The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

OLFACTORY RESPONSES OF *DASINEURA DIELSI* RÜBSAAMEN (DIPTERA: CECIDOMYIIDAE) FEMALES TO HOST PLANT VOLATILES

University of Cape Town

M.J. KOTZE

Thesis presented for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Zoology of

UNIVERSITY OF CAPE TOWN

June 2012

Dedicated to

my mother, Hester WJ Kotze

5 April 1927 – 4 June 2011

University of Cape Town

ACKNOWLEDGEMENTS

There are several people without whom this thesis and the work it describes would not have been possible at all. It gives me great pleasure to thank all those people who have contributed towards the successful completion of this work.

My sincere thanks go to Prof. John Hoffman, my supervisor for this project. John, I arrived on your doorstep with a slightly out-of-the-ordinary story, and you took me on as a student. I am grateful that you were my supervisor. Thank you for giving me freedom to work my project in my own personal style, but at the same time growing my skills as researcher. Thanks for allowing me to investigate some of the side paths that inevitably presented itself, and at the same time reminding me of the “storyline”. Thanks specifically for your excitement when I reported the results as it became unveiled; your enthusiasm fuelled my own excitement.

To Dr. Andreas Jürgens, co-supervisor of the project: I am very thankful for your input in my project. Your patience is remarkable, you truly have a “teacher’s heart”, you are a great coach. The general discussions we have had, the specific project related discussions we have had, your guidance, training and support was great. Whenever I struck a bleak spot in the experimentations and had to adjust or fine tune the techniques and start over, Andreas would just say: This is called “Method development....”!

Prof. Steve Johnson, thank you for allowing me to work in your lab and use your equipment and material. But above all, thanks for your input in my project, your comments and suggestions on papers, your help with the statistics, and your experience in general and the discussions we have had.

University of Cape Town

I collected most of the plant material and insects used in this project at Eskom's Nature Reserve at the Koeberg Power Station in the Western Cape. The Conservation Unit at Koeberg maintains a small site of *Acacia cyclops* in the Reserve specifically for research purposes. My sincere thanks to Mr Gert Greeff, Conservation Manager and Ms Jurina le Roux, Conservation Officer at Koeberg Nature Reserve for granting me access to the site for an extended period of time.

I received a lot of advice, professional input and technical assistance from a number of people. In this regard my appreciation is extended to especially Carien Kleinjan (UCT), Dr. Goddy Prinsloo (ARC), Dr.Taina Witt (UKZN), Fiona Impson (UCT/ARC), Dr. Carina Cilliers, Petra Muller (UCT), Miranda Waldron (UCT) and Dr. Des Conlong (SASRI). Often I contacted researchers in related disciplines from all over the world and received very valuable responses. I have found the international scientific community and resources very helpful.

Many of my friends and family have helped in special ways. Special thanks go to Grit and Tersia. A special word of thanks goes to Tannie Poppie, my aunt of 88. She enjoyed our excursion to collect galls so much, and true to her nature, wanted to know when we can go again! Thanks Blanche and Carina for your unwavering support throughout the whole study period. I am very grateful towards Francois Venter, my manager at the company I work for. Francois supported me to fulfil my personal dream to obtain my Ph.D. degree by allowing me to work part time for a long period and then later, by allowing me absence of leave for a number of months. My permanent employment is in a discipline unrelated to my field of study.

My thanks and appreciation also to two institutions for contributing funding towards my studies, the Drakenstein Trust and to the DST/NRF through the Centre of Excellence in Tree Health Biotechnology (CTHB).

University of Cape Town

Many other people provided encouragement, support and help in little things and the not-so-little things throughout the whole study period. I am grateful to them all.

ABSTRACT

In 2001, *Dasineura dielsi* (Diptera: Cecidomyiidae), a gall midge, was introduced into South Africa as a biological control agent on the invasive alien plant species, *Acacia cyclops* (Mimosaceae). The midges oviposit in the open flowers of *A. cyclops*, their primary host. Periodically they also lay eggs and induce galls on other invasive alien *Acacia* species including *A. melanoxylon*, *A. longifolia* and *A. saligna*, especially when they are growing in close proximity to *A. cyclops*. Galls are however, never found on African acacias. Nothing is known about the cues the females use in order to find suitable oviposition sites. An investigation was launched to test the following hypotheses: 1) the midges respond to the scent of *A. cyclops* to locate suitable oviposition sites; 2) the floral scent of *A. melanoxylon*, *A. longifolia* and *A. saligna* resembles that of *A. cyclops* and this explains the insects' use of these plants too; and 3) the floral scents of African acacias are distinctly different from *A. cyclops* and therefore has no attraction for *D. dielsi*.

Headspace samples of leaves and reproductive units at different stages (early buds, late buds, open flowers and senescing flowers) of *A. cyclops* were analysed using gas chromatography-mass spectrometry (GC-MS). In total, 87 different compounds were detected, of which 76 were identified. Leaf volatiles were distinct from those in the different reproductive units. The volatile profile changes from the early bud stage as the flowers mature until the senescing stage. The volatile profile of the open flowers, the stage *D. dielsi* uses for oviposition, was dominated by (*Z*)-3-Hexenyl acetate, 4-Oxoisophorone, (*Z*)-*β*-Ocimene, an unknown aliphatic compound (henceforth called UC1693), Heptadecane and Nonadecane. 4-Oxoisophorone and UC1693 in the ratio 2:1 may be characteristic of the floral scent of *A. cyclops*. Electroantennograms (EAG) showed antennae responded positively to (*Z*)-3-Hexenyl acetate, 4-Oxoisophorone, Limonene and *β*-Linalool; whereas gas chromatography coupled to electroantennal detection (GC-EAD) showed antennae responded positively to (*Z*)-3-Hexenyl acetate, 4-Oxoisophorone, UC1693, Limonene, Heptadecane, and Tricosane.

University of Cape Town

Behavioural bioassays with a 4-arm olfactometer showed that the midges are attracted to the scent of the open flowers of *A. cyclops*, but not to the scent of any other flower stage or the leaves, confirming that *D. dielsi* females employ olfactory cues to locate suitable flowers for oviposition. The midges also showed a behavioural attraction to 4-Oxoisophorone alone. The lack of a positive behavioural response to the simulated scent of *A. cyclops* containing the prominent compounds found in the open flower scent, but lacking UC1693, may be indicative of the importance of UC1693 as a co-attractant with 4-Oxoisophorone. This study is the first to show positive antennal and behavioural responses by a dipteran to 4-Oxoisophorone. Furthermore, the midges were repelled by *β*-Linalool which was tested at a very low concentration.

University of Cape Town

Headspace samples of the leaves and the reproductive units at different stages (late buds, open flowers and senescing flowers) of *A. melanoxylon*, *A. longifolia* and *A. saligna* were also analysed with GC-MS. As with *A. cyclops*, the leaf volatiles were distinct from those in the reproductive stages, and the volatile profile changed as the reproductive stages matured. In the floral scent of *A. melanoxylon*, 75 compounds were detected with (*Z*)-*β*-Ocimene and *p*-Anisaldehyde being most abundant. In the floral scent of *A. longifolia*, 69 compounds were detected with (*Z*)-*β*-Ocimene and Heptadecane being most dominant. In *A. saligna*, 54 compounds were detected in the floral scent with Benzyl alcohol and (*Z*)-3-Hexen-1-ol dominating. Benzyl alcohol made up almost 50% of the scent and may be characteristic of the scent of *A. saligna*. The floral volatile profiles of these three species were distinctly different from each other, as well as from *A. cyclops.* Bioassays with a 4-arm olfactometer showed the midges were not attracted to floral scents simulations of *A. melanoxylon*, *A. longifolia* and *A. saligna*. Other reasons for the attractiveness of these plants to *D. dielsi* need to be investigated.

Dasineura dielsi does not oviposit on African acacias. Headspace samples of the leaves and the open flowers of *A. karroo*, *A. sieberiana* var. *woodii* and *A. xanthophloea* were analysed with GC-MS. In

these cases the leaf scent profile of each species was distinct from the floral volatile profiles. In the floral scent of *A. karroo*, 69 compounds were detected with the aliphatics and benzenoids being the dominant compound classes. Seventy five compounds were detected in *A. sieberiana* var. *woodii* and 60 compounds in *A. xanthophloea*. In the latter two cases, compounds were mostly monoterpenoids. On the level of the compound classes there was some similarity in the profiles of *A. sieberiana* var. *woodii* and *A. xanthophloea*, and some similarity between the profiles of *A. karroo* and *A. saligna*. However, on the individual compounds level, there was no similarity pertaining to the compounds in combination with the ratios in which it occurred in the scent profiles of any of the African species tested.

University of Cape Town

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iii
ABSTRACT	vi
TABLE OF CONTENTS	ix
CHAPTER 1: GENERAL INTRODUCTION	1
1.1. Introduction	1
1.2. *Acacia* (Mimosaceae) species	3
1.3. The Cecidomyiidae galling insects	5
1.4. Plant volatiles mediate insect-host interaction	7
1.5. Hypotheses	9
1.6. Structure of the thesis	9
CHAPTER 2: VOLATILE CHARACTERISATION OF *A. CYCLOPS, A. MELANOXYLON, A. LONGIFOLIA* AND *A. SALIGNA*	12
2.1. Introduction	12
2.2. Methods and Materials	14
2.2.1 Collection of volatile samples from different flowering stages and leaves	14
2.2.2 Gas Chromatography-Mass Spectrometry (GC-MS) analyses	16
2.2.3 Statistical analyses	17
2.3. Results	18
2.3.1 Volatile profile of *Acacia cyclops*	18
2.3.2 Volatile profile of *Acacia melanoxylon*	27
2.3.3 Volatile profile of *Acacia longifolia*	35
2.3.4 Volatile profile of *Acacia saligna*	41
2.4. Discussion	48
2.4.1 Scent variation of leaves and different floral parts	48
2.4.2 Floral scent in the context of pollination biology	49
2.4.3 Leaf and floral scent in the context of chemical defence	54
2.4.4 Scent variation of senescing flowers	59

University of Cape Town

Page

CHAPTER 3: VOLATILE CHARACTERISATION OF *A. KARROO*, *A.SIEBERIANA* var. *WOODII* AND *A. XANTHOPHLOEA* 61

3.1. Introduction 61

3.2. Methods and Materials 62

3.3. Results 64

3.3.1 Volatile profile of *Acacia karroo* 64

3.3.2 Volatile profile of *Acacia sieberiana* var. *woodii* 65

3.3.3 Volatile profile of *Acacia xanthophloea* 66

3.3.4 Scent variation between species 71

3.4. Discussion 72

3.4.1 Leaf and flower volatiles in plant defence 73

3.4.2 Flower volatiles in pollinator attraction 74

CHAPTER 4: ANTENNAL RESPONSE OF *DASINEURA DIELSI* TO FLORAL SCENT COMPOUNDS OF ITS HOST 77

4.1. Introduction 77

4.2. Methods and Materials 78

4.2.1 Electroantennograms (EAG) 78

4.2.2 Gas chromatography coupled to electroantennographic detection (GC-EAD) 81

4.2.3 Gas Chromatography-Mass Spectrometry (GC-MS) analyses 82

4.2.4 Statistical analyses 83

4.3. Results 83

4.3.1 Electroantennograms (EAG) 83

4.3.2 Gas Chromatography-Mass Spectrometry (GC-MS) 85

4.3.3 Gas chromatography coupled to electroantennographic detection (GC-EAD) 87

4.4. Discussion 88

University of Cape Town

Page

CHAPTER 5: BEHAVIOURAL STUDIES 93

5.1. Introduction 93

5.2. Methods and Materials 94

5.2.1 Insects 94

5.2.2 Olfactometer setup 95

5.2.3 Odour sources tested 97

5.2.3.1 Fresh plant material 97

5.2.3.2 Individual authentic standard compounds 98

5.2.3.3 Biotests with mixtures of chemicals as found in the scent of four *Acacia* species 98

5.2.4 Behavioural observations 100

5.2.5 Digital record analysis 101

5.2.6 Cleaning procedures 103

5.2.7 Statistical analyses 103

5.3. Results 105

5.3.1 Time spent in treatment arm 105

5.3.2 Ratio of male to female emergence 108

5.4. Discussion 109

5.4.1 Attraction of *D. dielsi* to plant volatiles of *A. cyclops* 109

5.4.2 Behavioural response to antennal active compounds 113

5.4.3 Attraction of *D. dielsi* to simulations of the floral scent of four *Acacia* species 115

CHAPTER 6: GENERAL DISCUSSION 117

6.1. Introduction 117

6.2 Responses of *D. dielsi* females to plant volatiles from *A. cyclops* 118

6.3 Volatile profiles of non-standard hosts compared to *A. cyclops* 123

6.4 Volatile profiles of non-host African *Acacia* species compared to the Australian acacias 124

6.5 Conclusion 126

REFERENCES 127

University of Cape Town

CHAPTER 1: GENERAL INTRODUCTION

1.1. Introduction

Invasive alien species are known to cause substantial problems in areas where they became invasive and are a major threat to ecological services and global biodiversity (Enright, 2000; Mack et al., 2000; Van Driesche et al., 2008). Alien plants are plants that do not occur naturally in a specific area, have been brought either deliberately or unintentionally into a new area, and without their natural enemies present, may become invasive and spread aggressively and become weeds in usable land, watercourses, conservation areas and urban areas (Harley & Forno, 1992; Enright, 2000; Mack et al., 2000; Van Driesche et al., 2008).

Thirteen Australian *Acacia* species are invasive in South Africa (*A. baileyana* F. Muell., *A. cyclops* A. Cunn. ex G. Don, *A. dealbata* Link, *A. decurrens* Willd., *A. elata* A. Cunn. ex Benth., *A. implexa* Benth., *A. longifolia* (Andrews) Willd., *A. mearnsii* De Wild., *A. melanoxylon* R. Br., *A. paradoxa* DC., *A. podalyriifolia* A. Cunn. ex G. Don, *A. pycnantha* Benth. and *A. saligna* (Labill.) H. L. Wendl.) (Henderson, 2001) and cause harm to natural and agricultural ecosystems (Dennill et al., 1999; Kolesik et al., 2005). They form dense thickets which, besides being aesthetically unappealing, reduce agricultural productivity, create fire hazards, smother natural vegetation and associated fauna, threaten the conservation of biodiversity, and drastically reduce flow rates of rivers and streams, and alter or disrupt ecological processes and functions (Dennill et al., 1999; Hoffmann et al., 2002; Adair, 2005; Kolesik et al., 2005).

Many Australian acacias, including *A. cyclops, A. dealbata, A. decurrens, A. longifolia, A. mearnsii, A. melanoxylon, A. pycnantha* and *A. saligna*, were introduced to South Africa in the middle of the 19th century for commercial, cultural and horticultural purposes (Richardson & Kluge, 2008). Most of these species have been used in South Africa since the 1820s for stabilization of drift sands, timber

University of Cape Town

e.g. *A. melanoxylon* (black wood) and pulp production e.g. *A. decurrens* (green wattle), tannin extraction e.g. *A. mearnsii* (black wattle), and as garden ornamentals (Kolesik et al., 2005). *Acacia cyclops*, and *A. saligna*, to a limited extent, are collected from naturalized populations and widely used as a source of domestic firewood (Henderson, 2001; Hoffmann et al., 2002; Adair, 2005; Kolesik et al., 2005). Naturalization of these invasive species has been widespread and most now form a conspicuous part of the South African landscape (Henderson, 2001; Kolesik et al., 2005).

For many years, considerable resources and effort have been directed towards mechanical, chemical and biological control operations to curb these invasive weeds (Dennill et al., 1999; Van Wilgen et al., 2008; Van Wilgen & De Lange, 2011). More recently, these control efforts have escalated because the detrimental effects of invasive species on supplies of water within South Africa have been recognized (Zimmerman et al., 2004; Impson et al., 2011; Van Wilgen & De Lange, 2011). South Africa is deemed a water-stressed (and soon to be water-scarce) country (Jobson, 1999; Turton, 2001; Richardson & Kluge, 2008) and the invasive alien plants, and in particular Australian acacias, impact water supplies by reducing streamflow and impact on the ability of the water catchments areas to store water for steady release throughout the year (Van Wilgen et al., 2001; Le Maitre et al., 2002; Richardson & Van Wilgen, 2004, Van Wilgen & De Lange, 2011).

University of Cape Town

Since its inception, the biological control programme against invasive Australian *Acacia* species in South Africa has been influenced by a protracted conflict of interest between those who want to reduce the abundance of the plants and those who are benefiting from its cultivation e.g. *A. cyclops* being used for firewood and *A. melanoxylon* being used for furniture timber (Hoffmann et al., 2002; Zimmermann & Olckers, 2003; Impson et al., 2011). This conflict of interest has limited the extent to which biological control could be implemented (Zimmermann & Olckers, 2003; Impson et al., 2011). As a compromise, the choice of biological control agents has been restricted to those that reduce

the reproductive capacity, but not the vegetative growth (i.e. the useful attributes) of the plants (Adair, 2005; Kolesik et al., 2005; Impson et al., 2008; Impson et al., 2011; Klein, 2011). Biological control agents have been released on ten of the invasive *Acacia* species in South Africa. The agents are five seed weevils, *Melanterius* spp. (Coleoptera: Curculionidae) on *A. cyclops*, *A. melanoxylon*, *A. longifolia*, *A. saligna* and six others, two gall wasps, *Trichilogaster* spp. (Hymenoptera: Pteromalidae) on *A. longifolia* and *A. pycnantha*, two gall midges, *Dasineura* spp. (Diptera: Cecidomyiidae) on *A. cyclops* and *A. mearnsii* and a rust fungus, *Uromycladium tepperianum* (Pucciniales: Pileolariaceae) on *A. saligna* (Impson et al., 2011; Klein, 2011).

