (f) An undescribed Erotylidae sp. nov. showing smooth and ungrooved elytra. Scale bars=500 µm.

receptive cones were enclosed with calico cloth bags and insects dusted with orange fluorescent dye (Day-Glo color Corp. — Cleveland, OH, USA) were released into the enclosures. The cones were collected after 48 h and dissected under ultra violet light. The pattern of insect movement within the cone could be discerned from the trail of the visible dye. The presence of dye on the micropyle could also be detected so that the number of ovules visited by the insect could be calculated. Insect abundance in cones varied depending on the species and time of day, so the dye treatments were repeated with different numbers of insects: Erotylidae sp. nov — 15, 25, or 30 individuals per cone; *M. encephalarti* — 25, 35, or 50 individuals per cone; and *P. hispidus* — 10, 15 or 25 individuals per cone. There were three replicates for each abundance class and a total of nine replicates for each insect species.

2.5. Enclosure and exclosure experiments

To determine whether insects are necessary for pollination and to identify insect pollinators, female cones were covered with calico cloth bags that were used to either exclude all insect pollinators or to enclose specific pollinators that were released onto female cones using the methods of Donaldson et al. (1995), Donaldson (1997) and Terry et al. (2005). Female cones were covered with calico cloth bags before the cone was receptive for pollination. The bags were secured at the base of the cone and, because *E. friderici-guilielmi* cones don't have a peduncle, the area around the base of the cone was coated with a sticky insect barrier (Formex® Ciba Geigy South Africa) to prevent insects from crawling into the bag. The calico cloth was able to act as a barrier to insect movement and wind-borne pollen.

For enclosure experiments, the bagged cones were monitored until they reached pollen reception stage, when potential pollinators were introduced into the enclosed cones. Specimens of either Erotylidae sp. nov., *M. encephalarti*, or *P. hispidus* were collected from pollen shedding male cones and released into the mesh bags enclosing the female cone. There were 10 replicates for each beetle species. To determine whether there was a saturation point for pollinating all the ovules, different numbers of insects were introduced into the cones: Erotylidae

Table 1
Mean (±s.e) number of insects of different species collected from male cones of *E. friderici-guilielmi* at the pollen dehiscent stage.

Insect species	No. of cones	No. of insects per cone
Erotylidae sp.	10	202.2±19.87
Metacucujus encephalarti	8	207.8±32.97
Porthetes hispidus	8	56.5±6.97
Amorphocerus rufipes	10	27.8±2.97
Platymerus sp	10	194.9±18.58
Scarabidae spp	9	16.3±1.30

sp. nov. and *M. encephalarti* — 25, 30, or 40 beetles per cone; *P. hispidus* — 10, 15, or 20, beetles per cone. Insects were excluded from a total of 13 cones (in three coning events) and 14 open cones were used as natural controls. The bags were left on the cones for the whole reproduction period and removed five months later when the seeds were mature and ready for collection to determine if pollination was successful. The relationships between the number of beetles added to cones and seed set was determined using regression. Both linear and non-linear curves were fitted to data and the curve that explained the highest percentage of variance was selected.

The success of pollination was determined using a flotation method. All seeds were placed in a water bath and the fertile seeds sank to the bottom of the water bath while the infertile ovules floated. The accuracy of the method was confirmed by dissecting a sample of 40% of the seeds to detect the presence of an embryo.

3. Results

3.1. Insect visitors to *E. friderici-guilielmi* cones

No insects were found in pre-dehiscent male cones although Erotylidae sp. nov. were found in the woolly tomentum in the crown of the plant (at the base of the cone). Six species of insects (all Coleoptera) were present in male cones during dehiscence, namely *Amorphocerus rufipes* (Boheman) (Curculionidae), Erotylidae sp. nov., *M. encephalarti*, *Platymerus* spp (Brentidae), *Porthetes hispidus*, and an unidentified species of Scarabaeidae (Table 1). Larvae of Erotylidae sp. nov., *M. encephalarti*, and *P. hispidus* were present in the microsporophylls. After dehiscence, the male cone dried out rapidly, but adults of all six insect species were present in the cones together with unidentified species of ants, pyralid moth larvae, cockroaches, and spiders that were associated with the decaying cones. The next generations of adult beetles of species associated with dehiscent cones were also emerging from the cones at this stage. In observations of insect activity, *P. hispidus* was observed on the outside of male cones between 12:00 and 17:00, whereas Erotylidae sp. nov. and *M. encephalarti* were active in the evening (19:00–20:30).

Sampling of female cones showed that, prior to the pollen receptive stage, the only insects present on the cones were *Platymerus* sp., all moving over the cone surface. As the cones matured and become receptive, they were inhabited by all six species of insects in low numbers. The *Platymerus* sp; which occurred in high numbers, persisted in the cone up to the post pollen receptive stage. At post pollen receptive stage, all six insect species were present as well as other inhabitants such as scarabaeidae, ants and cockroaches. *Porthetes hispidus* was active during the day while Erotylidae and *M. encephalarti* were active in the evening, similar patterns to those observed on male cones.

3.2. Pollen loads and pollen delivery to the micropyles of the ovule

Beetles collected from the male cones at the pollen dehiscent stage were completely covered with pollen. Pollen loads ($\bar{x}$±1S.E) of *P. hispidus* leaving male cones were 1356.89±427.74 grains per weevil (n=18) and individuals that were foraging or arriving on female cones had pollen loads of 1019.50±277.66 grains, (n=6). Erotylidae sp. nov carried less pollen than *P. hispidus* when collected from either male cones ($\bar{x}$=662.98±145.47 grains per beetle, n=43) or female cones ($\bar{x}$=491.00±293.76 grains per beetle, n=13). Pollen loads of *M. encephalarti* from male cones ($\bar{x}$=2183.11±866.16 grains per beetle; n=27) were substantially greater than loads from specimens on female cones ($\bar{x}$=391.17±321.22 grains per beetle; n=12). The difference in pollen loads of *M. encephalarti* between male and female cones was statistically significant (ANOVA, Duncan test, $p<0.05$) and indicates that *M. encephalarti* loses more pollen when flying between cones than does *P. hispidus* or Erotylidae sp. nov. For *P. hispidus* and Erotylidae sp. nov., there was a slight loss of pollen but this was not statistically significant ($p>0.05$).

When specimens of Erotylidae sp. nov., *M. encephalarti*, and *P. hispidus* were dusted with fluorescent dye, traces of dye were

Table 2
Mean (±s.e) number of ovules per cone and mean (±s.e) percentage of ovules per cone with fluorescent dye deposited on micropyles by two beetle species released onto the cones.

Insect species	No. of insects per cone	Ovules per cone	% Ovules with dye per cone
Porthetes hispidus	10	142.33±9.60	46.18±5.76
Porthetes hispidus	15	170.67±7.69	34.91±1.49
Porthetes hispidus	25	186.33±15.86	36.52±4.45
Metacucujus encephalarti	25	160.00±7.94	42.04±7.88
Metacucujus encephalarti	35	202.67±18.94	18.04±3.63
Metacucujus encephalarti	50	190.33±13.96	32.47±5.51

The data are presented for different numbers of *Porthetes hispidus* and *Metacucujus encephalarti* released onto the cones at the pollen receptive stage. No significant differences were recorded between treatments ($ANOVA_{5,12}=3.51$; $p<0.05$).