1.2. *Acacia* (Mimosaceae) species

The Mimosaceae (= Fabaceae or Leguminosae) family is one of the largest plant families with more than 19 000 species categorised in three subfamilies, including the Mimosoideae subfamily to which the acacias belong (Miller et al., 2011). There are more than 1350 *Acacia* species worldwide (Maslin & McDonald, 2004) with most in Australia where there are 1028 species of which 1012 belong to a single clade, until recently known as *Acacia* subgenus *Phyllodineae* (Miller et al., 2011). According to records, 386 species have been moved outside Australia by human intervention. Of these, 71 species have become weedy or naturalised, including 23 species that are deemed invasive worldwide (Richardson et al., 2011). Thirteen species have been declared invasive in South Africa (Henderson, 2001).

The phylogeny of the 1028 Australian acacias was recently revised and five clades were defined (Miller et al., 2011). Clades A (*A. victoriae* and *A. pyrifolia* clade) and C (*A. murrayana* clade) do not contain any *Acacia* species that are invasive in South Africa. Clade B (the Pulchelloidea clade), contains *A. saligna*, Clade D (the *melanoxylon* clade) contains *A. melanoxylon*, *A. longifolia, A. cyclops* and *A. implexa*, and Clade E (the *mearnsii* clade) contains *A. mearnsii*, *A. pycnantha, A. baileyana*, *A. dealbata*, *A. decurrens, A. elata* and *A. podalyriifolia*, accounting for 12 of the 13

University of Cape Town

declared invaders in South Africa (Henderson, 2001; Miller et al., 2011). The outstanding species, *A. paradoxa*, could not be placed confidently in any of these clades and awaits future molecular phylogenetic research (Miller et al., 2011).

Of the 13 invasive species in South Africa (Henderson, 2001), *A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. saligna* are deemed to be the most problematic due to the rapid growth rate, large seed production and rapid rate of dispersal (Milton & Moll, 1982; Dennill & Donnelly, 1991; Richardson & Kluge, 2008; Gibson et al., 2011). These four species are all without thorns (Henderson, 2001), and except for *A. melanoxylon* which is a tall upright tree, the other three species are large shrubs to small trees (Van Wyk & Van Wyk, 1997; Henderson, 2001; Maslin & McDonald, 2004; Coates Palgrave, 2005).

The African acacias on the other hand, are part of a much smaller group. Forty-eight species, subspecies and varieties of African acacias occur in South Africa (Smit, 2008). In constrast to Australian acacias, all African acacias have thorns which are diagnostic of the different species (Smit, 2008). Until recently the type species for the genus *Acacia* was an African species, *A. nilotica* (L.) Willd. ex Delile, which has been changed in a controversial manner to the Australian species, *A. penninervis* Sieber ex DC. (Carruthers & Robin, 2010; Miller et al., 2011). New generic names resulted from the retypification and *Acacia* sensu lato is now divided in *Acacia* sensu stricto, *Vachellia*, *Senegalia*, *Acaiella* and *Mariosousa* (Bouchenak-Khelladi et al., 2010). The African acacias should now be renamed *Vachellia*.

Little is known about the volatile chemistry of *Acacia* species. Seigler (2003) presented a comprehensive report on the phytochemistry of *Acacia* and listed a number of secondary metabolites such as amines and alkaloids, cyanogenic glycosides, fatty acids and seed oils, gums, nonprotein amino acids, terpenes (including essential oils, diterpenes, phytosterol and triterpene

University of Cape Town

genins and saponins), hydrolyzable tannins, flavonoids and condensed tannins, but only mentions plant volatiles from *A. farnesiana*. Otherwise floral volatiles have been described from *A. farnesiana*, *A. berlandieri*, and *A. rigidula* (all three native to North America) (Flath et al., 1983), *A. praecox, A. caven* var. caven*, A. aroma* (all three from Argentina) (Zygadlo et al., 1996; Lamarque et al., 1998), and from *A. karroo*, an African species (Kaiser, 1997). Subsequently, work has been done on the reclassified *Acacia* species, *Vachellia seyal seyal*, *V. seyal fistula, V. etbaica, V. brevispica, V. drepanplobium* and *Senegalia mellifera* (Willmer et al., 2009). No information on the volatile chemistry from invasive Australian species is available, except for Kotze et al. (2010), a part of this study, and a study on *A. dealbata* published in 1959 (Lamarque et al., 1998).

University of Cape Town

1.3. The Cecidomyiidae galling insects

The family Cecidomyiidae is a large family of nematocerous Diptera, generally known as galling insects. This family comprises some of the most destructive agricultural pests on grains, fruits and vegetables, and covers a diverse array of feeding habits, e.g. herbivory, florivory and fungivory. It also contains species that are prominent predators of mites, scale insects and aphids (Gagné, 2010). Approximately four-fifths of the 6 131 known species in 783 genera in the family are associated with flowering plants (Gagné, 2010), making them one of the most abundant groups amongst galling arthropods (Yukawa et al., 2005).

Galling insects are deemed to have inherent potential as agents for biological control of weeds because of their usually narrow host ranges and thus they are unlikely to affect non-target plants (Harris & Shorthouse, 1996). They can strongly curb growth, and reduce the fitness, of their host plants (Almeida et al., 2006). A diverse array of phytophagous Cecidomyiidae species is associated with Australian acacias in their native habitat (Adair, 2005; Impson et al., 2008). Bud, flower, fruit and seed-feeding Cecidomyiidae occur on all Australian *Acacia* species (except for *A. saligna*), that are invasive in South Africa (Adair et al., 2000).

The genus *Dasineura* which belongs to the supertribe Lasiopteridi: tribe Dasineurini, was first described by Rondani in 1840 (Gagné, 2010). The genus is polyphyletic and 466 species are currently known. It contains mostly flower-dwelling or leaf-rolling species, sharing certain primitive characteristics, as well as many species that form galls (Gagné, 2010). One of these, *D. dielsi* Rübsaamen, which originated from *A. cyclops* in Western Australia, was introduced into South Africa as biological control agent of *A. cyclops.* Besides using *A. cyclops* as a standard host, galls of *D. dielsi* have been found on *Acacia* spp. (Mimosaceae) both in Australia and South Africa (Adair, 2005; Kolesik et al., 2005; Gagné, 2010; Post et al., 2010), with some difference in hosts that are used on the two continents. In both regions, *A. cyclops* is the primary host (Adair, 2004; Kolesik et al., 2005; Post et al., 2010), but in Australia secondary galling occurs on *A. papyrocarpa*, *A. sophorae*, and *A. oswaldii* at localities where these species are in close proximity to *A. cyclops* (Kolesik et al., 2005). Similarly, in South Africa secondary galling occurs on *A. melanoxylon*, *A. longifolia*, and *A. saligna*, also predominantly in localities where these species are in close proximity to *A. cyclops* (Post et al., 2010)

Dasineura dielsi is a small insect with a wing length of approximately 2 mm (Kolesik et al., 2005). The adults are short-lived, and although they have mouthparts they are unknown to feed and must spend their time finding mates and the females finding suitable oviposition sites to achieve their maximum fitness (Yukawa, 2000; Adair, 2004; Kolesik et al., 2005). Females oviposit in open *Acacia* flowers and deposit eggs within the perianth tube, usually against the exterior surface of the ovary, but often amongst staminal filaments (Adair, 2004; Kolesik et al., 2005). Flower galls then develop in place of normal fruits (Adair et al., 2000). Larvae cause the ovary to evaginate and form developmental chambers around the larvae. Infested flower heads are transformed into clusters of galls, which may vary in number of individual galls depending on the number of eggs deposited within the flower (Kolesik et al., 2005). Each larval chamber contains a solitary larva (Kolesik et al., 2005). Feeding and gall development usually takes place over a period of a few weeks. Pupation

University of Cape Town

occurs in cocoons within the gall chamber and is also completed in the gall (Adair et al., 2000; Adair, 2004). Females of *Dasineura* commence oviposition within a few hours of emergence. With time, the galls desiccate and the woody remnants may persist on the plants for up to two years (Adair, 2004).

Dasineura dielsi is multivoltine and, in South Africa, is known to complete up to five generations per annum (Adair, 2004, 2005). Emergence of adults is variable with some individuals pupating and emerging as adults immediately and others pupating and emerging as adults at irregular intervals lasting up to 12 months after oviposition (Impson et al., 2011). The delayed adult emergence results from some larvae entering a quiescent phase at the end of the third larval instar (Impson et al., 2011). The result is that adults are present throughout the year, with a peak in emergence during the main flowering season (October to April) of *A. cyclops* (Henderson, 2001; Post et al., 2010; Impson et al., 2011). *Acacia cyclops* flowering has being recorded throughout the year (Henderson, 2001). The staggered emergence of *D. dielsi* fits well with the extended and unpredictable flowering period of *A. cyclops* (Adair et al., 2000; Impson et al., 2011). The non-standard hosts used by *D. dielsi*, *A. melanoxylon, A. longifolia* and *A. saligna*, have discrete, short flowering seasons and are therefore unavailable to *D. dielsi* adults that emerge beyond the limits of the flowering periods of these species (Henderson, 2001; Post et al., 2010).

University of Cape Town

1.4. Plant volatiles mediate insect-host interaction

Many, if not all plants, emit a diverse set of chemical compounds (Dudareva & Pichersky, 2000; Schoonhoven et al., 2005) which result from a number of biosynthetic pathways that are part of the secondary plant metabolism (Dudareva & Pichersky, 2000; Levin et al., 2003). The compounds are emitted from both reproductive and vegetative structures (Levin et al., 2003). The scent varies among plant species in terms of identity, number and relative amounts of the constituent volatile compounds and insects are able to distinguish between individual compounds in a blend to

discriminate between host and non-host plants (Dudareva & Pichersky, 2000; Schoonhoven et al., 2005).

Plant volatiles play an almost ubiquitous role in plant-insect interactions (Andrews et al., 2007; Bruce & Pickett, 2011) and have many functions e.g. pollinator attraction, serve as herbivore attractants or repellents, protection against abiotic stresses or attracting the natural enemies of herbivores (De Moraes et al., 1998; Paré & Tumlinson, 1999; Pichersky & Gershenzon, 2002; Levin et al., 2003; Knudsen et al., 2006; Andrews et al., 2007).

Female insects intent on finding a suitable host plant for oviposition need to monitor the chemistry of potential candidate plants very accurately (Harborne, 2001). For herbivorous insects, sensory inputs are most important in host finding and host selection and usually a sequence of behaviours is mediated by a combination of visual and olfactory cues (Murchie et al., 1997; Van Driesche et al., 2008). Some of the important steps in host finding and selection include the orientation towards the host at long range (often called host habitat location), distinguishing the plant from other plants at closer range (or host location), and finding a suitable place on the host plant for feeding and/or oviposition (host finding) (Bernays & Chapman, 1994; Van Driesche et al., 2008). For successful offspring development of insects in which the pre-adult stages have little opportunity to move away from their oviposition sites, e.g. such as in the case of gall insects, it is critically important that the ovipositing female selects a suitable oviposition site (Gouinguené & Städler, 2006; Castells & Berenbaum, 2008; Dötterl et al., 2009). Often secondary plant chemicals provide behavioural orientation for insects specific to a restricted range of plants (Murchie et al., 1997). Gall midges also rely to a large extent on olfaction in their search for oviposition sites (Pettersson, 1976; Birkett et al., 2004b).

University of Cape Town

1.5. Hypotheses

In South Africa, *D. dielsi* uses *A. cyclops* as its standard host (Adair, 2004; Post et al., 2010; Impson et al., 2011). Successful oviposition and galling also occurs less frequently on other *Acacia* species including *A. melanoxylon*, *A. longifolia* and *A. saligna*, especially when they are growing in close proximity to *A. cyclops* (Post et al., 2010). Although there are gall-forming African cecidomyiids associated with African acacias (Adair, 2004), *D. dielsi* has not been successful in producing galls on African acacias (Adair, 2004). A number of studies exist that report female gall-forming midges respond to host odour cues (Amarawardana, 2009 and references therein). However, nothing is known about the cues *D. dielsi* females use in order to find suitable oviposition sites. An investigation was therefore launched to test the following hypotheses:

1) *Dasineura dielsi* females respond to the scent of *A. cyclops* to find suitable flowers for oviposition;

2) the scents of *A. melanoxylon*, *A. longifolia* and *A. saligna* resemble the scent of *A. cyclops* and the occasional use of these plants by *D. dielsi* can be explained by an olfactory attraction similar to *A. cyclops;* and

3) the scent of the African acacias is distinctly different from that of *A. cyclops* and therefore is not attractive to *D. dielsi*.

1.6. Structure of the thesis

This thesis is presented in four chapters that cover the different hypotheses related to the putative role of volatile chemicals for host attraction and host finding of *D. dielsi.*

University of Cape Town

1) The volatile profiles of *A. cyclops* (the standard host of *D. dielsi*; Adair, 2004; Post et al., 2010) and of *A. melanoxylon*, *A. longifolia*, and *A. saligna* (all non-standard hosts; Post et al., 2010) were determined by taking headspace samples from leaves and from reproductive units at different stages, early bud (*A. cyclops* only), late bud, early flowering, and senescing flowering stages. The samples were analysed using gas chromatography-mass spectrometry (GC-MS). The variability of the profiles was analysed calculating Bray-Curtis similarities and the data was visualised using non-metric multidimensional scaling (NMDS) (Clarke & Warwick, 2001).

2) *Dasineura dielsi* does not utilise African acacias as hosts (Adair, 2004, 2005). In order to compare the volatile profiles of the African acacias to the Australian acacias, headspace samples from leaves and flowers from *A. karroo*, *A. sieberiana* var. *woodii* and *A. xanthophloea* were analysed using gas chromatography-mass spectrometry (GC-MS). The variability of the profiles was analysed by calculating Bray-Curtis similarities and the data was visualised using non-metric multidimensional scaling (NMDS) (Clarke & Warwick, 2001).

3) Electroantennogram (EAG) experiments were performed on male and female midges to test the antennal responses to single floral scent compounds that have a relative abundance of more than 5% selected from the floral volatile profile of *A. cyclops.* Electroantennographic detection coupled to gas chromatography (GC-EAD) was employed to test antennal responses of female midges to floral volatiles from *A. cyclops*. The objective was to screen for compounds that are electrophysiologically active in order to test them in subsequent behaviour bioassays.

4) Behavioural bioassays using a 4-arm olfactometer were performed to test the behavioural responses of the female midges to three sets of odour sources. The first set of experiments used fresh plant material (leaves, yellow buds, open flowers and senescing flowers) of *A. cyclops*. The objective was to determine whether chemical cues might play a role in the selection of a

University of Cape Town

particular plant organ for oviposition. The second set of experiments used those single authentic standards that elicited positive antennal responses in the EAGs and GC-EAD experiments. The objective was to analyse whether these compounds had an attractant or repellent effect on the behaviour of the midges. Since fresh plant material of *A. melanoxylon*, *A. longifolia*, and *A. saligna* was not available at the time of the year when *A. cyclops* flowers were available, the third set of experiments used mixtures of authentic standard compounds in the same ratios as the most prominent compounds that were found in the floral scent of *A. cyclops, A. melanoxylon*, *A. longifolia*, and *A. saligna* to simulate the scent of these species. The objective was to determine whether the female midges showed a positive behavioural response to any of the simulated scents.

University of Cape Town

CHAPTER 2: VOLATILE CHARACTERISATION OF *A. CYCLOPS*, *A. MELANOXYLON*, *A. LONGIFOLIA* AND *A. SALIGNA*

2.1. Introduction

Plants interact with various organisms in their environment for survival and reproduction purposes (Schiestl, 2010). From a survival perspective, plants need to defend themselves against herbivores, predators and pathogens and have evolved herbivore-induced signals that either deter herbivores (as direct defence mechanisms) or attract enemies of herbivores, e.g. predators and parasitoids, to attack herbivores (as indirect defence mechanism) (Mumm & Dicke, 2010; Schiestl, 2010). From a reproduction perspective, plants employ visual signals, e.g. shape and colour of flowers, and olfactory signals to attract pollinators and other beneficial insects (Schiestl, 2010). Diverse blends of volatile secondary compounds are therefore continually being emitted from the leaves, flowers and fruits of plants into the atmosphere (Dudareva & Negre, 2005).

University of Cape Town

Generally, the function of pollinator attraction can be ascribed to the floral volatiles whilst the function of defence is associated with vegetative volatiles, especially those emitted after herbivory (Pichersky & Gershenzon, 2002). Terpenoids and specifically monoterpenes and green leaf volatiles seem to have evolved primarily for defence, whereas aromatics or benzenoids seem to have evolved for pollinator attraction (Schiestl, 2010; Büchel et al., 2011). However, the evolution of floral scent has also been interpreted in the context of herbivore defence, since facultaltive and/or damaging flower visitors play also an important role for plant fitness (Pellmyr & Thien, 1986; Junker & Blüthgen, 2010).

Plant volatiles have been characterised predominantly for beneficial plants (e.g. agricultural crops, forestry trees and ornamental flowers) (Jürgens et al., 2003; Blackmer et al., 2004; Zhang & Schlyter, 2004), extraordinary plants (e.g. carnivorous plants, deceptive orchids) (Ayasse, 2006; Jürgens et al.,

2009), threatened plant species on conservationist red-data lists (Kamatou et al., 2010) and plant products (e.g. wine, honey, perfume) (Flath et al., 1983; Bianchi et al., 2005; Tao & Li, 2009). Plant-insect interaction studies where volatile cues play a role, are a natural follow-up on plant volatile identification studies and have focused on the interactions of beneficial insects (e.g. pollinators, natural enemies of herbivores) and non-beneficial insects (e.g. herbivores) on plants (see Knudsen et al., 2006; Junker & Blüthgen, 2010). Our knowledge of plant volatiles emitted by non-beneficial plants, e.g. invasive species or weeds, is still limited. However, especially in invasive plant species such information is important for a better understanding of insect responses to olfactory signals for establishing beneficial interactions with biological control insects in a new geographical and ecological context (Jordon-Thaden & Louda, 2003; Wheeler, 2006).

University of Cape Town

Acacia cyclops (rooikrans), *A. melanoxylon* (blackwood), *A. longifolia* (long-leafed wattle) and *A. saligna* (Port Jackson willow) are invasive alien plants of particular interest in South Africa with all of them subjected to biological control programs since as long ago as 1982 (Impson et al., 2011). Adult plants of most Australian acacias have phyllodes, which are flattened petioles which resemble and function as leaves and are dull-green to bright green in colour (Van Wyk & Van Wyk, 1997; Henderson, 2001; Coates Palgrave, 2005). All have small tubular flowers arranged in flower heads which are either spherical (*A. cyclops*, *A. melanoxylon* and *A. saligna*), or elongate (*A. longifolia*), with pollen presented on the inflorescence surface (Stone et al., 2003). These species do not produce floral nectar and pollinators are rewarded either with only pollen (*A. cyclops*) or pollen and nectar from extra-floral nectaries (*A. melanoxylon, A. longifolia* and *A. saligna*) (Milton & Moll, 1982; Stone et al., 2003). Their flowers are bright yellow, except for *A. melanoxylon* which has cream to pale-yellow flowers (Van Wyk & Van Wyk, 1997; Henderson, 2001).

The peak flowering period for *A. cyclops* is mid-summer, but flowering can extend from October to April, with occasional flower production at other times of the year (Henderson, 2001; Post et al.,

2010). Flowering in *A. melanoxylon, A. longifolia* and *A. saligna* is more discrete with flowering seasons during August-September, July-September and August-November respectively (Henderson, 2001). There is thus hardly any overlap in the flowering times of *A. cyclops* and the other three species.

Not much is known or confirmed about the pollinators of *A. cyclops*, *A. melanoxylon, A. longifolia* and *A. saligna*, but it is generally accepted that bees, flies, wasps and beetles are part of the generalist pollinator assemblage (Milton & Moll, 1982; Turnbull, 1997; Stone et al., 2003; Gibson et al., 2011). The floral morphology supports supposition that the species have a generalist pollination syndrome as the flowers readily provide access to the pollen (Gibson et al., 2011).

Since there has not been much investigation of the plant volatiles of invasive tree species, and *Acacia* trees in particular, the primary objective of this study was to identify the plant volatiles emitted from different plant parts, vegetative and floral, of *A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. saligna*. It is generally accepted that the current knowledge about plant volatiles and their functions is adequate to attempt predictions about the pollinators of plants given the compounds found (Dobson, 2006; Jürgens et al., 2006; Goodrich & Raguso, 2009). Therefore, the secondary objective is to evaluate the compound emission patterns observed and to predict the function of those compounds for plant defence or pollinator attraction, and to predict the pollinator assemblages of these plants.

University of Cape Town

2.2. Methods and Materials

2.2.1. Collection of volatile samples from different flowering stages and leaves

The aim was to describe and compare headspace volatiles from different species and flowering stages. However, the globular flower heads of *A. cyclops* are individually interspersed among the leaves and therefore in-situ headspace collection of *A. cyclops* flower volatiles only was impractical.

Headspace samples of this species inadvertently included scent from leaves and a whole range of flower stages, with the flower scent concentration after GC-MS analysis of the samples too weak to compare the scent compositions of each of the flower stages separately. Therefore, despite the risk of obtaining potentially high levels of green leaf volatiles due to tissue damage (Grison et al., 1999; Arimura et al., 2001), flowers, buds and leaves had to be cut off the plant and collected separately for the headspace volatile collection. In order to maintain full comparability of the results across all species, it was decided to treat the flowers, buds and leaves of *A. melanoxylon*, *A. longifolia* and *A. saligna* in the same manner despite the fact that their inflorescence architectures were different.

Plant cuttings comprising leaves, and buds and flowers of different stages were collected from 25 randomly selected trees of each of the four study species, *A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. saligna*. From *A. cyclops* leaves, yellow buds, open flowers and senescing (old, with styles wilted) flowers were collected on 21 January 2010, and green buds were collected on 26 March 2010 from trees at the Koeberg Nature Reserve (S 33° 39' 14.8", E 18° 26' 00.4") near Melkbosstrand in the Western Cape, South Africa. Material from *A. melanoxylon* was collected on 27 August 2009 in Houtbay, in the Western Cape, South Africa (S 34° 00' 40.4" E 18° 23' 18.1"). Material from *A. longifolia* was collected on 17 July 2009 along the M63 road between Constantia Neck and Houtbay, South Africa (S 34° 00' 41.9" E 18° 23' 45.6") and material from *A. saligna* trees was collected on 29 August 2009 alongside the M19 road between the N7 highway and Melkbosstrand in the Western Cape, South Africa (S 33° 43' 32.7" E 18° 32' 26.9"). The plant cuttings with the desired leaf and inflorescence material were transported to a laboratory where on arrival the required plant parts (leaves, yellow buds, open flowers and senescing flowers, and green buds in the case of *A. cyclops*) were carefully removed from the cuttings with a pair of forceps. For each plant part of each species five samples were created containing the particular plant part (leaves or the selected flower stages) from five individual trees. The leaves, buds and flowers removed from the plant cuttings were enclosed within a polyacetate oven bag (25 cm × 30 cm; Kalle Bratschlauch, Wiesbaden, Germany)

University of Cape Town

for dynamic headspace collection as described by Dötterl et al. (2005b). For *A. cyclops*, on average 110 green buds, 106 yellow buds, 144 open flowers and 144 senescing flowers were bagged per sample. For *A. melanoxylon*, on average 160 yellow buds, 168 open flowers and 117 senescing flowers; for *A. longifolia*, on average 175 yellow buds, 208 open flowers and 166 senescing flowers and for *A. saligna*, on average 100 yellow buds, 168 open flowers and 96 senescing flowers were collected per sample.

Volatiles were trapped in an adsorbent tube by using a membrane pump (Spectrex PAS-500, Redwood City, California, USA) with a flow rate of 100 ml min^{-1} for 30 min. Air was drawn from the bag through the adsorbent tube which was filled with a mixture of 1.5 mg Tenax-TA® (mesh 60-80, Supelco, Bellefonte, USA) and 1.5 mg Carbotrap® (mesh 20-40, Supelco, Bellefonte, USA). To distinguish between plant volatiles and ambient contaminants, after each type of sample (leaf and each of the selected flower stages), surrounding air was collected separately as control samples for comparison purposes. After taking the headspace samples the buds and flowers in the sample bags were counted.

University of Cape Town

2.2.2. Gas Chromatography-Mass Spectrometry (GC-MS) analyses

The scent samples were analysed using a Varian CP-3800 Gas Chromatograph (Varian, Palo Alto, California) with a 30 m x 0.25 mm internal diameter (film thickness 0.25 µm) Alltech EC-WAX column coupled to a Varian 1200 quadrupole mass spectrometer in electron-impact ionization mode at 70eV (Shuttleworth & Johnson, 2010a). Cartridges were placed in a Varian 1079 injector equipped with a 'Chromatoprobe' thermal desorption device (Amirav & Dagan, 1997). The flow of helium carrier gas was 1 ml min^{-1}. The injector was held at 40 °C for 2 min with a 20:1 split and then increased to 200 °C at 200 °C min^{-1} in split-less mode for thermal desorption. The temperature of the GC oven was held for 3 min at 40 °C, whereafter it was ramped up to 240 °C at 10 °C min^{-1} and held there for 12 min. Identification of compounds included the use of the Varian Workstation software with the NIST05

mass spectral library (NIST/EPA/NIH Mass Spectral Library; data version: NIST 05; MS search software version 2.0 d) as well as verification by using retention times of authentic standards and published Kovats indices (references in the NIST 05 library; El-Sayed, 2011) wherever possible. Compounds present at similar amounts in both the specimen samples and control samples were considered to be contaminants and excluded from analysis. The emission rates per inflorescence per hour for each of the floral sample types (bud or flower) were quantified by injecting known amounts of Methyl benzoate into thermo-desorption cartridges that were then thermally desorbed using the same methods that were applied to the biological samples.

2.2.3. Statistical analyses

Statistical analyses of identified volatiles employed the statistical package PRIMER v6 (Clarke & Warwick, 2001; Clarke & Gorley, 2006). Since the total amount of volatiles emitted varied greatly among individual samples, differences in scent composition of each sample were assessed using percentages of individual compounds in the bouquet (relative amounts derived from total peak areas). All compounds from the samples taken from leaves, yellow buds, and open and senescing flowers, and green buds in the case of *A. cyclops*, were identified. To arrive at the relative amounts of compounds for leaves, green and yellow buds, and open and senescing flowers, the average relative amounts per compound were calculated and then square-root transformed. The significance level of differences in scent profiles of different plant parts was assessed with an analysis of similarities (ANOSIM) (Clarke & Gorley, 2006) with 10,000 random permutations based on the pair-wise Bray Curtis similarities between the individual samples. The extent to which individual compounds contributed to the overall dissimilarity among the plant parts was subsequently assessed with the SIMPER procedure (factor: plant parts) (Clarke & Warwick, 2001). Non-metric multidimensional scaling (based on the Bray–Curtis similarities) was used in PRIMER v6 to ordinate the scent samples of the different plant parts in order to visualize similarities among the individual samples. To analyse whether the compounds in the scent profiles showed an even occurrence in the

University of Cape Town

different floral stages and leaves the evenness index (E = H'/ ln C; where H' is the Shannon index (Shannon & Weaver, 1949) and C is the total number of compounds) was calculated (Stiling, 1999). The evenness index is constrained between 0 and 1 (Stiling, 1999); the higher the index the more even the occurrence of the compounds (the less some compounds dominate) in the scent profile. To determine if there were significant differences in the mean rates of volatile emission from various flowers stages, one-way ANOVA, followed by the Tukey multiple range test, was used after the data were log-transformed to improve normality and homescedasticity of the data.

2.3. Results

2.3.1. Volatile profile of *Acacia cyclops*

General findings - The chemical composition of leaves and floral stages (green buds, yellow buds, open flowers, and senescing flowers) of *Acacia cyclops* is listed in Table 2.1 (including additions and corrections to information given in Kotze et al., 2010). In total 87 compounds were found. They belong to six different chemical classes, namely aliphatic compounds (39), monoterpenoids (23), benzenoids (9), irregular terpenes (8), sesquiterpenoids (6), and nitrogen containing compounds (2). Of the 87 compounds found in all sample types together, 49 were present in leaf samples, 29 compounds were emitted by samples from green buds, 50 compounds were detected in yellow buds, 53 compounds in open flowers and 52 in senescing flowers. Nearly a third of all compounds (26 out of 87) occurred in only one sample type. The relative amounts of each of these unique 26 compounds were however very low, often only detectable in traces: In leaves, green buds, yellow buds and open flowers the relative amounts were far below 2% per compound and in senescing flowers they never exceeded 5% (Table 2.1). Only thirteen compounds (17% of the total number of compounds) were common across all five sample types. The total relative amount of these compounds was typically high, adding up to 88.3% in leaves, 94.2% in green buds, 74.9% in yellow buds, 74.2% in open flowers and 67.5% in senescing flowers. However, only few of these common compounds dominated the scent profile of each particular sample type. Considering only common

University of Cape Town

compounds that reached on average more than 5% in any sample type, (*Z*)-*β*-Ocimene dominated especially in green sample types (leaves 69.7%, green buds 44.9%), followed by yellow buds and flowers, whereas (*Z*)-3-Hexenyl acetate, was most dominant in all bud and flower stages (green buds 45%, followed by yellow buds and flowers, and finally leaves). In yellow buds and open flowers, 4-Oxoisophorone contributed about 20% and 23% respectively to the scent profile accompanied by an unidentified compound (KRI = 1693; henceforth called UC1693) (11.1%) in open flowers and Heptadecane (12.3%) in yellow buds. The relative amounts of the other common compounds was typically below 5%; often they were found only in traces. Only two other compounds were not found in all sample types, but nevertheless exceeded 5% in some: Nonadecane with 5.5% in open flowers and Hexan-1-ol with 5.7% in senescing flowers.

Comparison of volatile profiles of different sample types - The leaf and green bud scent profiles were dominated by two main compounds, (*Z*)-*β*-Ocimene (69.7% and 44.9% respectively) and (*Z*)-3-Hexenyl acetate (9.2% and 45% respectively). The dominance of these two compounds was further demonstrated by the Shannon evenness index, being 0.38 and 0.36 for leaves and green buds respectively. The scent bouquets of reproductive units close to maturity (yellow buds and open flowers) are richer in scent compounds, containing again (*Z*)-*β*-Ocimene (18.9% and 10.3% respectively) and (*Z*)-3-Hexenyl acetate (21% and 23.5% respectively), accompanied by 4-Oxoisophorone (19.5% and 23.2% respectively) and UC1693 (6.2% and 11.1% respectively), Heptadecane (12.3% and 8% respectively) and Nonadecane (2.9% and 5.5% respectively). Yellow buds and open flowers contained similar volatile profiles as can be inferred by the percentages. Qualitatively the scent of yellow buds to open flowers differed by only 1.4% (10 compounds unique to yellow buds) and of open flowers to yellow buds the difference was only 1.3% (13 compounds unique to open flowers). Accordingly, the Shannon evenness indices for the yellow bud and open flower profiles were both 0.61. The scent of senescing flowers was less strongly dominated by (*Z*)-*β*-Ocimene (17.7%) and (*Z*)-3-Hexenyl acetate (22.1%), and contained in all samples also Hexan-1-ol in

University of Cape Town

relatively high amounts (5.7%) and Nonadecane (1.5%) in two of the five samples. Furthermore, many compounds unique to senescing flowers were found in low relative amounts only. Consequently, the evenness of the compound occurrence in the senescing flowers chemical profile was relatively high with 0.75.

Table 2.1. Mean relative amounts (%) of compounds identified by GC-MS from headspace samples of leaves and different flower stages (green buds, yellow buds, open flowers, senescing flowers) of *Acacia cyclops*. The number of samples in which a compound occurred is given in brackets. Scent compounds are listed according to compound class and Kovats retention index (KRI). CAS = Chemical Abstracts Service Registry Number. tr = trace amount (<0.1 % of total sample). Compound identification criteria: a = comparison of MS with published data (e.g. NIST library); b = comparison of MS and retention time with published data; c = comparison of MS and retention time with published data and authentic standard. Unknowns that did not reach at least 1% of relative amount in any sample were pooled with the superscript digit indicating the number of pooled compounds. Mass fragments for unknowns are listed with the base peak first, followed by the other fragments in order of decreasing abundance.