Table 3
Mean (±s.e) number of ovules per cone, and percentage of fertile seeds obtained from treatments in which cones were covered with mesh bag to either enclose one of three species of potential insect pollinators Erotylidae sp. nov; *Metacucujus encephalarti*; *Porthetes hispidus* or to exclude all insects.

Treatment	No. of cones	No. of ovules per cone	% Fertile seeds per cone	Homogenous groups
Erotylidae	10	233.5±11.10	57.7±5.6	a
Metacucujus encephalarti	10	181.7±8.3	41.9±8.7	a
Porthetes hispidus	10	172.2±9.3	44.8±5.1	a
Exclude all insects	13	125.0±12.3	4.7±1.3	b
Open control	14	210.8±8.2	49.3±6.6	a

Open controls were not bagged. ANOVA showed a significant difference between treatments in the percentage of ovules that developed into fertile seeds. $F_{4,51}=13.2$; $p<0.001$. Pairwise comparison showed a difference between insect exclusion and all other treatments (Duncan test).

observed on the cone surface, indicating that they had crawled on the outside of the cone before crawling between the megasporophylls. Dye deposits left by the beetles showed that *M. encephalarti* and *P. hispidus* appeared to move freely within the cone as dye trails were detected along the cone axis and passing the micropyles of the ovules. Erotylid beetles did not appear to enter the cones but traces of dye were detected between the megasporophylls indicating that they attempted entering the cones. They might have not entered the cone either because it was not completely open. The mean (±s.e) number of ovules with dye deposits left by *P. hispidus* was 62.00±2.90 per cone ($n=9$), slightly higher than those of *M. encephalarti* (54.44±6.15 per cone, $n=9$). There was no significant difference between treatments of ovules dusted with dye when different numbers of individual insects were released onto the female cones (ANOVA $F_{5,12}=3.51$; $p<0.05$) (Table 2).

3.3. Enclosure experiments

The results show that all three insect species tested were capable of pollinating *E. friderici-guilielmi* (Table 3). In all the insect enclosure treatments, cones contained a relatively high mean percentage of fertile seeds (41–57%) that was comparable to mean seed set in open cones (49.2%), but was significantly higher than mean seed set in insect exclusion treatments (4.7%) (Single factor ANOVA, $F_{4,\ 52}=13.3$, $p<0.01$).

Where different numbers of insects were released onto female cones, there was a trend towards higher seed set when more *P. hispidus* beetles were added and reduced seed set when more individuals of *M. encephalarti* and Erotylidae sp. nov. were added (Fig. 2). Even at relatively low numbers of 10 individuals per cone, pollination by *P. hispidus* resulted in a mean (±s.e.) of 37.0±0.13% fertile seeds but this increased to a mean of 70.0±12.34% when 20 individuals were released onto the cone (Fig. 2). In the case of *M. encephalarti* and Erotylidae sp. nov, the highest levels of seed set were obtained when 25 individuals were released onto the cone ($\bar{x}=60\pm10.03\%$ for *M. encephalarti*; 70±4.21% for Erotylidae sp. nov.). Pollination success dropped off dramatically when the numbers of insects released onto cones increased to 40 insects ($\bar{x}=3.29\pm3.29\%$ for *M. encephalarti*; 41±1.66% for Erotylidae sp. nov.).

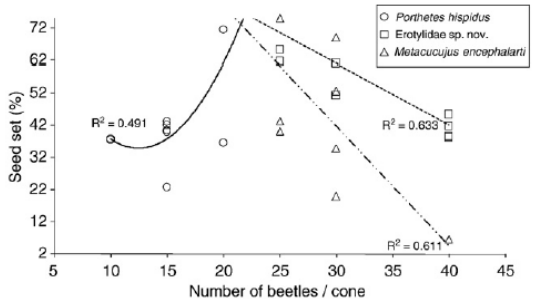

Fig. 2. Correlation between number of beetles per cone and percent seed set for three beetle species found on female cones of *E. friderici-guilielmi*. The correlation coefficient (R^2) is given for each data set.

4. Discussion

The results from this study show that insects were active on male and female cones at the time of pollination. Behavioural studies, using fluorescent dyes, showed that that at least two beetle species, *P. hispidus* and *M. encephalarti*, were capable of transferring pollen to the micropyles of receptive ovules. The results of the enclosure and exclosure experiments also showed that levels of seed set were considerably higher when insects were present and that enclosure of Erotylidae sp. nov., *P. hispidus* and *M. encephalarti* resulted in levels of seed set that were similar to natural levels. In contrast, exclusion of insects resulted in extremely low levels of seed set. Our results therefore confirm that *E. friderici-guilielmi* is entomophilous and that the pollinators include the weevil *P. hispidus* (Curculionidae), the Erotylidae sp. nov. (Cucujoidea) and *M. encephalarti* (Boganiidae).

We did not specifically test wind pollination in this study, as there is no simple experimental treatment to exclude small insects from the female cone while allowing wind borne pollen to reach the cone. However, the bags we used in our experiments to enclose potential pollinators were fine enough to also exclude wind-borne pollen. From the results, it can be concluded that wind is not essential for pollination. Partial pollination by wind has been mentioned in several cycad studies (Rattray, 1913; Niklas and Norstog, 1984; Norstog et al., 1986). The occurrence of some seed set when insects and wind were excluded from *E. friderici-guilielmi* cones (Table 3) is similar to observations in other studies of cycad pollination (e.g. Norstog et al., 1986; Tang, 1987; Donaldson et al., 1995; Donaldson, 1997; Proches and Johnson, 2009) where the cause has been ascribed either to a real but small contribution by wind borne pollen or an artifact of the experimental design. The main problem is that exclusion

bags do not penetrate into the cone tissue so that beetles can burrow under the bag to reach the cone.

We discuss these results in the context of a growing body of knowledge on cycad pollination.

4.1. Insect pollination in Encephalartos

The confirmation of insect pollination in *E. friderici-guilielmi* means that three species of *Encephalartos* have been shown to be entomophilous. In the case of *E. cycadifolius*, the pollinators were identified as *M. encephalarti* and Erotylidae sp. nov. (Donaldson et al., 1995), although it is not known whether this is the same erotylid species as the one on *E. friderici-guilielmi*. These two Erotylidae species remain unidentified. However, because their host plants have similar cone structures, including a dense woolly tomentum, they might have similar behaviours. For *E. villosus*, the pollinators were identified as *Porthetes* sp., *Metacucujus goodei* Endrödy-Younga, and Erotylidae sp. nov. (Donaldson, 1997), in which all the species were different to those recorded from *E. friderici-guilielmi*. These results indicate that species of *Metacucujus* and Erotylidae are pollinators of all the entomophilous species of *Encephalartos* that have been studied so far. Species of *Porthetes* are also important pollinators but they do not occur on all *Encephalartos* spp. The implication is that there may be at least two different cycad pollination systems within *Encephalartos*, one involving only cucujoid beetles (Boganiidae and Erotylidae) and a second that involves cucujoid beetles and *Porthetes*.

The further confirmation of the role of cucujoid beetles in pollination of *Encephalartos* species raises questions about earlier studies in which these taxa were discounted as possible pollinators of *E. lanatus* and *E. transvenosus* (Endrödy-Younga, 1991). It also raises questions about the possible role of cucujoid beetles in the pollination of many *Encephalartos* species in the northern part of South Africa and in eastern and central Africa where *Porthetes* appears to be absent from many populations (Donaldson, 1999).

It is not clear from this study whether any of the pollinator species is more important than any of the others. Erotylidae sp. nov, *M. encephalarti*, and *P. hispidus* differed in their abundance in male and female cones and their ability to carry pollen The difference in pollen load between species was not surprising because of differences in size and body structure. *Porthetes hispidus* has grooved elytrae and setae covering the body (Fig. 1c), which would facilitate pollen retention, while Erotylidae sp. nov has smooth elytrae with no setae (Fig. 1f), and only carried pollen on the legs and tarsi as has been recorded in similar studies (Donaldson, 1997). *Metacucujus* spp also have elytra covered with fine setae (Endrödy-Younga, 1991) which might help trap pollen (Fig. 1d). There is no clear reason why *M. encephalarti* retained less pollen when moving from male to female cones but the setae lie flat against the elytrae and may therefore provide less structural protection for trapped pollen when the beetle is in flight.

Our results showed that greater numbers of *Porthetes* resulted in higher seed set whereas seed set was greatest with intermediate numbers of Erotylidae sp. nov. and *M. encephalarti*. This could be due to differences in feeding behaviour. Larvae of *Porthetes* (Curculionidae) develop and feed within the sporophylls of the male cones and the adults have snouts for boring into sporophyll tissue. They do not appear to feed on pollen (Donaldson, 1997). In contrast, members of the Cucujoidea (Erotylidae and Boganiidae) have chewing and biting mouth structures (Labandeira, 2000) and both adults and larvae may feed on pollen (Leschen, 1997; Chavez and Genaro, 2005). This suggests that when Erotylidae sp. nov. and *M. encephalarti* occur in large numbers, their feeding habits may lead to a reduction of pollen on the female cone.

All of the potential pollinators studied here were found in male cones at the post dehiscent stage and larvae were observed in the cones, indicating that male cones are used as brood sites. This is similar to other pollinator–cycad mutualisms where pollinators mate and lay eggs on the microsporophylls (Norstog et al., 1986; Tang, 1987; Norstog and Fawcett, 1989; Donaldson, 1997; Terry et al., 2005; Wilson, 2002; Hall et al., 2004). The role of *Platymerus* spp., which were found as adults on male cones and which occur on the megasporophylls of the female cones of *E. friderici-guilielmi* (Oberprieler, 1995), was not resolved in this study and their role in pollen transfer and deposition is currently uncertain. It is also not known to what extent plant chemistry affects the behaviour of the different insects. Cycad cone tissues typically contain methylazoxymethanol (MAM) glycosides (e.g. cycasin, neocycasin, and macrozamin), which are common in cycads (Moretti et al., 1983). Studies of other cycads have shown that exposure of insects to MAM glycosides may vary in male and female cone tissues because the MAM glycosides are contained in idioblast cells in male microsporophylls but occur freely in female megasporophylls (Norstog et al., 1992; Vovides et al., 1993). The implication that insects do not feed on female cone tissues as a result of the greater exposure to MAM glycosides has yet to be tested in the species of *Encephalartos* where there are several insect species (*Platymerus* spp. and *Amorphocerus* spp) that feed on megasporophyll tissues.

In summary, the results of this study form part of a growing body of evidence for insect pollination in cycads. These cycad–insect mutualisms are important for the long term viability of cycad populations but may be especially vulnerable because cycads typically provide larval brood sites for their pollinators.

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Appendix 3

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Scent chemistry and patterns of thermogenesis in male and female cones of the African cycad *Encephalartos natalensis* (Zamiaceae)

T.N. Suinyuy [a,d,c,*], J.S. Donaldson [a,b,d], S.D. Johnson [c]

[a] *KRC, SA National Biodiversity Institute, Private Bag X7, Claremont 7735, Cape Town, South Africa*
[b] *Research Associate, Fairchild Tropical Botanic Garden, Miami, FL, USA*
[c] *School of Biological and Conservation Sciences, University of KwaZulu-Natal Pietermaritzburg, Private Bag X01, Scottsville 3201, South Africa*
[d] *Department of Botany, University of Cape Town, South Africa*

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Abstract

Most, if not all, extant cycads are pollinated by insects which use the cones as larval brood sites. These interactions appear to be mediated by cone volatiles, and, in some species, by patterns of thermogenesis. We investigated the chemical composition of volatile emissions and patterns of thermogenesis in cones of the South African cycad *Encephalartos natalensis,* using a gas chromatograph–mass spectrometer (GC–MS) and miniature temperature data loggers (ibuttons), respectively. This was done during various developmental stages (before and during receptivity and pollen release) for both female and male cones. A total of 31 compounds were identified in headspace samples; 17 of which were common to both sexes, 12 found only in male cones, and two found only in female cones. The major volatiles in pollen and female cones are (3*E*)-1,3-octadiene (averaging 54.25% and 15.82% of total emissions), (3*E*,5*Z*)-1,3,5-octatriene (averaging 13.37% and 47.66%), and α-pinene (averaging 16.29% and 12.24%). Female cones were not thermogenic before and during receptivity whereas pollen cones were thermogenic during pollen shedding. Thermogenesis of male cones occurred between 1400 h and 1530 h on successive afternoons, reaching an average of c. 10.5 °C above ambient temperature. Volatile emissions and thermogenesis occurred in association with insect activity on the cones suggesting that they both play a role in regulating insect behaviour.

Keywords: African cycads; Gas chromatography–mass spectrometry; Insect visitors; KwaZulu-Natal; Pollination; Thermogenesis; Volatile emissions; Zamiaceae

1. Introduction

Several highly specialized plant–pollinator interactions have been shown to be mediated by emission of volatiles in conjunction with heat production (Bronstein et al., 2006; Jürgens, 2009; Pellmyr and Thien, 1986; Pellmyr, 1992; Seymour and Schultze-Motel, 1997). Although this area of research has focused mostly on flowering plants, there is an emerging body of work on cycads, which shows that volatile emissions and thermogenesis also influence pollinator behaviour in these early seed plants (Donaldson, 1997; Seymour et al., 2004; Tang, 1987a,b; Terry et al., 2004, 2007). Studies of cycads can provide important insights into the evolution of plant–pollinator interactions. Firstly, cycads are the oldest group of extant seed plants and the evolution of insect pollination mutualisms in cycads may have pre-dated the evolution of insect pollination in angiosperms (Norstog and Nicholls, 1997; Stevenson et al., 1998). Secondly, it has been hypothesized that plant volatiles originated as herbivore deterrents and later served a function as attractants (Pellmyr and Thien, 1986). All known cycad–pollinator interactions involve insect herbivores that feed on cycad reproductive structures, and this means that cycads are a good model to test this hypothesis. Finally, studies of cycad–insect associations indicate that some cycad–pollinator interactions could have originated through a shift from angiosperm host plants to cycads (Oberprieler, 2004). Therefore, studies of cycads may contribute to a better understanding of the evolution and function of volatile compounds in herbivore and pollinator attraction, and the occurrence of host shifts.