Compound	KRI	CAS	Leaves	Green buds	Yellow buds	Open flowers	Sene-scing flowers
Number of samples			5	5	5	5	5
Number of plants used per sample			5	5	5	5	5
Mean scent emission per inflorescence (ng h^{-1})			-	38.55	48.24	31.34	7.17
Number of compounds out of 87			49	29	50	53	52
Evenness index			0.61	0.71	0.65	0.48	0.61
Aliphatic compounds							
Aliphatic aldehydes							
(*E*)-2-Hexenal[b]	1242	6728-26-3	-	0.8 (3)	tr	0.4 (2)	1.0 (3)
(*E,Z*)-2,6-Nonadienal[b]	1608	557-48-2	-	-	tr	-	0.7 (3)
Aliphatic esters							
Hexyl acetate[b]	1288	142-92-7	-	1.1 (2)	2.0 (5)	3.0 (5)	4.2 (5)
Methyl (*E*)-2-hexenoate[b]	1308	13894-63-8	0.4 (5)	-	-	-	-
(*Z*)-3-Hexenyl acetate[b]	1333	3681-71-8	9.2 (5)	45.0 (5)	21.0 (5)	23.5 (5)	22.1 (5)
(*E*)-2-Hexenyl acetate[b]	1348	2497-18-9	0.2 (1)	0.6 (2)	tr	-	-
(*E*)-3-Hexenyl butyrate[b]	1474	53398-84-8	0.1 (5)	-	-	-	-
(*Z*)-3-Hexenyl isovalerate[b]	1480	35154-45-1	-	tr	-	tr	-
(*Z*)-3-Hexenyl hexanoate[b]	1704	31501-11-8	tr	-	-	-	-
Unidentified aliphatic esters			tr[1]	0.4[1]	0.1[1]	-	-
Aliphatic alcohols							
Hexan-1-ol[c]	1352	111-27-3	-	-	0.2 (2)	0.3 (2)	5.7 (5)
(*Z*)-3-Hexen-1-ol[b]	1387	928-96-1	1.5 (4)	2.1 (5)	1.1 (3)	0.6 (2)	2.5 (3)
(*E*)-2-Hexen-1-ol[b]	1388	928-95-0	-	-	0.3 (1)	0.1 (1)	1.2 (1)

University of Cape Town

Compound	KRI	CAS	Leaves	Green buds	Yellow buds	Open flowers	Sene-scing flowers
(*Z*)-2-Hexen-1-ol[b]	1420	928-94-9	tr	0.6 (5)	-	-	-
4-Methyl-1-heptanol[a]	1529	817-91-4	-	-	-	-	0.2 (1)
6-Methyl-1-heptanol[a]	1531	1653-40-3	-	-	-	-	0.8 (3)
2,3-Butanediol[b]	1580	513-85-9	-	-	-	-	0.2 (1)
Aliphatic alkanes							
Undecane[c]	1100	1120-21-4	-	-	tr	0.9 (4)	-
Dodecane[c]	1200	112-40-3	-	-	0.2 (2)	-	0.3 (1)
Tridecane[c]	1300	629-50-5	0.4 (4)	-	0.1 (2)	tr	0.7 (4)
Tetradecane[c]	1400	629-59-4	0.2 (1)	-	0.4 (1)	tr	0.6 (1)
Pentadecane[c]	1500	629-62-9	-	-	2.1 (2)	1.1 (2)	-
Hexadecane[c]	1600	544-76-3	0.3 (4)	-	0.5 (2)	0.3 (2)	0.4 (2)
Heptadecane[c]	1700	629-78-7	0.4 (5)	-	12.3 (5)	8.0 (3)	2.5 (4)
Octadecane[c]	1800	593-45-3	-	-	0.2 (4)	0.2 (4)	tr
Nonadecane[c]	1900	629-92-5	0.2 (1)	-	2.9 (3)	5.5 (4)	1.5 (2)
Eicosane[c]	2000	112-95-8	-	-	tr	tr	1.0 (1)
Heneicosane[c]	2100	629-94-7	-	-	tr	0.3 (5)	0.3 (3)
Docosane[c]	2200	629-97-0	-	-	-	-	0.3 (1)
Tricosane[c]	2300	638-67-5	-	-	tr	0.2 (5)	0.6 (5)
Tetracosane[c]	2400	646-31-1	-	-	-	-	0.4 (1)
Pentacosane[c]	2500	629-99-2	-	-	-	tr	tr
Heptacosane[c]	2700	593-49-7	tr	-	-	tr	-
Nonacosane[c]	2900	630-03-5	0.4 (3)	-	tr	0.2 (4)	-
Aliphatic ketones							
5-Methyl-5-vinyldihydro-2(3H)-furanone[b]	1683	1073-11-6	-	tr	-	0.2 (2)	0.4 (4)
Benzenoids							
Benzaldehyde[c]	1551	100-52-7	0.4 (5)	0.6 (5)	0.7 (5)	0.5 (5)	1.9 (5)
Methyl benzoate[c]	1650	93-58-3	0.2 (5)	0.4 (5)	0.2 (5)	-	-
Benzyl acetate[b]	1755	140-11-4	-	0.1 (5)	tr	-	-
Methyl salicylate[c]	1806	119-36-8	2.6 (5)	0.7 (5)	0.5 (4)	-	0.2 (3)
Benzyl alcohol[c]	1902	100-51-6	0.4 (4)	0.4 (5)	0.7 (2)	0.2 (1)	4.0 (4)
2-Phenylethyl alcohol[c]	1938	60-12-8	0.1 (5)	0.2 (5)	0.2 (5)	tr	0.6 (5)
Dimethyl salicylate[b]	2093	606-45-1	tr	-	tr	tr	tr
Benzyl tiglate[a]	2139	37526-88-8	-	-	tr	tr	0.4 (4)
Cinnamyl alcohol[b]	2300	104-54-1	-	-	tr	-	-
Unidentified benzonoid			-	-	-	tr[1]	-
Monoterpenoids							
α-Pinene[c]	1087	80-56-8	-	-	-	-	2.9 (2)
3-Carene[b]	1122	13466-78-9	0.8 (2)	-	-	-	-
β-Pinene[c]	1137	127-91-3	0.1 (4)	-	-	-	-
Limonene[c]	1225	138-86-3	1.2 (5)	0.1 (4)	1.8 (5)	1.6 (5)	3.1 (5)
(*Z*)-*β*-Ocimene[b]	1252	3338-55-4	69.7 (5)	44.9 (5)	18.9 (5)	10.3 (5)	17.7 (5)

University of Cape Town

Compound	KRI	CAS	Leaves	Green buds	Yellow buds	Open flowers	Sene-scing flowers
(*E*)-*β*-Ocimene[b]	1253	3779-61-1	0.1 (2)	-	-	-	-
2,6-Dimethyl-6-octanol[a]	1437	78-69-3	tr	tr	-	-	-
(*E*)-Linalool oxide (furanoid)[c]	1454	34995-77-2	0.8 (5)	-	0.6 (5)	0.7 (5)	2.5 (5)
(*E,E*)-2,6-Dimethyl-1,3,5,7-octatetraene[a]	1466	460-01-5	1.3 (5)	tr	-	tr	-
2,6-Dimethyl-7-octen-2-ol[a]	1474	18479-58-8	-	-	-	tr	-
(*Z*)-Linalool oxide (furanoid)[c]	1486	5989-33-3	-	-	0.2 (3)	tr	-
2,7-Dimethyl-2,6-octadien-1-ol[a]	1522	16736-42-8	-	-	-	tr	-
β-Linalool[c]	1556	78-70-6	0.9 (5)	0.1 (5)	0.4 (4)	0.3 (5)	1.1 (5)
β-Citronellal[b]	1609	432-25-7	-	-	-	-	0.1 (1)
Isobornyl acetate[a]	1610	125-12-2	tr	-	tr	0.1 (5)	tr
1-Menthol[b]	1651	2216-51-5	-	-	-	tr	-
α-Terpineol[b]	1708	98-55-5	0.1 (4)	tr	-	0.2 (5)	0.3 (4)
(*Z*)-Linalool oxide (pyranoid)[a]	1720	14049-11-7	0.4 (5)	-	0.4 (5)	0.3 (5)	1.0 (4)
Borneol[b]	1725	507-70-0	tr	tr	-	-	-
6,6-Dimethyl-2-methylene-Bicyclo[3.1.1]heptan-3-ol[a]	1731	5947-36-4	tr	-	-	-	-
Verbenone[a]	1740	18309-32-5	tr	-	-	tr	0.2 (5)
(*Z,Z*)-2,6-Dimethyl-3,5,7-octatriene-2-ol[a]	1824	1174030-42-2	tr	-	-	-	tr
(*E,E*)-2,6-Dimethyl-3,5,7-octatriene-2-ol[a]	1836	206115-88-0	0.8 (5)	tr	0.2 (4)	tr	-
Unidentified monoterpenes			0.4[2]	-	-	-	tr[1]
Sesquiterpenoids							
α-Bulnesene[a]	1599	3691-11-0	-	-	-	0.3 (3)	-
Longifolene[a]	1600	475-20-7	tr	-	0.2 (3)	-	0.7 (3)
β-Caryophyllene[c]	1625	87-44-5	0.4 (5)	0.2 (5)	0.4 (5)	0.8 (5)	3.6 (5)
α-Farnesene[b]	1765	502-61-4	2.3 (5)	tr	0.7 (5)	0.5 (5)	2.3 (4)
Caryophyllene oxide[b]	2021	1139-30-6	0.1 (1)	-	tr	0.2 (5)	0.3 (4)
Unidentified sesquiterpene			-	-	-	0.3[1]	0.3[1]
Irregular terpenes							
6-Methyl-5-hepten-2-one[b]	1354	110-93-0	2 (5)	0.6 (5)	3.4 (5)	1.6 (3)	4.2 (4)
Unidentified irregular terpene m/z: 138,96,68,67,95,39,41,42,40,69[a]	1537		-	-	0.3 (1)	1.4 (4)	-
4-Methoxy-2,2,6-trimethyl-cyclohexanone[a]	1630	17429-03-7	-	-	tr	0.2 (4)	-
4-Oxoisophorone[b]	1722	1125-21-9	0.2 (4)	tr	19.5 (5)	23.2 (5)	2.8 (5)
2,2,6-Trimethyl-1,4-cyclohexanedione[a]	1808	20547-99-3	-	-	0.4 (5)	0.7 (5)	tr
Nitrogen containing compounds							
Unidentified nitrogen containing compound m/z: 73,56,59,41,86,55,39,69,72,57[a]	1504		0.3 (5)	0.4 (1)	-	-	-
Indole[c]	2478	120-72-9	0.5 (5)	-	tr	-	tr

University of Cape Town

Compound	KRI	CAS	Leaves	Green buds	Yellow buds	Open flowers	Sene-scing flowers
Unidentified compound							
m/z: 56,85,125,43,41,69,153,55, 83,39[a] (=UC1693)	1693		0.1 (4)	tr	6.2 (5)	11.1 (5)	1.4 (5)

Patterns of variation of volatile compounds - There were distinct patterns in the relative amounts of the seven prominent compounds (relative amounts > 5%) already highlighted across the flowering stages of the inflorescences and leaves (Fig. 2.1).

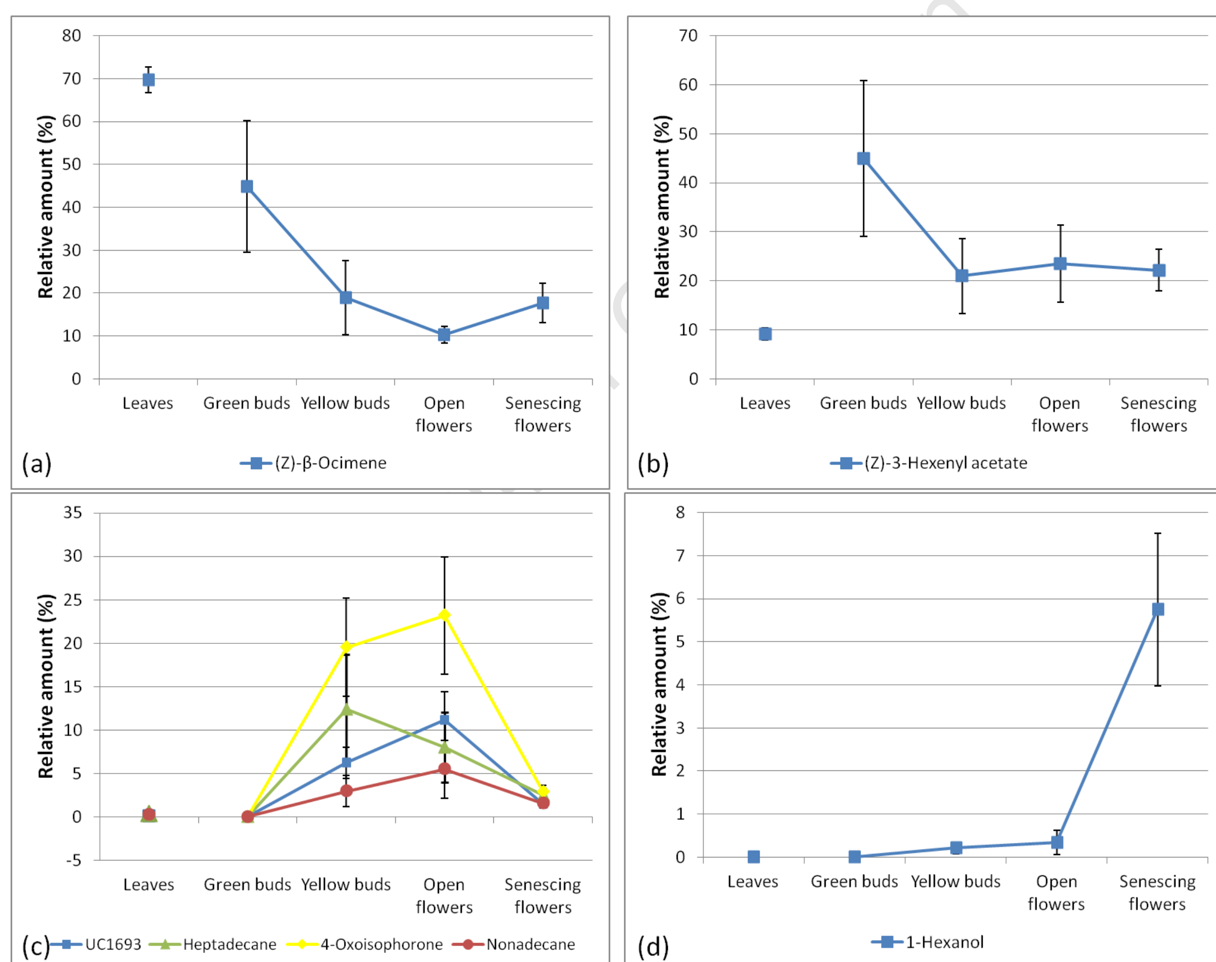


Fig. 2.1. Patterns of relative amounts (mean ± SE) of seven volatile compounds found in leaves and floral stages of *A. cyclops* inflorescences.

Comparing all sample types, the relative amount of (*Z*)-3-Hexenyl acetate was lowest in leaves (9.2%) and highest in green buds (45%). During bud and flower development its relative amount

decreased to 21-23.5%, though it was still dominant in the open flower scent (Fig. 2.1a). (*Z*)-*β*-Ocimene was present in the highest relative amount in the leaves (69.7%), and was less dominant in the green bud stage. All later floral stages had considerably lower relative amounts ranging from 10.3-18.9% (Fig. 2.1a). Relative amounts of both 4-Oxoisophorone and UC1693 were close to trace amounts in leaves and green buds and highest in open flowers (23.2% for 4-Oxoisiphorone and 11.1% for UC1693) followed by yellow buds (Fig. 2.1b). Both Heptadecane and Nonadecane were present in trace amounts in leaves, absent in green buds, but peaked in more-mature flower stages (maxima 12.3% Heptadecane in yellow buds and 5.5% Nonadecane in open flowers) (Fig. 2.1b). Contrary to the other dominant compounds described, Hexan-1-ol was much less pronounced and reached its maximum proportion (5.7%) only in senescing flowers (Fig. 2.1c).

of Cape Town

Compound classes - There were pertinent differences in the proportion of compound classes that made up the volatile profiles of the different samples (Fig. 2.2).

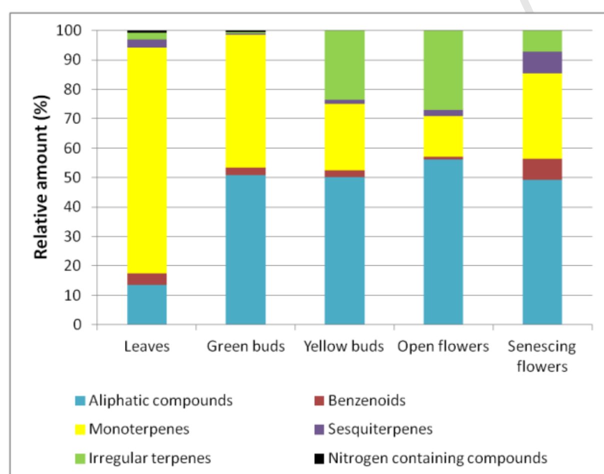


Fig. 2.2. Mean proportions of compound classes in headspace samples of leaves and different floral stages of *A. cyclops*.

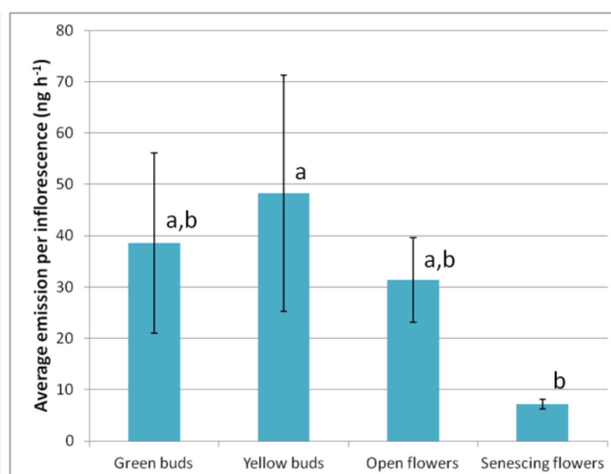


Fig. 2.3. Volatile emission rates (ng h^{-1}) per inflorescence (mean ± SE) of different floral stages of *A. cyclops*. Different letters indicate significant differences at $p < 0.05$ (Tukey HSD, $F_{(3,16)} = 3.860$; $p = 0.03$; $n = 5$).

All floral stages emitted a relatively-constant, high proportion of about 50% of aliphatic compounds (range: 49.3% - 56.2%) compared to leaves (<15%). In contrast monoterpenes dominated in the leaf

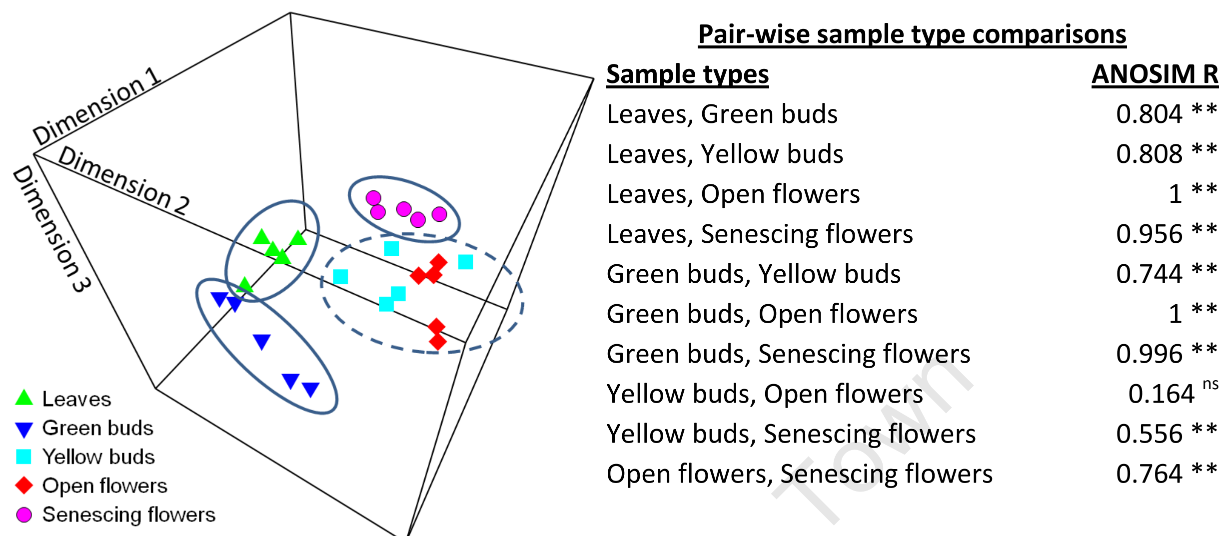
volatile profile (77%). Through floral development, the relative amount of monoterpene decreased from ca. 45% in green floral buds to ca. 14% in open flowers, while the proportion of irregular terpenes was higher in the scent profiles of yellow buds and open flowers. The proportions of benzenoid and sesquiterpene volatiles were similarly low in leaves and all floral stages (respective ranges: 0.9% - 3.8%; and 0.3% - 2.8%), but increased to 7.1% and 7.3% respectively in the senescing flowers' profile. Closer inspection revealed that these compound class differences were mainly due to changes in single dominating compounds (Table 2.1): (1) Leaves were distinct from reproductive plant parts, especially open flowers, by their high relative amount of (*Z*)-*β*-Ocimene; (2) with respect to reproductive units, (*Z*)-3-Hexenyl acetate had its maximum relative amount in the green buds samples and was much lower in the following floral stages; (3) in contrast, 4-Oxoisophorone peaked in yellow buds and open flowers with little of it found in younger or older stages, and UC1693 and Hepta- and Nonadecane exhibited a similar trend albeit on a much lower level, with UC1693 and Nonadecane peaking in open flowers, and Heptadecane in mature yellow buds.

Total volatile emission - The senescing flower stage was characterised by low volatile emissions (mean 7 ng h^{-1} per inflorescence) (Table 2.1; Fig.2.4). This contrasted with the >30 ng h^{-1} per flower of the buds and open flower stages. The yellow buds and senescing flower stages differed significantly in emission rates (Fig. 2.3).

Statistical comparison of volatile profiles of different sample types - The overall separation of the plant volatile profiles, based on Bray-Curtis similarities of the leaves and different floral stages was highly significant (3D-NMDS stress value = 0.06; ANOSIM R = 0.788, $p < 0.001$) (Fig. 2.4). Pair-wise comparison revealed highly significant differences between leaves and the four floral stages (range of R-values: 0.804 - 1), between green buds and the three yellow floral stages (range of R-values: 0.744 - 1) and between open flowers and senescing flowers (R-value = 0.764). The pair-wise comparison however revealed no differences between yellow buds and open flowers (R-value =

University of Cape Town

0.164; p = 0.143), but a significant difference (p < 0.01), between yellow buds and senescing flowers (R-value = 0.556).



Pair-wise sample type comparisons

Sample types	ANOSIM R
Leaves, Green buds	0.804 **
Leaves, Yellow buds	0.808 **
Leaves, Open flowers	1 **
Leaves, Senescing flowers	0.956 **
Green buds, Yellow buds	0.744 **
Green buds, Open flowers	1 **
Green buds, Senescing flowers	0.996 **
Yellow buds, Open flowers	0.164 ^{ns}
Yellow buds, Senescing flowers	0.556 **
Open flowers, Senescing flowers	0.764 **

Fig. 2.4. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarities of the odour composition (87 compounds) of the leaves and different floral stages of *A. cyclops*. 3D stress value = 0.06. ANOSIM Global R = 0.791, p < 0.001. Pair-wise sample type comparisons shown with ** indicate significance at p < 0.01; ^{ns} = not significant at p = 0.143.

The dissimilarity indices calculated by the SIMPER method (Table 2.2) revealed dissimilarity between the green stages (leaves and green buds) of 47.6 (meaning greater similarity than dissimilarity). The main compounds contributing to this dissimilarity were (*Z*)-3-Hexenyl acetate (12.6%) and (*Z*)-*β*-Ocimene (9.8%). The chemical profile of open flowers showed a higher dissimilarity to the green stage profiles, leaves (60.5) and green buds (64.3), than to the yellow stages, yellow buds (38.0) and senescing flowers (48.3). Dissimilarity indices for yellow buds and senescing flowers displayed the same trend (Table 2.2). The dissimilarities between the yellow floral stages (yellow buds, open flowers and senescing flowers) and green buds (range: 58.0 - 64.3) are higher than between the yellow floral stages and the leaves (range: 52.8 - 60.5). The already-described similarity in the profiles of yellow buds and open flowers is confirmed by a low dissimilarity index of 38.0 (or similarity index of 62.0). Quantitative differences of Heptadecane, (*Z*)-3-Hexenyl acetate, (*Z*)-*β*-Ocimene, 4-Oxoisophorone, Nonadecane and UC1693 are contributing most to the dissimilarity.

University of Cape Town

Table 2.2. Mean dissimilarity indices based on SIMPER procedure (factor: plant parts) (Clarke & Warwick, 2001) between chemical profiles of leaves and different floral stages of *A. cyclops*.

Plant part	Leaves	Green buds	Yellow buds	Open flowers	Senescing flowers
Leaves	-				
Green buds	47.6	-			
Yellow buds	52.8	58.0	-		
Open flowers	60.5	64.3	38.0	-	
Senescing flowers	56.1	60.9	46.7	48.3	-

2.3.2. Volatile profile of *Acacia melanoxylon*

General findings - The chemical composition of leaves and of floral stages (yellow buds, open flowers, and senescing flowers) of *Acacia melanoxylon* is summarised in Table 2.3. In total 75 compounds were found belonging to six different chemical classes, namely aliphatic compounds (29), monoterpenoids (25), benzenoids (9), sesquiterpenoids (8), irregular terpenes (3), and a nitrogen containing compound (1). Of the 75 compounds found in all samples, only 30 were present in leaf samples, whereas flower stages emitted many more compounds: 48 were detected in yellow bud samples, 43 in senescing flowers, but a maximum of 66 was found in samples from open flowers. Nearly 30% of all compounds (21 of the 75) were unique for a certain sample type. The total relative amount of the unique compounds however was generally low. In leaves and senescing flowers it never exceeded 0.1%, whereas in yellow buds and open flowers it reached 2.3% and 3.9% respectively (Table 2.3).

University of Cape Town

A total of 22 compounds (=29.3%) were common across all four sample types. The total relative amount of these compounds was typically high, adding up to 96.3% in leaves, 75.6% in yellow buds, 65.2% in open flowers and 79% in senescing flowers. However, only eight of these compounds made up more than 5% of the relative amount in any sample type and only some of these clearly dominated a particular sample type. (*Z*)-*β*-Ocimene was found in high relative amounts (c. 30%) in all four sample types. Limonene was most dominant in leaves (21.5%) and senescing flowers (15.2%),

whereas Ethyl acetate was found in similarly high amounts only in leaves (19.6%). Highest relative amounts of (*Z*)-3-Hexenyl acetate (10.2%) and *β*-Linalool (7.2%) were found in yellow buds, while Benzaldehyde was most prominent in open flowers (6.5%). In senescing flowers, *β*-Caryophyllene (6.7%) and (*Z*)-3-Hexen-1-ol (5.7%) were detected in the highest relative amounts. The summated relative amounts of the other 14 compounds ranged from 15.3% to 26.7%. From the compounds that were not common to all sample types only three exceeded 5% in a particular sample type: *p*-Anisaldehyde and Nonadecane were prominent in open flowers with 9.5 and 5.7% respectively, while *β*-Pinene was present with up to 5% in buds and flowers. Altogether, there was a relatively high level of evenness of compound distribution in the chemical bouquets of all four sample types, as indicated by the Shannon evenness indices ranging from 0.67 in leaves to 0.72 in yellow buds (Table 2.3).

University of Cape Town

Comparison of volatile profiles of different sample types – The leave profiles were clearly dominated by (*Z*)-*β*-Ocimene (28.4%), Limonene (21.5%) and Ethyl acetate (19.6%). In yellow buds the common (*Z*)-*β*-Ocimene (29.8%) was mainly accompanied by (*Z*)-3-Hexenyl acetate (10.2%) followed by *β*-Linalool (7.6%), Limonene (6.9%), Nonadecane (5.7%), and Ethyl acetate (5.5%). Open flowers showed, apart from the usual proportion of (*Z*)-*β*-Ocimene (28.5%), a combination of *p*-Anisaldehyde (9.5%), Limonene (6.8%), Benzaldehyde (6.5%), *β*- Linalool and Nonadecane (each 5.7%). Senescing flowers emitted also mostly (*Z*)-*β*-Ocimene (30.4%), accompanied by high proportions of Limonene (15.2%), *β*-Caryophyllene, *β*-Linalool (5.8%), (*Z*)-3-Hexen-1-ol (5.7%), and *β*-Pinene (5%).

Table 2.3. Mean relative amounts (%) of compounds identified by GC-MS from headspace samples of leaves and different flower stages (green buds, yellow buds, open flowers, senescing flowers) of *Acacia melanoxylon.* The number of samples in which a compound occurred is given in brackets. Scent compounds are listed according to compound class and Kovats retention index (KRI). CAS = Chemical Abstracts Service Registry Number. tr = trace amount (<0.1 % of total sample). Compound identification criteria: a = comparison of MS with published data (e.g. NIST library); b = comparison of MS and retention time with published data; c = comparison of MS and retention time with published data and authentic standard. Unknowns that did not reach at least 1% of relative amount in any sample were pooled with the superscript digit indicating the number of pooled compounds. Mass fragments for unknowns are listed with the base peak first, followed by the other fragments in order of decreasing abundance.

Compound	KRI	CAS	Leaves	Yellow buds	Open flowers	Senescing flowers
Number of samples			5	5	5	5
Number of plants used per sample			5	5	5	5
Mean scent emission per inflorescence (ng h^{-1})			-	20.40	16.70	10.31
Number of compounds out of 75			30	48	66	43
Evenness index			0.67	0.72	0.71	0.70
Aliphatic compounds						
Aliphatic aldehydes						
(*E*)-2-Hexenal[b]	1242	6728-26-3	-	-	0.4 (1)	0.6 (4)
Aliphatic esters						
Ethyl acetate[a]	882	141-78-6	19.6 (3)	5.5 (1)	0.5 (1)	2.5 (1)
Isobutyl acetate[a]	975	110-19-0	1.2 (1)	2.2 (2)	-	-
(*Z*)-3-Hexenyl acetate[b]	1333	3681-71-8	0.3 (3)	10.2 (5)	1.7 (5)	2.4 (5)
Dimethyl pentanedioate[b]	1690	1119-40-0	tr	0.1 (1)	-	-
Unidentified aliphatic ester[a]			-	-	0.1[1]	-
Aliphatic alcohols						
Butan-1-ol[b]	1160	71-36-3	-	-	1.3 (1)	-
Hexan-1-ol[c]	1352	111-27-3	-	2.6 (5)	0.8 (3)	3.6 (5)
(*Z*)-3-Hexen-1-ol[b]	1387	928-96-1	3.6 (2)	3.7 (5)	1.9 (4)	5.7 (5)
3-Methoxy-3-methylbutanol[c]	1453	56539-66-3	-	-	0.7 (1)	0.2 (1)
Aliphatic alkanes						
Undecane[c]	1100	1120-21-4	-	1.8 (1)	-	-
Dodecane[c]	1200	112-40-3	-	-	0.7 (2)	0.8 (2)
Tridecane[c]	1300	629-50-5	-	1.9 (3)	0.2 (1)	0.7 (2)
Tetradecane[c]	1400	629-59-4	-	0.4 (1)	-	-
Hexadecane[c]	1600	544-76-3	-	-	0.2 (1)	-
Heptadecane[c]	1700	629-78-7	0.7 (2)	0.7 (1)	3.1 (5)	0.8 (3)
Octadecane[c]	1800	593-45-3	-	tr	0.3 (5)	-
Nonadecane[c]	1900	629-92-5	-	0.4 (1)	5.7 (4)	-
Eicosane[c]	2000	112-95-8	-	-	0.3 (5)	-
Heneicosane[c]	2100	629-94-7	-	tr	1.0 (4)	tr
Tricosane[c]	2300	638-67-5	0.6 (4)	0.2 (5)	1.1 (5)	0.2 (5)

University of Cape Town

Compound	KRI	CAS	Leaves	Yellow buds	Open flowers	Senescing flowers
Tetracosane[c]	2400	646-31-1	1.6 (4)	0.4 (4)	0.4 (5)	0.3 (3)
Pentacosane[c]	2500	629-99-2	1.9 (4)	0.4 (5)	0.6 (5)	0.6 (5)
Hexacosane[c]	2600	630-01-3	1.9 (5)	0.3 (4)	0.2 (5)	0.5 (4)
Heptacosane[c]	2700	593-49-7	-	0.3 (5)	0.5 (5)	0.4 (5)
Octacosane[c]	2800	630-02-4	1.4 (5)	0.2 (4)	0.2 (5)	0.3 (4)
Nonacosane[c]	2900	630-03-5	1.0 (5)	0.2 (5)	0.5 (5)	0.3 (5)
Aliphatic ketones						
5-Methyl-5-vinyldihydro-2(3H)-furanone[b]	1683	1073-11-6	0.4 (2)	0.1 (2)	0.3 (2)	0.1 (1)
Benzonoids						
Benzaldehyde[c]	1551	100-52-7	2.2 (5)	2.4 (5)	6.5 (5)	3.4 (5)
Methyl benzoate[c]	1650	93-58-3	-	-	0.1 (4)	0.3 (5)
Benzyl acetate[b]	1755	140-11-4	-	0.2 (5)	tr	tr
Benzyl alcohol[c]	1902	100-51-6	1.6 (5)	1.1 (5)	0.9 (1)	1.7 (5)
2-Phenylethyl alcohol[c]	1938	60-12-8	0.5 (5)	1.6 (5)	1.9 (5)	0.8 (5)
p-Anisaldehyde[a]	2015	123-11-5	-	2.3 (5)	9.5 (5)	0.7 (5)
Benzenepropanol[a]	2045	122-97-4	-	-	tr	-
Eugenol[b]	2197	97-53-0	-	-	tr	-
Unidentified benzonoid[a]			-	0.2[1]	-	-
Monoterpenoids						
α-Pinene[c]	1087	80-56-8	-	-	1.7 (3)	3.5 (4)
β-Pinene[c]	1137	127-91-3	-	4.3 (4)	2.5 (5)	5.0 (4)
Limonene[c]	1225	138-86-3	21.5 (5)	6.9 (4)	6.8 (5)	15.2 (5)
β-Phellandrene[a]	1227	555-10-2	-	0.6 (4)	tr	-
(*Z*)-β-Ocimene[b]	1252	3338-55-4	28.4 (5)	29.8 (5)	28.5 (5)	30.4 (5)
1,3,8-p-Menthatriene[b]	1385	21195-59-5	-	-	tr	-
(*E*)-Linalool oxide (furanoid) [c]	1454	34995-77-2	-	0.7 (4)	0.7 (5)	1.3 (5)
(E,E)-2,6-Dimethyl-1,3,5,7-octatetraene[a]	1466	460-01-5	-	0.4 (4)	0.8 (2)	-
(*Z*)-Linalool oxide (furanoid)[c]	1486	5989-33-3	-	2.9 (5)	1.6 (5)	2.6 (5)
Camphor[c]	1545	76-22-2	-	-	tr	-
β-Linalool[c]	1556	78-70-6	3.7 (5)	7.6 (5)	5.7 (5)	5.8 (5)
(*Z*)-β-Terpineol[c]	1564	7299-41-4	-	-	tr	-
6,6-Dimethyl-2-methylene-bicyclo[2.2.1]heptan-3-one[c]	1599	16812-40-1	-	-	0.3 (2)	-
Isobornyl acetate[a]	1610	125-12-2	0.4 (5)	-	0.1 (4)	-
Myrtenal[a]	1642	564-94-3	-	-	0.1 (5)	-
1-Menthol[b]	1651	2216-51-5	-	0.1 (5)	tr	-
α-Terpineol[b]	1708	98-55-5	0.8 (5)	0.3 (5)	0.2 (5)	0.4 (5)
α-Terpineol acetate[c]	1716	80-26-2	tr	-	-	tr
(Z)-Linalool oxide (pyranoid)[a]	1720	14049-11-7	-	-	tr	0.2 (4)
Borneol[b]	1725	507-70-0	0.3 (4)	0.1 (3)	-	tr
Verbenone[a]	1740	18309-32-5	-	-	tr	-