* Corresponding author. KRC, SA National Biodiversity Institute, Private Bag X7, Claremont 7735, Cape Town, South Africa.
E-mail address: T.Suinyuy@sanbi.org.za (T.N. Suinyuy).

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Early observations of African cycads indicated that male cones emitted volatiles and produced heat (thermogenesis) during pollen shed, and that insects visited male and female cones at the time of odour emission and heat production (Jacot-Guillarmod, 1958; Pearson, 1906; Rattray, 1913; Tang, 1987a). However, the role of insects and the function of volatile emissions and thermogenesis in cycad pollination went untested for a long time because of the prevailing paradigm that all cycads were wind-pollinated (e.g. Chamberlain, 1935). Subsequent studies have shown that at least four African cycad species, *Encephalartos cycadifolius*, *E. villosus*, *E. friderici-guilielmi* and *Stangeria eriopus,* are pollinated by beetles (Donaldson et al., 1995; Donaldson, 1997; Proches and Johnson, 2009; Suinyuy et al., 2009). These studies, together with those of other extant species (e.g. *Zamia furfuracea*, Norstog et al., 1986; *Z. pumila*, Tang, 1987b; *Macrozamia communis*, Terry, 2001; *Bowenia*, Wilson, 2002; *Lepidozamia peroffskyana*, Hall et al., 2004; *M. machinii* and *M. lucida*, Terry et al., 2005; and *Cycas*, Kono and Tobe, 2007) provide experimental evidence for insect pollination in seven cycad genera and strong circumstantial evidence for insect pollination in the remaining three genera, *Ceratozamia* and *Dioon* (Vovides, 1991), and *Microcycas* (Vovides et al., 1997). Wind pollination, if it occurs, is likely to be an exception to this general pattern of insect pollination, or may occur in conjunction with insect pollination (Niklas and Norstog, 1984). Although insect pollination is apparently common in extant cycads, the role of cone volatiles and thermogenesis in mediating cycad–insect interactions is still poorly known.

Early studies of other African *Encephalartos* cycads have identified thermogenesis in male cones of *E. altensteinii* and *E. lehmannii* (Jacot-Guillarmod, 1958) and in male and/or female cones of *E. barteri*, *E. bubalinus*, *E. ferox*, *E. gratus*, *E. hildebrandtii*, *E. longifolius*, and *E. manikensis* (Tang, 1987a). Gas chromatography–mass spectrometry (GC–MS) analyses of volatiles have been conducted only for *E. altensteinii* and *E. villosus* (Pellmyr et al., 1991; Suinyuy et al., in press). Therefore, further studies are required to determine the possible role that cycad cone volatile and thermogenesis plays in regulating insect behaviour in *Encephalartos*.

In this study, we examined cone volatiles, and thermogenesis, as well as insect visitors, in *Encephalartos natalensis* Dyer and Verdoorn, a cycad in which both female and male cones are known to be visited by a variety of insect species (Oberprieler, 1995; Vorster, 1995). In our study, we measured thermogenesis, analysed volatile compounds, and surveyed insect visitors in both male and female cones at different stages of development (before and during pollen shedding and receptivity). We compare the results from these studies to the available data for other cycads and discuss the possible function of cone volatiles and thermogenesis for pollination.

2. Materials and methods

2.1. Study species and system

E. natalensis, commonly called the Natal cycad, is endemic to South Africa and is widely distributed in KwaZulu-Natal. The species occurs mostly inland and is often associated with rocky outcrops, cliffs and escarpments in hilly terrain (Jones, 1993). The climate in this area is characterised by hot and wet summers with cold and dry winters and occasional frost. Different forms exist that are differentiated mostly by leaf characteristics and the extent of woolly tomentum in the crown (Giddy, 1978; Goode, 2001). We sampled *E. natalensis* specimens of unknown origin at the Pietermaritzburg campus of the University of KwaZulu-Natal (UKZN), as well as plants occurring naturally at the KwaZulu-Natal National Botanical Garden, Pietermaritzburg, and the conservation area of Hilton College just north of Pietermaritzburg. Mature specimens have an erect or reclining stem and older plants can be between 4 and 6 m tall (Fig. 1a). Female and male plants produce between one and five cones per stem. Female cones are 50 to 60 cm long and 25 to 30 cm in diameter while male cones are 45 to 40 cm long and 10 to 12 cm in diameter (Fig. 1a,b). Female and male cones have a woolly tomentum early in their development but gradually lose their woolliness as they mature (Giddy, 1978). Male cones are pale yellow when mature and the cone extends so that the sporophylls separate and pollen is freely dispersed from the cone (Tang, 1987a).

2.2. Sampling of volatile compounds

Headspace sampling was used to collect volatiles from male and female cones before and during pollen release and receptivity, respectively. Polyacetate bags (Nalo Bratfolie Kalle GmbH-Germany) were placed over the cone just prior to sampling in

Fig. 1. (a) Female *Encephalartos natalensis* with receptive cones; (b) male *Encephalartos natalensis* with male cones at pollen shed stage.

order to concentrate the volatile compounds. Air from inside the bags was suctioned into a chromatoprobe trap containing 2 mg of a 50:50 mixture of Tenax TA® (Alltech Associates, USA) and activated charcoal (Carbotrap™, Supelco, USA) using a portable field battery operated pump (Spectrex Personal Air Sampler PAS 500, USA) calibrated at 200 ml/min. Cone volatiles were collected in the afternoon (between 1330 h and 1630 h) before and at pollen shed stage for male cones, and before and at receptive stage (gaps present between upper sporophylls) for female cones. Air samples were simultaneously collected from empty polyacetate bags as controls to identify background contamination. The adsorbent Tenax TA used in the study is commonly used to trap volatile compounds and has a high thermal stability up to 350 °C, which allows for thermal desorption in GC analysis (Tholl and Röse, 2006).