University of Cape Town

Compound	KRI	CAS	Leaves	Yellow buds	Open flowers	Senescing flowers
(*Z,Z*)-2,6-Dimethyl-3,5,7-octatriene-2-ol[a]	1824	1174030-42-2	-	-	0.1 (2)	-
(*E,E*)-2,6-Dimethyl-3,5,7-octatriene-2-ol[a]	1836	206115-88-0	0.4 (4)	0.4 (4)	1.0 (5)	0.5 (5)
2,6-Dimethyl-3,7-Octadiene-2,6-diol[b]	1945	13741-21-4	-	-	tr	-
Unidentified monoterpenes[a]			tr[1]	-	0.9[1]	-
Sesquiterpenoids						
α-Humulene[c]	1697	6753-98-6	-	0.2 (4)	-	0.2 (2)
α-Bulnesene[a]	1599	3691-11-0	1.2 (5)	0.8 (4)	0.2 (1)	-
Longifolene[a]	1600	475-20-7	-	-	0.2 (1)	-
β-Caryophyllene[c]	1625	87-44-5	1.8 (5)	3.1 (5)	2.7 (5)	6.7 (5)
α-Farnesene[b]	1765	502-61-4	1.6 (4)	0.2 (4)	0.3 (5)	0.3 (2)
Caryophyllene oxide[b]	2021	1139-30-6	-	0.3 (5)	0.4 (3)	0.3 (4)
Unidentified sesquiterpene[a]			-	-	0.2[1]	-
Irregular terpenes						
2,6-Dimethyl-6-octanol[a]	1437	78-69-3	0.5 (5)	0.2 (3)	0.1 (4)	-
2,6-Dimethyl-7-octen-2-ol[a]	1474	18479-58-8	0.6 (5)	0.3 (5)	0.2 (5)	0.2 (5)
4-Oxoisophorone[b]	1722	1125-21-9	-	1.1 (4)	1.3 (5)	0.1 (2)
Nitrogen containing compounds						
Indole[c]	2478	120-72-9	-	-	0.1 (4)	0.1 (4)
Unidentified compound						
m/z: 56,85,125,43,41,69,153,55, 83,39[a] (=UC1693)	1693		-	0.5 (5)	1.1 (5)	-

Patterns of variation of volatile compounds - Patterns emerged when looking at the eleven compounds that exceeded relative amounts of 5% in the volatile profiles of of *A. melanoxylon* (Fig. 2.5). Ethyl acetate (19.6%) and Limonene (21.5%) showed the highest relative amount in the leaf volatile profile, both being clearly higher than in the yellow bud and open flower stage, and then Ethyl acetate with a slight increase and Limonene with a sharp increase, from the open flower stage to the senescing flower stage reaching relative amounts of 2.5% and 15.1% respectively (Fig.2.5a). Both (*Z*)-3-Hexenyl acetate and *β*-Linalool (Fig. 2.5b) had their highest relative amount (10.1% and 7.6% respectively) in the yellow bud volatile blend. Benzaldehyde (6.5%), Nonadecane (5.7%) and *p*-Anisaldehyde (9.5%) peaked in relative amount in the open flower scent of *A. melanoxylon* (Fig. 2.5c). *β*-Pinene, (*Z*)-3-Hexen-1-ol, *β*-Caryophyllene and especially (*Z*)-*β*-Ocimene have relative

University of Cape Town

amounts in a narrow range across the four sample types, with all having a maximum level in the senescing flower volatile profile (Fig. 2.5d).

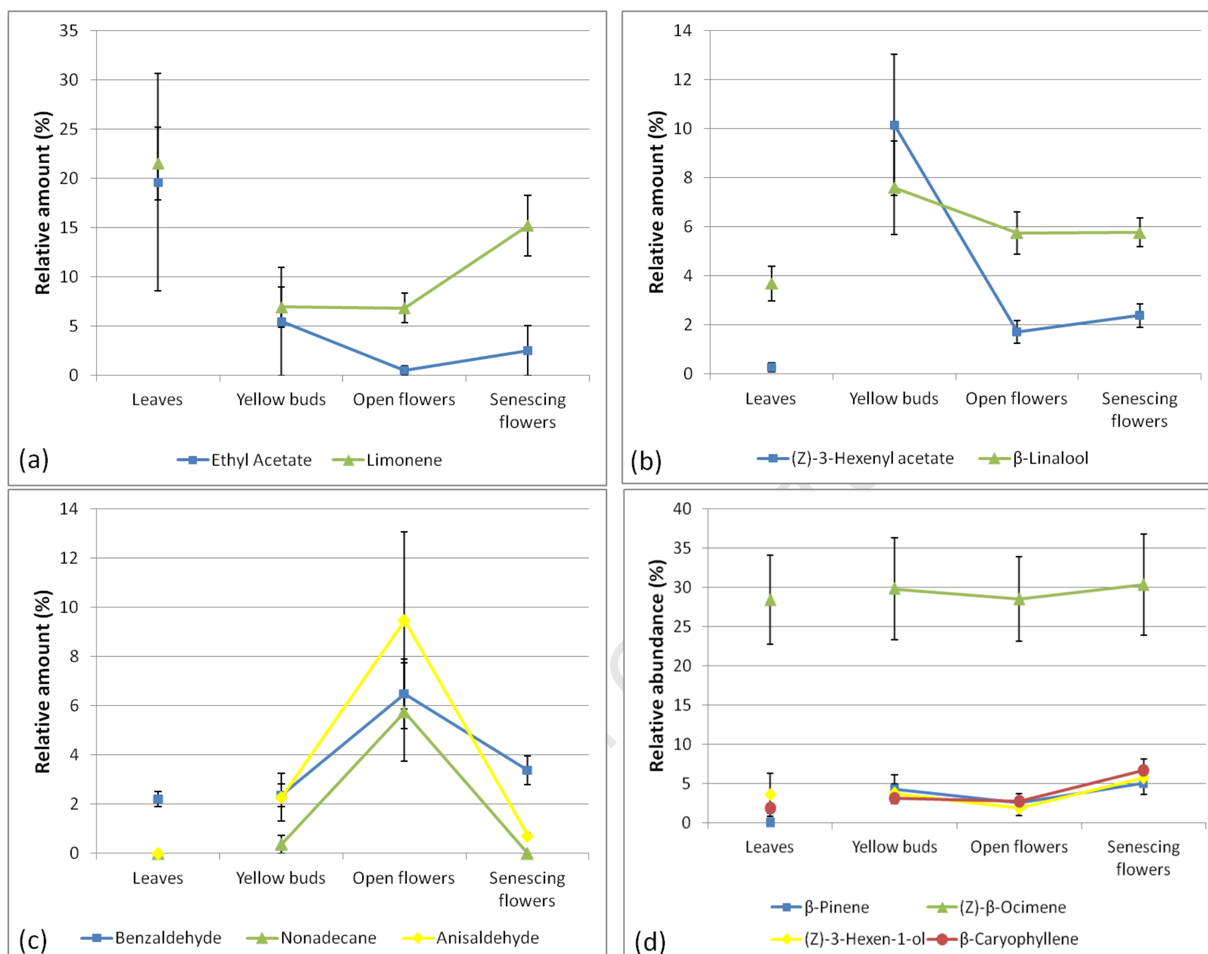


Fig. 2.5. Patterns of relative amounts (mean ± SE) of eleven volatile compounds found in leaves and floral stages of *A. melanoxylon*.

Compound classes - There were pertinent differences in the compound classes between the volatile profiles of the different sample types (Fig. 2.6). Monoterpenes (range: 51.8% - 65.2%) dominated in all four volatile profiles and decreased slightly from the yellow bud profile, with their lowest level in the open flower profile, and then increased steeply in the senescing flower profile. The aliphatic compounds were most prominent in leaves, and decreased steadily from the yellow bud profile to the senescing flower stage (range: 32.2% - 20.1%). The benzenoids were lowest in leaves, and peaked in the open flower profile (at 19% relative amount) with almost similar levels in the yellow bud and senescing flower profiles. The relative amount of sesquiterpenes was very similar when

comparing scent profiles of leaves with open flowers (range: 4.7% - 3.9%) and peaking in the senescing flowers (at 7.5%). Irregular terpenes showed low relative amount in all of the profiles (range: 0% - 1.1%) and the relative amount of the nitrogen containing compounds was negligible. Unlike *A. cyclops* where there was pronounced variation in the summarized abundance levels per compound class, the compound class profiles of *A. melanoxylon* were similar across the four sample types except for the open flowers profile where the variance can be attributed to the higher relative amounts of the benzenoids, Benzaldehyde (6.5%) and *p*-Anisaldehyde (9.5%).

Total volatile emission – Despite apparent highest volatile emission rate by the yellow buds of *A. melanoxylon* and the apparent lowest volatile emission rate in the senescing flower stage, the statistical methods used did not detect any significant difference in emission rates (Fig. 2.7).

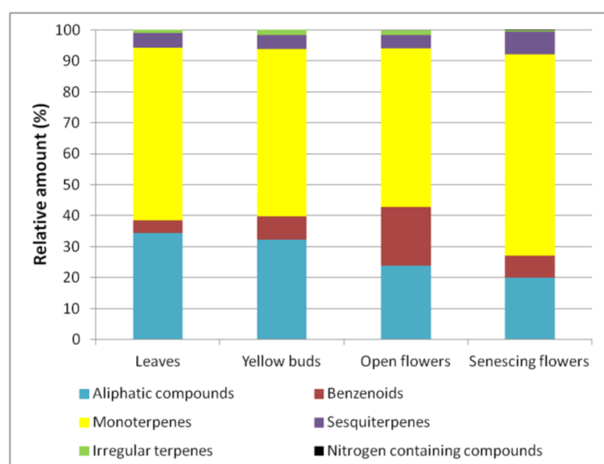


Fig. 2.6. Mean proportions of compound classes in headspace samples of leaves and different floral stages of *A. melanoxylon*.

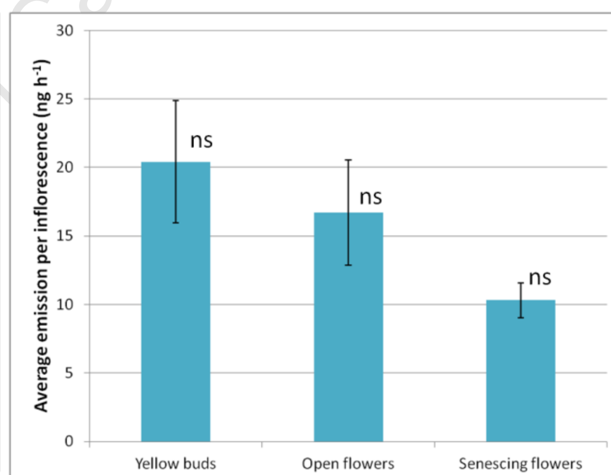


Fig. 2.7. Volatile emission rates (ng h^{-1}) per inflorescence (mean ± SE) of different floral stages of *A. melanoxylon*. ns = no significant difference between emission rates ($F_{(2, 12)}$ = 0.884; p = 0.439; n = 5).

Statistical comparison of volatile profiles of different sample types - The overall separation of the plant volatile profiles, based on Bray-Curtis similarities of the leaves and different floral stages was highly significant (Fig. 2.8; 3D-NMDS stress value = 0.1; ANOSIM R = 0.710, p < 0.001). Pair-wise

comparisons between sample types revealed highly significant differences between all sample types (Fig. 2.8).

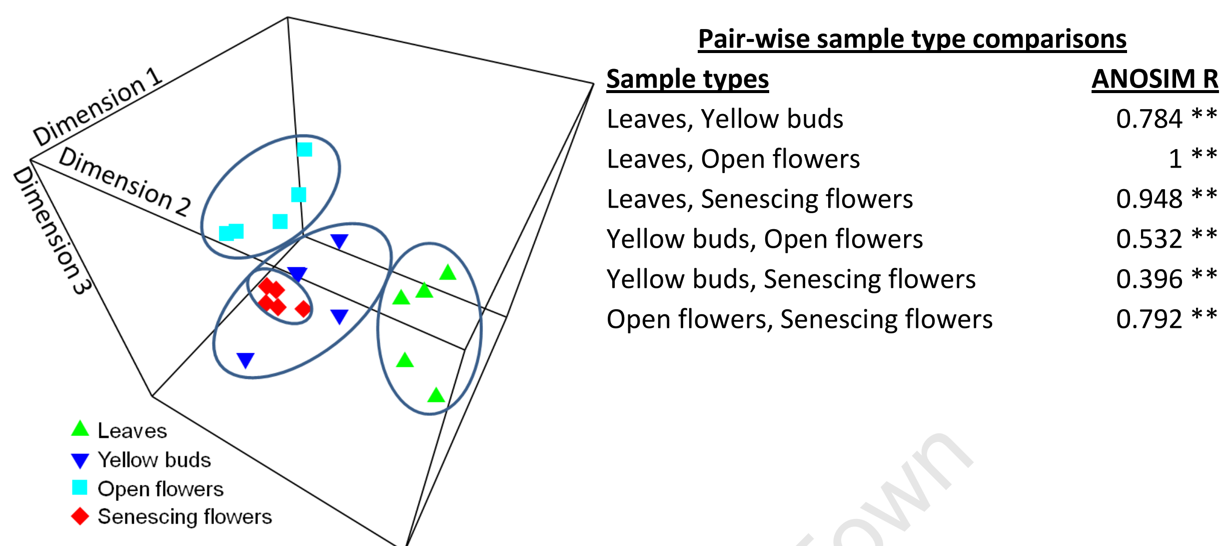


Fig. 2.8. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarities of the odour composition (75 compounds) of the leaves and different floral stages of *A. melanoxylon*. 3D stress value = 0.1, ANOSIM Global R = 0.710, $p < 0.001$. Pair-wise group comparisons shown with ** indicate significance at $p < 0.01$.

University of Cape Town

The dissimilarity indices calculated by the SIMPER method (Table 2.4) revealed a higher similarity than dissimilarity amongst all but one of the pair-wise comparisons of the different sample types (dissimilarity index range: 35.3 - 48.6). A dissimilarity index of greater than 50 was calculated only between leaves and open flowers. Ethyl acetate and *p*-Anisaldehyde contributed most to this result with a total of only 13.3% of the total amount of volatiles emitted by these plant parts. The vegetative parts had a higher dissimilarity to the floral stages (range: 46.3 - 54.5) than the floral stages had amongst themselves (range: 35.3 - 39.8). The dissimilarity index results correlate well with the distribution of compounds per compound class as seen in Fig. 2.6.

Table 2.4. Mean dissimilarity indices based on SIMPER procedure (factor: plant parts) (Clarke & Warwick, 2001) between chemical profiles of leaves and different floral stages of *A.melanoxylon*.

Plant part	Leaves	Yellow buds	Open flowers	Senescing flowers
Leaves	-			
Yellow buds	48.6	-		
Open flowers	54.5	41.3	-	
Senescing flowers	46.3	35.3	39.8	-

2.3.3. Volatile profile of *Acacia longifolia*

General findings - The chemical composition of leaves and the floral stages (yellow buds, open flowers, and senescing flowers) of *Acacia longifolia* is summarised in Table 2.5. In total there were 69 compounds belonging to six different chemical classes, namely aliphatic compounds (39), monoterpenoids (17), benzenoids (6), sesquiterpenoids (3), irregular terpenes (3), and a nitrogen containing compound (1). Of the 69 compounds found in all of the sample types, similarly low numbers were found in the leaves (38), yellow buds (39), and senescing flowers (38), while a maximum number of compounds was found in the open flower samples (60). Similarly to *A. cyclops* and *A. melanoxylon*, there were qualitative differences among the sample types with 22 of the 69 compounds (or 32% of the compounds) being unique or occurring in only one sample type. Again, the total relative amount of these 22 unique compounds was low with the total in leaves and senescing flowers being just over 0.1%, in yellow buds the total was just over 1% and in open flowers the total was 5.6% (Table 2.5).