Volatile samples were analysed using a coupled Varian 3800 gas chromatograph (Varian Palo Alto, California, USA) and Varian 1200 mass spectrometer (GC–MS). The GC was equipped with a Carbowax column (DB-wax) of 30 m × 0.32 mm internal diameter x 0.25 μm film thickness (Alltech, Deerfield, Illinois, USA). Helium was used as the carrier gas at a flow rate of 1 mL/min. Traps containing the adsorbent and volatiles were placed in a Varian 1079 injector by means of a 'Chromatoprobe' fitting and thermally desorbed. After a 3 min hold at 40 °C, the GC oven was ramped up to 240 °C at 10 °C/min and held there for 12 min. Compound identification was carried out using the NIST05 mass spectral library and comparisons with retention times of authentic standards, where available, as well as comparisons between calculated Kovats retention indices and those published in the literature. A homologous series of alkanes (C8–C20) was used to determine Kovats retention indices. All reference compounds used for retention time comparisons were obtained from Sigma Aldrich Inc. GmbH, Germany, except (3*E*)-1,3-octadiene which was obtained from ChemSampco, USA. Compounds present at higher or similar percentages in controls were considered as contaminants and excluded from the analysis.

2.3. Thermogenesis

Temperatures of male and female cones of *E. natalensis* were monitored *in situ* for thermogenesis events before and during pollen release and receptivity, respectively. Temperature was measured using ibuttons (Fairbank technology USA). An ibutton was inserted between the cone sporophylls to measure cone temperature and a second ibutton was placed in between the leaves of the same cycad to measure ambient temperature. Both ibuttons were positioned to avoid direct exposure to sunlight. The ibuttons were set to record the temperature at 10 minute intervals for the duration of the pollination period of the cones.

2.4. Insect visitors to male and female cones of E. natalensis

To determine which insects visited *E. natalensis*, female and male cones were sampled before and during receptivity and pollen shedding respectively. Cones on cycads growing naturally at Hilton College Conservation area and the forest areas of KZN National Botanical Garden were examined around the same time that volatiles were sampled (1300 h–1530 h). To survey insects from male cones, the cone was placed over a beating sheet and tapped to dislodge all the insects onto the sheet. Seven male cones were surveyed and all the insects were counted, recorded and stored in 70% ethanol. Only insects crawling on the surface of female cones were sampled as these could be collected and stored in alcohol without damaging the female cone.

2.5. Statistical analysis

We used the Primer 6 programme (Clarke and Gorley, 2006) to analyse and compare scent profiles from female and male cones before and during receptivity and pollen shedding. Non-metric multidimensional scaling (NMDS), based on Bray–Curtis similarities of square root transformed data, was used to detect similarities among samples. To evaluate how well or poorly the particular configuration reproduces the observed distance matrix, the stress value is given. The smaller the stress value, the better the fit of the reproduced ordination to the observed distance matrix (Clarke, 1993). Differences in scent profiles between stages and sex was assessed by ANOSIM (Clarke and Gorley, 2006) with 10 000 random permutations.

3. Results

3.1. Volatile emission

The volatiles emitted by female and male cones of *E. natalensis* before and during receptivity and pollen release are summarised in Table 1. The compounds are identified by common names and CAS (Chemical Abstract Service) registry numbers and listed according to estimated Kovats retention index (KRI). In total, 31 compounds were found, which included 13 fatty acid derivatives (four unsaturated hydrocarbons, four aldehydes, four ketones, and one alcohol), seven benzenoids, ten terpenoids (nine mono-terpenes and one sesquiterpene), and one nitrogen-containing compound (Table 1). In general, there were fewer compounds from female cones, with 20 of the 31 compounds recorded from pre-receptive or receptive cones. In contrast, 29 of the 31 compounds were found in male cone samples taken either before or during pollen shed. Analysis of odour components showed that most volatile compounds were specific to particular cone stages, while others were sex specific, or even sex and cone stage specific. Before pollen release and receptivity, the major compounds, in male and female cones respectively, were benzaldehyde (50.3 and 63.1%), heptanal (35.2 and 12.8%) and linalool (3.1 and 10.4%) (Table 1). The most frequently occurring compounds in cones before pollen shed and receptivity were benzaldehyde and heptanal (found in all seven pre-pollen shedding and all eight pre-receptivity cones), 1-octen-3-ol (in six male cones), phenol (six male and eight female cones), linalool (five male and seven female cones) and benzyl alcohol and dihydro-2(3)-furanone (in all eight female cones). Two compounds were frequently emitted in relatively small amounts in both male and female cones, respectively: 1-octen-3-ol (4.2 and 0.3%) and phenol, (3.5 and 2.0%). The emission of dihydro-2(3)-furanone by the female

Table 1
Occurrence and relative amounts of volatile compounds emitted by male and female cones of *E. natalensis* before and during pollen shedding and receptivity, respectively. Compounds are identified by common names and CAS (Chemical Abstracts Service) registry number, and listed according to estimated Kovats retention index (KRI) within each compound class. Values are mean (±S.E) percentages of the total peak area. The number of samples in which the compound was identified is given in parentheses.

Compound	CAS	KRI	Male cones		Female cones	
			Pre-pollen shed	Pollen shed	Pre-receptive	Receptivity
			n=7	*n*=16	*n*=8	*n*=11
Aliphatics						
Unsaturated hydrocarbons						
(3*E*)-1,3-Octadiene [a]	1002-33-1	1062	–	54.25±10.27 (11)	–	15.82±9.60 (7)
(3*E*,5*Z*)-1,3,5-Octatriene [b]	40087-61-4	1148	–	13.37±4.60 (11)	–	47.66±13.21 (8)
(*E*,*E*,*E*)-2,4,6-Octatriene [b]	15192-80-0	1198	–	0.25±0.18 (6)	–	–
1,2-Dimethyl-1,4-cyclohexadiene [b]	17351-28-9	1215	–	0.04±0.03 (6)	–	1.06±0.43 (5)
Aldehyde						
Heptanal [a]	111-71-7	1209	35.23±5.73 (7)	1.64±0.93 (5)	12.83±2.62 (8)	1.82±1.15 (5)
(*Z*)-2-Heptenal [b]	57266-86-1	1340	–	0.07±0.03 (11)	–	–
(2*E*,4*E*)-Hepta-2,4-dienal [b]	4313-05-3	1482	–	–	–	0.97±0.39 (6)
2,4-Octadienal [b]	30361-28-5	1580	–	0.01±0.01 (7)	–	–
Ketone						
2-Nonanone [a]	821-55-6	1401	–	4.37±3.31 (4)	–	–
2,5-Hexanedione [b]	110-13-4	1524	–	0.01±0.01 (5)	–	–
2,2,5-Trimethyl-2,6-heptadien-4-one [b]	546-49-6	1584	–	0.02±0.01 (8)	–	–
Dihydro-2(3H)-Furanone [b]	96-48-0	1659	–	–	3.79±1.02 (8)	–
Alcohol						
1-Octen-3-ol [a]	3391-86-4	1461	4.17±0.91 (6)	0.01±0.01 (1)	0.34±0.34 (1)	–
Benzenoids						
Anisole [a]	100-66-3	1357	–	6.75±2.85 (6)	–	14.86±6.61 (4)
Benzaldehyde [a]	100-52-7	1553	50.31±6.02 (7)	0.41±0.13 (14)	63.12±4.86 (8)	2.80±0.60 (11)
Methyl benzoate [a]	93-58-3	1638	–	0.01±0.00 (3)	–	0.01±0.01 (3)
Methyl salicylate [a]	119-36-8	1808	–	tr (3)	–	tr (2)
Benzylalcohol [a]	100-51-6	1896	–	0.02±0.01 (10)	7.48±0.71 (8)	0.08±0.04 (8)
2-Methylphenol [b]	95-48-7	2023	–	0.01±0.00 (5)	–	–
Phenol [a]	108-95-2	2032	3.55±0.72 (6)	0.04±0.01 (13)	2.03±0.30 (8)	0.27±0.09 (11)
Terpenoids						
Monoterpenoids						
α-Pinene [a]	7785-70-8	1095	–	16.29±6.52 (5)	–	12.24±6.46 (3)
β-Pinene [a]	127-91-3	1194	3.55±2.31 (2)	1.52±0.77 (9)	–	1.62±0.97 (3)
β-Myrcene [a]	123-35-3	1199	–	0.04±0.04 (2)	–	0.28±0.28 (3)
Limonene [a]	138-86-3	1224	–	0.28±0.15 (4)	–	0.35±0.23 (3)
cis-β-Ocimene [a]	3338-55-4	1275	–	0.16±0.12 (2)	–	–
p-Cymene [a]	99-87-6	1383	–	0.05±0.02 (8)	–	–
cis-Linalool Oxide (Furanoid) [a]	5989-33-3	1467	–	0.10±0.06 (3)	–	0.13±0.08 3)
Linalool [a]	78-70-6	1562	3.19±0.88 (5)	0.12±0.05 (11)	10.41±2.43 (7)	0.03±0.02 (3)
β-Cyclocitral [b]	432-25-7	1647	–	0.01±0.00 (5)	–	–
Sesquiterpenoid						
β-Caryophyllene [a]	87-44-5	1636	–	tr (6)	–	tr (2)
Nitrogen-containing compound						
2-Methoxy-3-methylpyrazine [c]	2847-30-5	1392	–	0.17±0.07 (5)	–	–

[a] Identifications based on mass spectrum, Kovats retention index and authentic standard.
[b] Identifications based on mass spectrum and Kovats retention index.
[c] Identification based on mass spectrum only. tr=trace amounts (<0.01%).

cones is the major difference between female and male cones before pollination.

During pollen release, the major compounds in male cones were unsaturated hydrocarbons, (3*E*)-1,3-octadiene (54.2%) and (3*E*,5*Z*)-1,3,5-octatriene (13.4%), the monoterpene α-pinene (16.3%), and the benzenoid anisole (6.7%) (Table 1). In receptive female cones, the same major compounds were detected but the ranking was slightly different, comprising (3*E*,5*Z*)-1,3,5-octatriene (47.7%) and (3*E*)-1,3-octadiene (15.8%), anisole (14.9%), and α-pinene (12.2%).

The NMDS analysis of volatile compounds emitted from female cones before and during receptivity, and from male cones before and during pollen shed (Fig. 2), showed a significant separation between life stages and sexes (NMDS stress value 0.06;

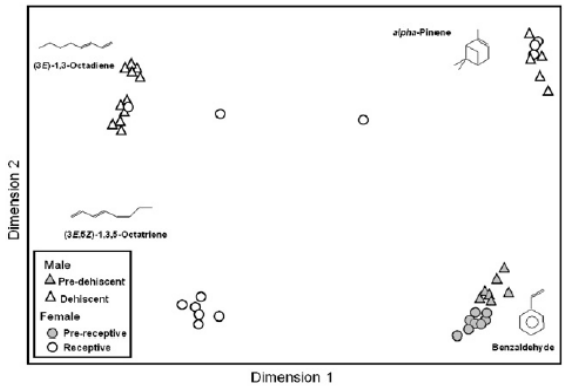

Fig. 2. Non-metric multidimensional scaling (NMDS) based on Bray–Curtis similarities of the odour composition of male and female cones *E. natalensis* before and during pollination. (NMDS 2D stress value=0.06).

ANOSIM, $R=0.402$, $P<0.01$). There was no distinction between pre-receptive female cones and pre-dehiscent male cones, which were characterized by the presence of benzaldehye together with smaller amounts of benzyl alcohol, heptanal, 1-octen-3-ol, dihydro-2(3)-furanone, and linalool. Most receptive female cones formed a cluster which was characterised by the dominance of (3*E*,5*Z*)-1,3,5-octatriene. Male cones formed two clusters, one dominated by (3*E*)-1,3-octadiene and a second by α-pinene, with a few female cones also falling into these clusters (Fig. 2). Other compounds emitted in small relative amounts contributed to the separation of the stages. For example, the compounds (*Z*)-2-heptenal, 2,4-octadienal, 1,2-dimethyl-1,4-cyclohexadiene, 2-nonanone, 2,5-hexanedione, 2,2,5-trimethyl-2,6-heptadien-4-one, 2-methoxy-3-methylpyrazine, and *p*-cymene are emitted only by pollen shedding cones whereas (2*E*,4*E*)-hepta-2-4-dienal is emitted only by receptive female cones.

3.2. Thermogenesis

There was little or no increase in temperature in female cones before and during receptivity. In non-receptive and receptive female cones, cone temperatures never rose above the maximum ambient air temperature, indicating that there was no thermogenesis. There was however a lag in the cooling of female cones as ambient temperatures dropped in the late afternoon resulting in cone temperatures that were consistently above ambient from 1400 h to 1900 h (Fig. 3a). Heating pattern of male cones before pollen shed was similar to that of female cones before receptive phase. Cone temperatures were consistently below ambient temperature from morning to late afternoon (Fig. 3b). In contrast, cone temperature of male cones consistently rose above the maximum concurrent ambient temperature during pollen shed (Fig. 3b), with a peak in the early afternoon (around 1400 h). During pollen shed, daily maximum mean cone temperature ranged between 28.0 and 31.8 °C and exceeded mean ambient temperature by between 10.1 and 11.1 °C.

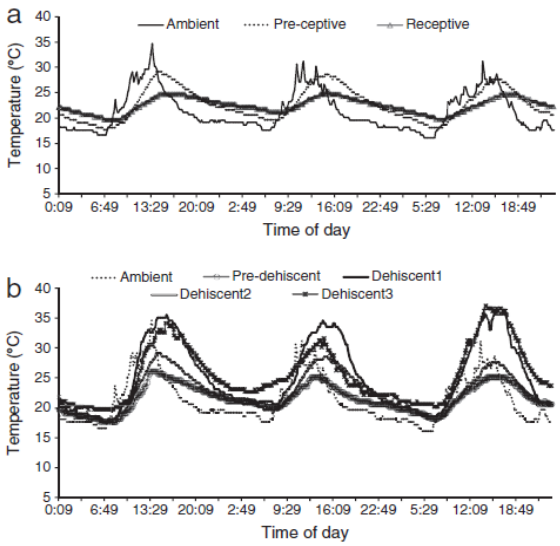

Fig. 3. Pattern of thermogenesis in relation to ambient temperature: (a) female cones (one each) before and during receptivity; (b) male cones before (one example shown) and during pollen shed (three examples shown).