A total of 25 compounds (or 36% of the total number of compounds) were common across all four sample types. Although 16 of these common compounds never exceeded 5% relative amount in any sample type, the total relative amount of these compounds was usually high reaching 82.8% in leaves, 89.7% in yellow buds, 76.4% in open flowers and 90.4% in senescing flowers. In fact, per sample type, only a small number out of these 25 common compounds clearly dominated the scent profile. As for *A. melanoxylon*, Limonene and (*Z*)-*β*-Ocimene were found in highest relative amounts (respective ranges: 7.9% - 16.8% and 28.8% - 33.9%) in all four sample types. In addition to these two compounds (*Z*)-3-Hexen-1-ol added significantly to the scent blend of senescing flowers (13.3%) and yellow buds (9.4%). (*Z*)-3-Hexenyl acetate was highest in yellow buds (9.7%), and Hexan-1-ol in senescing flowers (5.7%). Highest proportions of *β*-Linalool were detected in the leaf profile (6.5%). Aside from Limonene and (*Z*)-*β*-Ocimene, Benzyl alcohol and 2-Phenylethyl alcohol contributed to the scent of open flowers (6.4% each). The total relative amount of the remaining common 17

University of Cape Town

compounds summed up to 18% (in senescing flowers) to 26.7% (in yellow buds). Altogether, this indicates a relatively high level of evenness of compound distribution in the chemical bouquets of these four sample types, as is indeed confirmed by the Shannon evenness indices of the four sample types ranging from 0.65 to 0.68 (Table 2.5).

Table 2.5. Mean relative amounts (%) of compounds identified by GC-MS from headspace samples of leaves and different flower stages (green buds, yellow buds, open flowers, senescing flowers) of *Acacia longifolia.* The number of samples in which a compound occurred is given in brackets. Scent compounds are listed according to compound class and Kovats retention index (KRI). CAS = Chemical Abstracts Service Registry Number. tr = trace amount (<0.1 % of total sample). Compound identification criteria: a = comparison of MS with published data (e.g. NIST library); b = comparison of MS and retention time with published data; c = comparison of MS and retention time with published data and authentic standard. Unknowns that did not reach at least 1% of relative amount in any sample were pooled with the superscript digit indicating the number of pooled compounds. Mass fragments for unknowns are listed with the base peak first, followed by the other fragments in order of decreasing abundance.

Compound	**KRI**	**CAS**	**Leaves**	**Yellow buds**	**Open flowers**	**Senescing flowers**
Number of samples			5	5	5	5
Number of plants used per sample			5	5	5	5
Mean scent emission per inflorescence (ng h^{-1})			-	15.69	25.38	10.02
Number of compounds out of 69			38	39	60	38
Evenness index			0.68	0.68	0.65	0.67
Aliphatic compounds						
Aliphatic aldehydes						
(*E*)-2-Hexenal[b]	1242	6728-26-3	1.2 (2)	3.4 (5)	1.4 (4)	2.3 (5)
(*E,Z*)-2,6-Nonadienal[b]	1608	557-48-2	-	-	tr	0.1 (3)
Aliphatic esters						
Ethyl acetate[a]	882	141-78-6	2.6 (3)	-	1.2 (2)	2.2 (1)
Isobutyl acetate[a]	975	110-19-0	3.7 (3)	-	0.2 (1)	-
Ethyl butanoate[b]	1040	105-54-4	5.2 (2)	-	0.4 (1)	-
2-Methylbutyl acetate[b]	1134	624-41-9	0.8 (3)	-	0.1 (1)	-
(*Z*)-3-Hexenyl acetate[b]	1333	3681-71-8	0.3 (5)	9.7 (5)	2.6 (5)	0.4 (3)
(*E*)-3-Hexenyl butyrate[b]	1474	53398-84-8	-	0.4 (5)	0.3 (3)	tr
Dimethyl pentanedioate[b]	1690	1119-40-0	0.2 (5)	-	tr	-
Aliphatic alcohols						
Butan-1-ol[b]	1160	71-36-3	-	0.6 (2)	-	-
Hexan-1-ol[c]	1352	111-27-3	3.1 (5)	2.8 (5)	1.4 (5)	5.7 (5)
(*E*)-3-Hexen-1-ol[b]	1374	928-97-2	0.1 (1)	-	tr	0.3 (5)
(*Z*)-3-Hexen-1-ol[b]	1387	928-96-1	4.4 (5)	9.4 (5)	3.1 (4)	13.3 (5)
(*Z*)-2-Hexen-1-ol[b]	1420	928-94-9	-	-	0.4 (3)	-

University of Cape Town

Compound	KRI	CAS	Leaves	Yellow buds	Open flowers	Senescing flowers
Unidentified aliphatic alcohol[a]	1427		-	-	tr	-
Aliphatic alkanes						
Dodecane[c]	1200	112-40-3	-	-	0.6 (2)	-
Tridecane[c]	1300	629-50-5	-	-	0.5 (1)	-
Tetradecane[c]	1400	629-59-4	-	-	0.7 (1)	-
Pentadecane[c]	1500	629-62-9	-	-	0.2 (1)	-
Hexadecane[c]	1600	544-76-3	-	0.2 (1)	0.2 (2)	0.1 (1)
Heptadecane[c]	1700	629-78-7	-	3.5 (5)	11.5 (3)	3.8 (5)
Octadecane[c]	1800	593-45-3	tr	tr	0.1 (4)	-
Nonadecane[c]	1900	629-92-5	-	-	2.8 (2)	-
Eicosane[c]	2000	112-95-8	-	tr	-	-
Heneicosane[c]	2100	629-94-7	-	-	tr	0.1 (4)
Docosane[c]	2200	629-97-0	-	-	tr	-
Tricosane[c]	2300	638-67-5	0.2 (4)	0.3 (4)	0.2 (4)	0.4 (5)
Tetracosane[c]	2400	646-31-1	0.3 (3)	0.3 (3)	0.2 (3)	0.6 (5)
Pentacosane[c]	2500	629-99-2	0.4 (4)	0.8 (4)	0.2 (3)	0.8 (5)
Hexacosane[c]	2600	630-01-3	0.3 (3)	0.2 (2)	0.2 (3)	0.8 (5)
Heptacosane[c]	2700	593-49-7	0.3 (4)	1.0 (3)	0.2 (3)	0.7 (5)
Octacosane[c]	2800	630-02-4	0.2 (4)	0.2 (2)	tr	0.6 (5)
Nonacosane[c]	2900	630-03-5	0.7 (4)	1.3 (3)	0.2 (3)	0.6 (5)
Aliphatic acids						
Butanoic acid[c]	1669	107-92-6	-	0.3 (1)	-	-
Aliphatic ketones						
2-Heptanone[a]	1200	110-43-0	-	4.7 (4)	-	1.2 (5)
6-Methyl-2-heptanone[c]	1689	928-68-7	-	tr	-	-
Unidentified aliphatic compound						
m/z: 43,69,55,117,97,83,57,56,41,95[a]	1526		-	-	tr	-
Benzenoids						
Benzaldehyde[c]	1551	100-52-7	3 (5)	2.7 (5)	2.5 (5)	3.3 (5)
Methyl benzoate[c]	1650	93-58-3	3.6 (5)	-	tr	-
Benzyl acetate[b]	1755	140-11-4	-	0.1 (3)	-	-
Benzyl alcohol[c]	1902	100-51-6	3.2 (5)	4.9 (5)	6.4 (5)	5.9 (5)
2-Phenylethyl alcohol[c]	1938	60-12-8	0.6 (5)	1.2 (4)	6.4 (5)	2.7 (5)
2-Phenylethyl 3-methylbutanoate[b]	1961	140-26-1	-	-	tr	-
2-Phenylethyl 2-methylbutanoate[b]	1988	24817-51-4	-	-	tr	-
Ethyl 4-ethoxybenzoate[c]	2193	23676-09-7	tr	-	0.3 (1)	-
Monoterpenes						
Limonene[c]	1225	138-86-3	16.8 (5)	15.2 (5)	7.9 (5)	15.8 (5)
(*Z*)-*β*-Ocimene[b]	1252	3338-55-4	33.8 (5)	28.8 (5)	33.9 (5)	31.7 (5)
(*E*)-Linalool oxide (furanoid)[c]	1454	34995-77-2	1.2 (4)	0.2 (2)	1.1 (5)	0.5 (2)
(*E,E*)-2,6-Dimethyl-1,3,5,7-octatetraene[a]	1466	460-01-5	0.8 (3)	0.3 (5)	0.6 (5)	tr

University of Cape Town

Compound	KRI	CAS	Leaves	Yellow buds	Open flowers	Senescing flowers
(*Z*)-Linalool oxide (furanoid)[c]	1486	5989-33-3	-	-	2.5 (5)	1.2 (5)
β-Linalool[c]	1556	78-70-6	6.5 (5)	2.8 (5)	1.5 (5)	1.0 (5)
Isobornyl acetate[a]	1610	125-12-2	-	tr	tr	tr
4-Terpineol[b]	1622	562-74-3	0.3 (5)	0.4 (5)	0.4 (4)	0.3 (4)
1-Menthol[b]	1651	2216-51-5	-	-	tr	-
α-Terpineol[b]	1708	98-55-5	0.2 (5)	0.2 (3)	tr	0.1 (3)
Unidentified monoterpene[a]	1714		-	-	0.1 (2)	-
(Z)-Linalool oxide (pyranoid)[a]	1720	14049-11-7	0.3 (5)	-	0.1 (5)	-
Borneol[b]	1725	507-70-0	0.2 (5)	tr	-	-
Verbenone[a]	1740	18309-32-5	-	-	tr	-
(*Z,Z*)-2,6-Dimethyl-3,5,7-octatriene-2-ol[a]	1824	1174030-42-2	0.2 (5)	0.1 (4)	0.2 (5)	-
(*E,E*)-2,6-Dimethyl-3,5,7-octatriene-2-ol[a]	1836	206115-88-0	1.4 (4)	0.6 (5)	1.4 (5)	0.5 (5)
2,6-Dimethyl-3,7-Octadiene-2,6-diol[b]	1945	13741-21-4	-	-	tr	-
Sesquiterpenes						
Longifolene[a]	1600	475-20-7	-	-	tr	0.2 (3)
β-Caryophyllene[c]	1625	87-44-5	0.3 (5)	tr	0.3 (5)	0.5 (5)
α-Farnesene[b]	1765	502-61-4	-	tr	0.4 (4)	0.2 (1)
Irregular terpenes						
6-Methyl-5-hepten-2-one[b]	1354	110-93-0	3.5 (5)	3.1 (5)	1.3 (5)	1.4 (4)
2,6-Dimethyl-6-octanol[a]	1437	78-69-3	0.1 (3)	-	-	-
2,6-Dimethyl-7-octen-2-ol[a]	1474	18479-58-8	-	-	-	0.1 (2)
Nitrogen containing compounds						
Benzyl nitrile[b]	1939	140-29-4	0.1 (5)	tr	2.9 (5)	0.5 (5)

Patterns of variation of volatile compounds - There were distinct patterns in the relative amounts of the ten compounds that had relative amounts >5% in the volatile profiles of the leaves and across the developmental stages of the inflorescences (Fig. 2.9). Limonene (Fig. 2.9a) showed the highest relative amount in the leaf volatile profile (16.8%), and though it was present in similar proportions in yellow buds and old flowers, it had its lowest relative amount in the open flower stage. Ethyl butanoate (Fig. 2.9a) occured in the leaf profile and in much lower percentage in the open flower profile. *β*-Linalool (Fig. 2.9a) was highest in green leaves, much less dominant in yellow buds and then steadily decreased through the floral stages. Like in *A. cyclops* and *A. melanoxylon*, (*Z*)-3-Hexenyl acetate (Fig. 2.9b) had the lowest relative amount in the leaf profile, and similar to *A. melanoxylon*, peaked in the yellow buds profile. (*Z*)-*β*-Ocimene (Fig. 2.9c) had by far the highest

University of Cape Town

relative amount of all compounds across all four sample types, varying only in a very narrow range (28.8% - 33.9%) with its highest proportions detected in the open flower profile. Heptadecane, Benzyl alcohol and 2-Phenylethyl alcohol (Fig. 2.9c) had their lowest relative amounts in the leaf profile, and their highest in the open flower profile. Hexan-1-ol and (*Z*)-3-Hexen-1-ol (Fig. 2.9d) both followed a similar pattern with the lowest relative amounts in the open flower profile, and highest proportions in the senescing flower profiles.

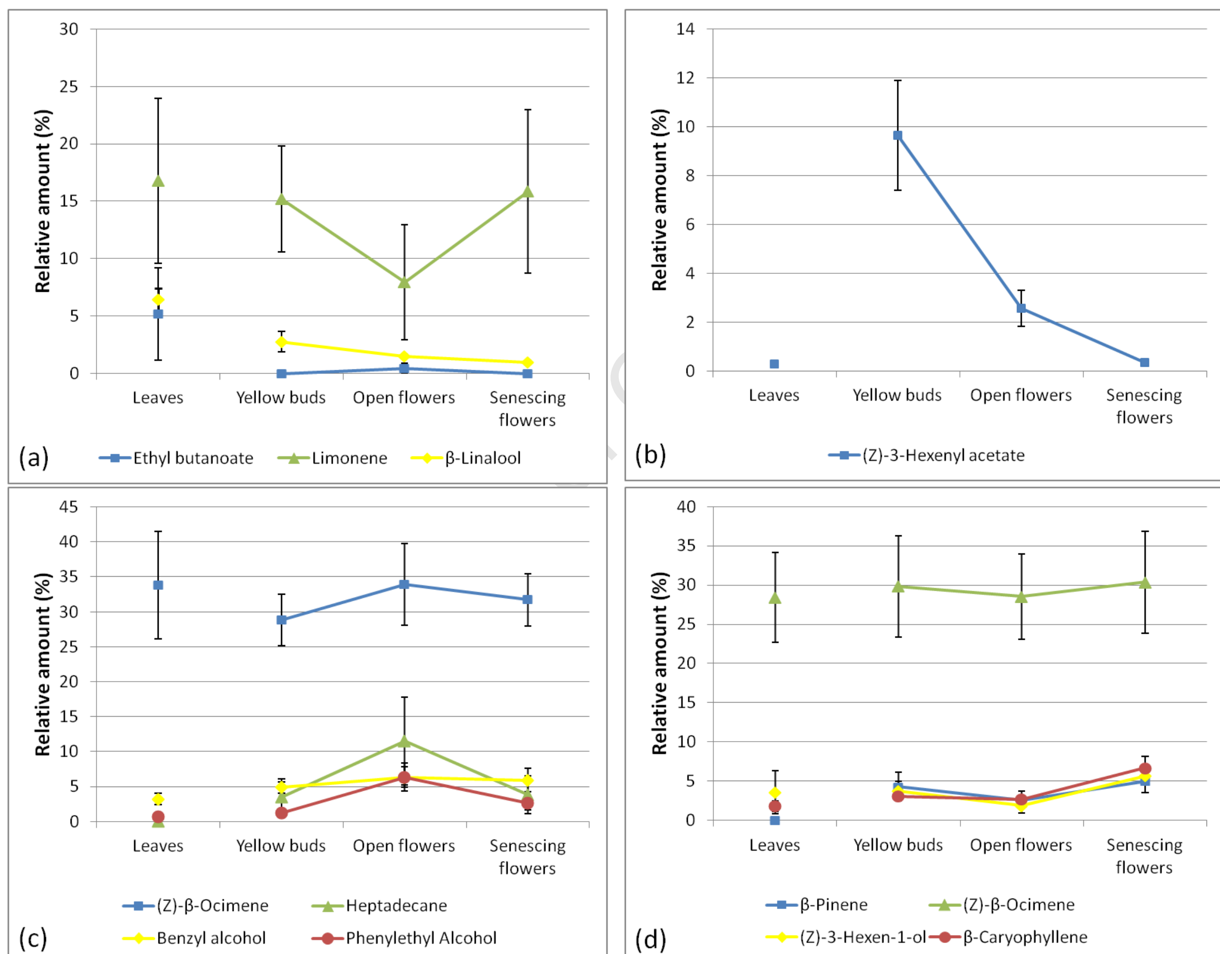


Fig. 2.9. Patterns of relative amounts (mean ± SE) of ten volatile compounds found in leaves and floral stages of *A. longifolia*.

Compound classes - The most pertinent differences in the proportions of the compound classes between the volatile profiles of the different sample types were between the profile for the vegetative green leaf tissue and those of the floral stages (Fig. 2.10). Monoterpenes which dominated in all four volatile profiles had their highest levels in the vegetative tissue (61.8%)

compared to floral stages (range: 48.7% - 51.4%). The aliphatic compounds are generally more dominant in the headspace profiles of the buds and the flowers (range: 29.4% - 39.1%) than in the headspace of the leaf profile (23.8%). Benzenoids ranged from 10.5% in the leaf volatile profile to 15.5% in the open flower volatile profile. The total relative amount of nitrogen containing compounds was generally very low and peaked with only 2.9% relative amount in the open flowers. Compared to the compound class composition of *A. cyclops* and *A. melanoxylon*, the level of sesquiterpenes in *A. longifolia* was very low never exceeding 0.9%.