3.3. Insect visitors to male and female cones

Before maturity, when sporophylls were not yet separated, no insects were found on male cones. At pollen shed, three beetle species (Coleoptera) were present in male cones, namely an undescribed Erotylidae, *Metacucujus goodei* Endrödy-Younga (Boganiidae), and an undescribed species of *Porthetes* (Curculionidae) that is probably the same taxon as *Porthetes* sp. 14.2 and 14.3 referred to by Downie et al. (2008) (Fig. 4a–c). Older male cones had beetle larvae in the microsporophylls. Adult *Porthetes* sp. were active on the cone surface while Erotylidae sp. nov. and *M. goodei* were mostly moving in between the sporophylls and along the cone axis. *Porthetes* sp. specimens were most abundant on the male cones (75.71±8.31 individuals; mean±S.E) and their abundance was significantly greater than *M. goodei* (36.14±2.93) and Erotylidae sp nov. (39.00±7.15) (ANOVA, $F_{2,\ 18}=6.02$: $P<0.01$). Sampling of female cones revealed that, prior to receptivity, female and male *Antliarhinus signatus* and *Antliarhinus zamiae* beetles (Fig. 4d–f) were crawling over the surface of the cones. On receptive cones, we also found Erotylidae sp. nov. and *Porthetes* sp., the same taxa as on pollen shedding cones. While *Antliarhinus* spp. could be seen mating and actively crawling over the cone surface, Erotylidae sp. nov. and *Porthetes* sp. were mostly found in between the tightly packed megasporophylls which hampered their collection.

4. Discussion

Our results show that pollen shedding in male *E. natalensis* cones was characterised by a distinct period of thermogenic activity accompanied by volatile emissions that were significantly different from the emissions preceding pollen shed. At

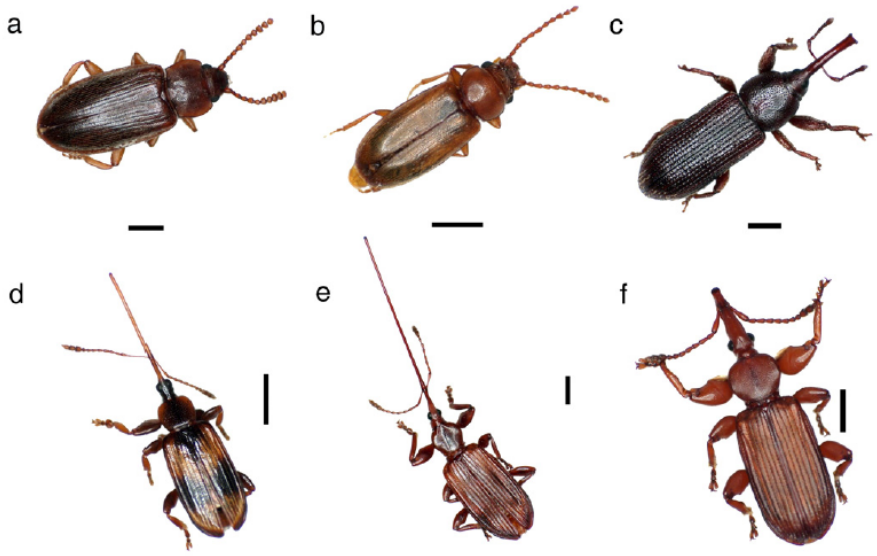

Fig. 4. (a–f) Insects which visit male and/or female cones of *Encephalartos natalensis*. (a) An undescribed Erotylidae sp nov. (male and female cones); (b) *Metacucujus* goodei (male cones); (c) *Porthetes* sp. (male and female cones); (d) female *Antliarhinus signatus* (female cones); (e) female *Antliarhinus zamiae* (female cones); (f) male *Antliarhinus zamiae* (female cones). Scale bars=1000 μm.

least three species of beetles were active on the male cones during pollen shed, all belonging to genera that are involved in the pollination of other *Encephalartos* species. In contrast, female cones did not appear to be thermogenic, but receptive female cones also emitted characteristic volatile profiles that were significantly different to those emitted by pre-receptive cones. The odour constituents of receptive female cones were mostly a subset of the odour constituents of male cones, but the relative amounts of major components differed substantially between sexes. Nevertheless, the beetles occurring on male cones were also present on female cones. This study thus provides new insights into the changes in volatile emissions associated with pollen shedding and receptivity in cycad cones.

Plant scents are made up of a complex blend of compounds that belong to different biosynthetic pathways; they may vary in number of constituents, composition, relative amounts, and in their temporal and spatial emission patterns (Raguso, 2004; Knudsen et al., 2006;). The complexity of these blends is evident in the differences between mature female and male cones. Odours from male cones comprised a greater number of compounds with (3*E*)-1,3-octadiene being the dominant compound followed by (3*E*,5*Z*)-1,3,5-octatriene, whereas odours from female cones comprised a subset of the compounds found in male cones and were characterised by high levels of (3*E*,5*Z*)-1,3,5-octatriene and (3*E*)-1,3-octadiene respectively (Table 1). Generally, it may be expected that there would be some congruence in the cues associated with male and female cones since the pollinator needs to interact with both sexes and other studies have shown that female cones emit similar odours to male cones but at lower concentrations (Terry et al., 2004). In addition to the overall blend of compounds, differences in the concentration of specific compounds can influence pollinator behaviour. In the Australian cycad, *Macrozamia lucida*, low concentrations of *β*-myrcene attracted pollen vectors whereas high concentrations of the same compound repelled them (Terry et al., 2004; Terry et al., 2007). This 'push–pull' interaction has not been confirmed in any other cycads but emphasizes the potential importance of differences in the relative and absolute concentrations of different compounds.

The NMDS analysis indicated that there were differences between samples, based primarily on volatile profiles of mature male cones. Some samples of *E. natalensis* were characterised by unsaturated hydrocarbons, made up of (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene, whereas other samples were dominated by the monoterpene, α-pinene. In male and female cones, (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene were associated with 1,2-dimethyl-1,4-cyclohexadiene and (*E*,*E*,*E*)-2,4,6-octatriene while α-pinene was associated with anisole, 2-nonanone, and *cis*-linalool oxide. Plants which emitted unsaturated hydrocarbons did not emit the terpenoid and vice versa. The emission patterns of these minor volatiles might reflect different biosynthetic pathways (Knudsen et al., 2006) for the unsaturated hydrocarbons and monoterpenes. The variation in the groupings might also be influenced by geographic origin. All *E. natalensis* sampled from the Pietermaritzburg campus at UKZN emitted α-pinene and anisole as the dominant compounds while those sampled from the KZN National Botanical Garden and conservation area of Hilton College Pietermaritzburg emitted the unsaturated hydrocarbons (3*E*)-1,3-octadiene and (3*E*,5*Z*)-1,3,5-octatriene. Different forms of *E. natalensis* have been recognised, based on leaf characteristics and the extent of woolly tomentum (Giddy, 1978; Goode, 2001; Grobbelaar, 2002) and the occurrence of different dominant volatiles suggest that a more critical

sampling approach is required to test for differences in volatile profiles between populations of *E. natalensis*, and between garden and natural populations.

With the exception of the unsaturated hydrocarbons, many of the volatiles found in *E. natalensis* are well known floral volatiles occurring in many flowering plants (Knudsen et al., 2006) and may have different biological roles. Cone volatiles have been analysed for only three African cycads species (Pellmyr et al., 1991; Proches and Johnson, 2009; Suinyuy et al., in press) and eight other cycad species (Pellmyr et al., 1991; Terry et al., 2004; Azuma and Kono, 2006) out of an estimated 300 species (Hill et al., 2004). As a result, any comparative analysis of cone volatiles from *E. natalensis* with chemical compounds from other cycads will need to be revised as additional information becomes available.

It is notable that *E. natalensis* shares only one major compound (3*E*)-1,3-octadiene with *E. altensteinii,* which is a morphologically similar and closely related species (Treutlein et al., 2005; Vorster 2004). A number of minor compounds, such as *β*-myrcene, *cis*-*β*-ocimene, limonene, benzyl alcohol, and methyl salicyclate also occur in both *E. natalensis* and *E. altensteinii* and a few other minor compounds are common to *E. natalensis*, *E. altensteinii* and *E. villosus*, e.g. α-pinene, linalool, and benzaldehyde. This study is the first to report the presence of (3*E*,5*Z*)-1,3,5-octatriene in cycads although this compound, together with (3*E*)-1,3-octadiene and anisole have also been recorded from some populations of *E. villosus* from KwaZulu-Natal (Suinyuy, unpublished data). Little is known about the biological function of (3*E*,5*Z*)-1,3,5-octatriene except that it has been reported as a chemo-attractant in marine brown algae (Kajiwara et al., 1980; Boland, 1995). Our identification of this isomer in the scent of *E. natalensis* is tentative and based on mass spectra and the Kovats retention index (Table 1). Roman Kaiser (pers. comm.) has also indicated that this particular isomer tends to be the one most commonly encountered in floral scents.

A large number of monoterpenes were detected in cone volatiles from *E. natalensis* and have been recorded from *E. altensteinii* (Pellmyr et al., 1991), *E. villosus (*Suinyuy et al., in press), as well as species of *Macrozamia* and *Zamia* (Pellmyr et al., 1991; Terry et al., 2004). Despite the prevalence of monoterpenes in cycad volatiles, relatively few have been studied in relation to their function in pollination (e.g. Terry et al., 2007) and this gap should be addressed in future studies. A similar situation exists for benzenoids, which were the second most numerous volatile compounds emitted by *E. natalensis* cones and which have been recorded in headspace samples from many flowering plants (Knudsen et al., 2006). Anisole and phenol have not been previously reported in cycad species, but are also emitted by members of the Araceae (Borg-Karlson et al., 1994; Skubatz et al., 1996). Anisole acts as a sex pheromone in some Coleoptera (Leal et al., 1996) and an attractant for bark beetles (Vrkocova et al., 2000) while phenol is an attractant of *Cyclocephala* sp. (Gruner and Marival, 1974) and an aggregation pheromone of the palm weevil *Rhynchophorus* (Oehlschlager et al., 1995). These compounds could therefore play a role in mediating the behaviour of cycad insects.

Temperature measurements in male cones of *E. natalensis* showed a single peak associated with thermogenesis occurring from early afternoon (1300 h) to late afternoon (1600 h). The cone temperature reached a peak of between 7.0 °C and 11.0 °C above ambient between 1400 h and 1530 h, before it started cooling down. The frequent co-occurrence of heat production and volatile emissions in cycads indicates that this is a syndrome which may regulate insect pollinator behaviour (Tang, 1987b; Seymour et al., 2004; Terry et al., 2004). Studies of other cycads suggest that thermogenesis enhances volatilization of odour, promotes male cone elongation and separation of sporophylls (Tang 1987a,b; Tang et al., 1987; Tang 1993), and may increase growth and survival in pollinating insects (Terry et al., 2004). Seymour and Schultze-Motel (1997) suggest that the heat generated by thermogenesis increases the activity of insect pollinators. In angiosperm systems, thermogenesis may enhance scent production to attract insects, increase insect activity, facilitate dehiscence of pollen sacs (Seymour and Matthews, 2006), and provide warm habitats (Azuma et al., 1999). The daily timing of peak temperatures in male cones differs between cycad species, from late morning to evening (e.g. Donaldson, 1997; Seymour et al., 2004; Suinyuy et al., in press; Tang, 1987b; Terry 2001; Terry et al., 2004) and may represent a species specific signal. The timing of peak heat production in *E. natalensis* was consistently earlier (1400 h–1530 h) than in *E. villosus* (1700 h–1830 h) (Donaldson, 1997; Suinyuy et al., in press) but was similar to some studies of *E. altensteinii* (Tang, 1987a).

The insects observed on pollen shedding cones of *E. natalensis* (Erotylidae nov. sp., *M. goodei*, and *Porthetes* sp.) all belong to genera or families where at least one species has been shown to pollinate other *Encephalartos* spp. (Donaldson, 1997; Donaldson et al., 1995; Suinyuy et al., 2009). The *Porthetes* is morphologically very similar to a species originally collected from *E. altensteinii* (Oberprieler, 1995) but molecular studies (Downie et al., 2008) indicated that similar specimens collected from *E. lebomboensis* male cones and the old leaf bases of *E. natalensis* were distinct from the specimens from *E. altensteinii* (*Porthetes* sp. 14.2, 14.3 in Downie et al., 2008). Our study represents the first record of this *Porthetes* from male and female cones of *E. natalensis*. All insects seen on male cones were covered with pollen so there is reasonable circumstantial evidence that these taxa also play a role in the pollination of *E. natalensis*. At this stage there is no experimental evidence to confirm their role in pollination. Although *M. goodei* was not recorded from female cones, the same species was considered to play some role in the pollination of *E. villosus* (Donaldson, 1997) where another species of *Porthetes* was the main pollinator.

In conclusion, this study forms part of a growing body of work on the pollination biology of cycads, including evidence for volatile emissions and thermogenesis. It is the first study to show thermogenesis in *E. natalensis* and to analyse the volatile odours emitted before and during pollination. It also provides the first report of (3*E*,5*Z*)-1,3,5-octatriene from cycads and includes new records for insects associated with the cones of *E. natalensis*. At present, there is only a circumstantial link between thermogenesis, volatile emissions and pollination in *E. natalensis* and further experiments are required to test how

insects respond to cone temperature and volatiles, as has been done for some other species (e.g. Suinyuy et al., in press; Terry et al., 2007). Nevertheless, identifying the volatile compounds is a first step that needs to be followed by studies to identify which compounds generate physiological responses, e.g. using gas chromatography–electroantennogram detection, and field tests to determine behavioural responses (Schiestl and Marrion-Poll, 2002).

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