Total volatile emission – Unlike in *A.cyclops* and *A.melanoxylon* which had the highest volatile emission rate in the buds, the highest volatile emission rate in *A. longifolia* occurred in the open flowers (Fig. 2.11). The open flower and senescing flower stages differed significantly in emission rates.

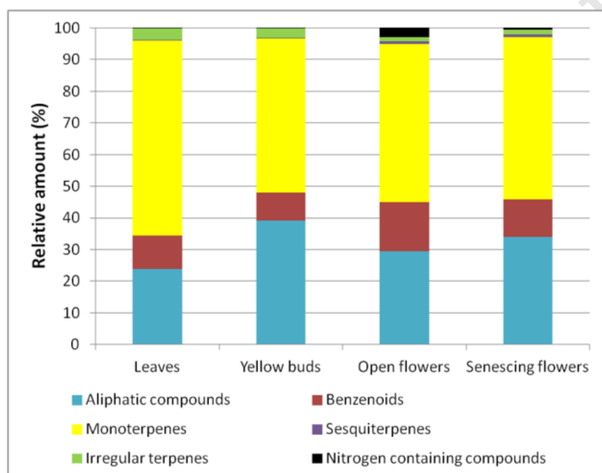


Fig. 2.10. Mean proportions of compound classes in headspace samples of leaves and different floral stages of *A. longifolia*.

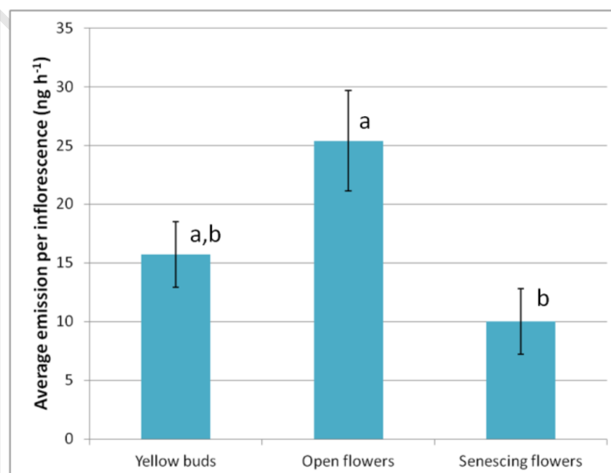
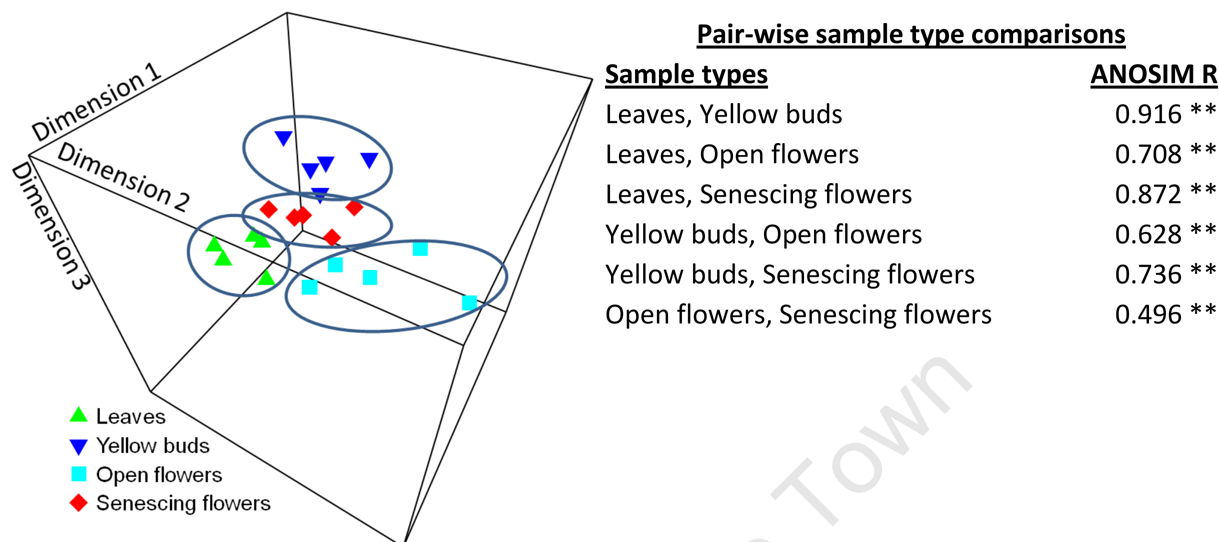


Fig. 2.11. Volatile emission rates (ng h^{-1}) per inflorescence (mean ± SE) of different floral stages of *A. longifolia*. Different letters indicate significant differences at $p < 0.05$ (Tukey HSD, $F_{(2, 12)} = 4.52$; $p = 0.034$; $n = 5$).

Statistical comparison of volatile profiles of different sample types - The overall separation of the plant volatile profiles, based on Bray-Curtis similarities of the leaves and different floral stages was highly significant (3D-NMDS stress value = 0.11; ANOSIM R = 0.69, p < 0.001) (Fig. 2.12). Pair-wise

comparisons between sample types revealed highly significant differences between all sample types (Fig. 2.12).



Pair-wise sample type comparisons

Sample types	ANOSIM R
Leaves, Yellow buds	0.916 **
Leaves, Open flowers	0.708 **
Leaves, Senescing flowers	0.872 **
Yellow buds, Open flowers	0.628 **
Yellow buds, Senescing flowers	0.736 **
Open flowers, Senescing flowers	0.496 **

Fig. 2.12. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarities of the odour composition (87 compounds) of the leaves and different floral stages of *A. longifolia.* 3D stress value = 0.11. ANOSIM Global R = 0.69, $p < 0.001$. Pair-wise group comparisons are shown with ** indicating significance at $p < 0.01$.

University of Cape Town

The dissimilarity indices calculated by the SIMPER method (Table 2.6) revealed overall a higher similarity than dissimilarity amongst the pair-wise comparisons of the different sample types (dissimilarity index range: 34.4 - 44.9).

Table 2.6. Mean dissimilarity indices based on SIMPER procedure (factor: plant parts) (Clarke & Warwick, 2001) between chemical profiles of leaves and different floral stages of *A.longifolia*.

Plant part	Leaves	Yellow buds	Open flowers	Senescing flowers
Leaves	-			
Yellow buds	41.6	-		
Open flowers	44.9	42.1	-	
Senescing flowers	40.9	34.4	39.5	-

2.3.4. Volatile profile of *Acacia saligna*

General findings - The chemical composition of leaves and floral stages (yellow buds, open flowers, and senescing flowers) of *Acacia saligna* is summarised in Table 2.7. In total there were 54

compounds belonging to five different chemical classes, namely aliphatic compounds (24), monoterpenoids (14), benzenoids (8), sesquiterpenoids (3) and irregular terpenes (5). Of the 54 compounds found in all the sample types, lowest numbers were present in leaf samples (28), in yellow bud samples (29) and in senescing flowers (30), whereas highest numbers were found in the open flower samples (45). Nearly 30% of all compounds (16 of the 54 compounds) occurred in only one sample type. The relative amount of each of these compounds was low and never exceeded 2.3% in any of the four sample types (Table 2.7).

Fifteen compounds (27.8% of all compounds) were common across all four sample types. The total relative amount of these compounds was typically high, adding up to 87.3% in leaves, 76.8% in yellow buds, 89.5% in open flowers and 83.6% in senescing flowers. However, only a few of these common compounds dominated the scent profile of each sample type. Limonene, (*Z*)-*β*-Ocimene and Benzyl alcohol together contributed 69.1% of the volatile abundance in the leaf profile (Shannon evenness index = 0.62). In the yellow buds profile in addition to the three compounds mentioned for the leaf profile, (*Z*)-3-Hexen-1-ol was also found to be in high abundance with the four compounds totalling 57.4% of the total. The compounds in this profile were evenly distributed as is demonstrated by an evenness index of 0.78 (Table 2.7). In the open flowers profile, (*Z*)-3-Hexen-1-ol and Benzyl alcohol dominated with 72.5% which was demonstrated by a Shannon evenness index of 0.48 (Table 2.7). The same four compounds as in the yellow buds profile, dominated in the senescing flowers profile with a combined total of 64.1% and an evenness index of 0.66 in the senescing flower profile. The total relative amount of the other 11 common compounds added up to less than 20% in all sample types (Table 2.7). Of the compounds not found to be common to all samples, only three contributed close to or more than 5% to the scent emission of any sample type: Hexan -1-ol (max. 4.5% in floral stages), *α*-Pinene (6.5% in leaf profiles) and UC1693 had its maximum relative amount in yellow buds (9.1%).

University of Cape Town

Table 2.7. Mean relative amounts (%) of compounds identified by GC-MS from headspace samples of leaves and different flower stages (green buds, yellow buds, open flowers, senescing flowers) of *Acacia saligna.* The number of samples in which a compound occurred is given in brackets. Scent compounds are listed according to compound class and Kovats retention index (KRI). CAS = Chemical Abstracts Service Registry Number. tr = trace amount (< 0.1 % of total sample). Compound identification criteria: a = comparison of MS with published data (e.g. NIST library); b = comparison of MS and retention time with published data; c = comparison of MS and retention time with published data and authentic standard. Mass fragments for unknowns are listed with the base peak first, followed by the other fragments in order of decreasing abundance.

Compound	**KRI**	**CAS**	**Leaves**	**Yellow buds**	**Open flowers**	**Senescing flowers**
Number of samples			5	5	5	5
Number of plants used per sample			5	5	5	5
Mean scent emission per inflorescence (ng h^{-1})			-	10.72	55.87	18.61
Number of compounds out of 54			28	29	45	30
Evenness index			0.62	0.78	0.48	0.66
Aliphatic compounds						
Aliphatic esters						
(*Z*)-3-Hexenyl acetate[b]	1333	3681-71-8	0.5 (1)	2.9 (5)	0.9 (5)	0.9 (3)
Methyl hexadecanoate[b]	2220	112-39-0	0.3 (5)	0.6 (5)	1.0 (5)	0.2 (5)
Aliphatic alcohols						
Hexan-1-ol[c]	1352	111-27-3	-	2.0 (3)	1.0 (5)	4.5 (5)
Compound	**KRI**	**CAS**	**Leaves**	**Yellow buds**	**Open flowers**	**Senescing flowers**
(*E*)-3-Hexen-1-ol[b]	1374	928-97-2	-	-	0.1 (5)	0.2 (5)
(*Z*)-3-Hexen-1-ol[b]	1387	928-96-1	3.1 (2)	17.1 (5)	23.7 (5)	20.7 (5)
3-Methoxy-3-methylbutanol[c]	1453	56539-66-3	1.2 (3)	-	-	-
n-Octanol[b]	1565	111-87-5	-	0.7 (5)	-	-
Aliphatic alkanes						
Dodecane[c]	1200	112-40-3	-	-	0.2 (3)	-
Tridecane[c]	1300	629-50-5	-	-	tr	-
Tetradecane[c]	1400	629-59-4	0.9 (1)	-	-	-
Heptadecane[c]	1700	629-78-7	1.0 (2)	2.6 (5)	1.7 (5)	0.8 (5)
Octadecane[c]	1800	593-45-3	0.8 (5)	0.8 (5)	1.3 (5)	0.3 (5)
Heneicosane[c]	2100	629-94-7	-	-	-	tr
Tricosane[c]	2300	638-67-5	0.3 (2)	-	tr	0.1 (2)
Tetracosane[c]	2400	646-31-1	tr	-	-	-
Pentacosane[c]	2500	629-99-2	0.3 (3)	-	tr	0.1 (5)
Hexacosane[c]	2600	630-01-3	0.2 (2)	-	tr	-
Heptacosane[c]	2700	593-49-7	0.2 (2)	-	tr	-
Octacosane[c]	2800	630-02-4	0.2 (2)	-	-	-
Nonacosane[c]	2900	630-03-5	0.4 (3)	-	tr	-
Aliphatic ketones						
5-Methyl-5-vinyldihydro-2(3H)-furanone[b]	1683	1073-11-6	0.4 (3)	-	tr	-

University of Cape Town

Compound	KRI	CAS	Leaves	Yellow buds	Open flowers	Senescing flowers
Unidentified aliphatic compound						
m/z: 43,69,55,117,97,83,57,56,41,95[a]	1526		-	-	tr	-
Benzenoids						
Benzaldehyde[c]	1551	100-52-7	4.0 (5)	4.2 (5)	4.2 (5)	4.7 (5)
Methyl benzoate[c]	1650	93-58-3	-	0.2 (5)	0.4 (5)	0.2 (5)
Benzyl acetate[b]	1755	140-11-4	-	0.3 (4)	tr	-
Methyl salicylate[c]	1806	119-36-8	-	-	tr	-
Benzyl alcohol[c]	1902	100-51-6	7.4 (5)	13.4 (5)	48.8 (5)	29.4 (5)
2-Phenylethyl alcohol[c]	1938	60-12-8	0.9 (5)	0.4 (5)	0.1 (5)	0.2 (5)
Benzenepropanol[a]	2045	122-97-4	-	0.3 (4)	2.2 (5)	1.1 (5)
Dimethyl salicylate[b]	2093	606-45-1	-	0.3 (5)	tr	-
Cinnamyl alcohol[b]	2300	104-54-1	-	-	tr	-
Monoterpenes						
α-Pinene[c]	1087	80-56-8	6.5 (5)	-	0.5 (3)	-
3-Carene[b]	1122	13466-78-9	-	-	0.8 (3)	-
β-Pinene[c]	1137	127-91-3	1.9 (5)	-	0.3 (2)	-
Limonene[c]	1225	138-86-3	41.3 (5)	17.1 (5)	4.7 (5)	14.1 (5)
(*Z*)-*β*-Ocimene[b]	1252	3338-55-4	20.4 (5)	9.8 (5)	2.2 (5)	9.0 (5)
Camphor[c]	1545	76-22-2	-	-	tr	0.2 (5)
β-Linalool[c]	1556	78-70-6	1.9 (5)	3.0 (5)	0.3 (4)	0.9 (5)
Isobornyl acetate[a]	1610	125-12-2	-	0.8 (5)	0.1 (5)	0.4 (5)
4-Terpineol[b]	1622	562-74-3	0.2 (1)	0.7 (3)	-	-
1-Menthol[b]	1651	2216-51-5	-	0.2 (5)	-	-
α-Terpineol[b]	1708	98-55-5	0.7 (5)	0.7 (5)	0.1 (3)	0.4 (5)
Borneol[b]	1725	507-70-0	-	-	tr	-
(*E,E*)-2,6-Dimethyl-3,5,7-octatriene-2-ol[a]	1836	206115-88-0	-	0.3 (5)	tr	-
(*Z*)-Jasmone[b]	1963	488-10-8	-	-	tr	-
Sesquiterpenes						
Longifolene[a]	1600	475-20-7	2.5 (5)	1.3 (5)	0.2 (5)	0.9 (5)
β-Caryophyllene[c]	1625	87-44-5	1.1 (5)	0.9 (3)	tr	0.3 (3)
α-Farnesene[b]	1765	502-61-4	-	-	0.5 (4)	0.3 (3)
Irregular terpenes						
6-Methyl-5-hepten-2-one[b]	1354	110-93-0	-	3.9 (4)	1.1 (5)	2.8 (5)
2,6-Dimethyl-6-octanol[a]	1437	78-69-3	-	1.2 (5)	0.2 (5)	0.8 (5)
2,6-Dimethyl-7-octen-2-ol[a]	1474	18479-58-8	1.5 (5)	2.1 (5)	0.3 (5)	1.0 (5)
4-Oxoisophorone[b]	1722	1125-21-9	-	2.9 (5)	0.8 (5)	2.4 (5)
2,2,6-Trimethyl-1,4-cyclohexanedione[a]	1808	20547-99-3	-	-	-	0.2 (5)
Unidentified compound						
m/z: 56,85,125,43,41,69,153,55,83,39[a] (=UC1693)	1693		-	9.1 (5)	1.6 (5)	3.0 (5)

University of Cape Town

Patterns of variation of volatile compounds - There were distinct patterns in the relative amounts of the six compounds already highlighted across the life cycles of the inflorescences and leaves (Fig. 2.13). *α*-Pinene was mainly found in leaves (6.5%), and in low proportions in open flowers only (0.5%). Limonene and (*Z*)-*β*-Ocimene were found in high relative amounts in leaves. In flowering stages the relative amounts were much lower, with their minimum in open flowers (Fig. 2.13a). UC1693 (together with 4-Oxoisophorone with which it also had occurred as "peak pair" in volatile profiles of *A. cyclops* and *A. saligna*) was absent from leaf headspace, and had their highest relative amounts in the yellow bud profile (Fig. 2.13b). Both (*Z*)-3-Hexen-1-ol and Benzyl alcohol followed a similar pattern, with lowest proportions in leaves, and highest relative amounts in open flowers (Fig. 2.13c).

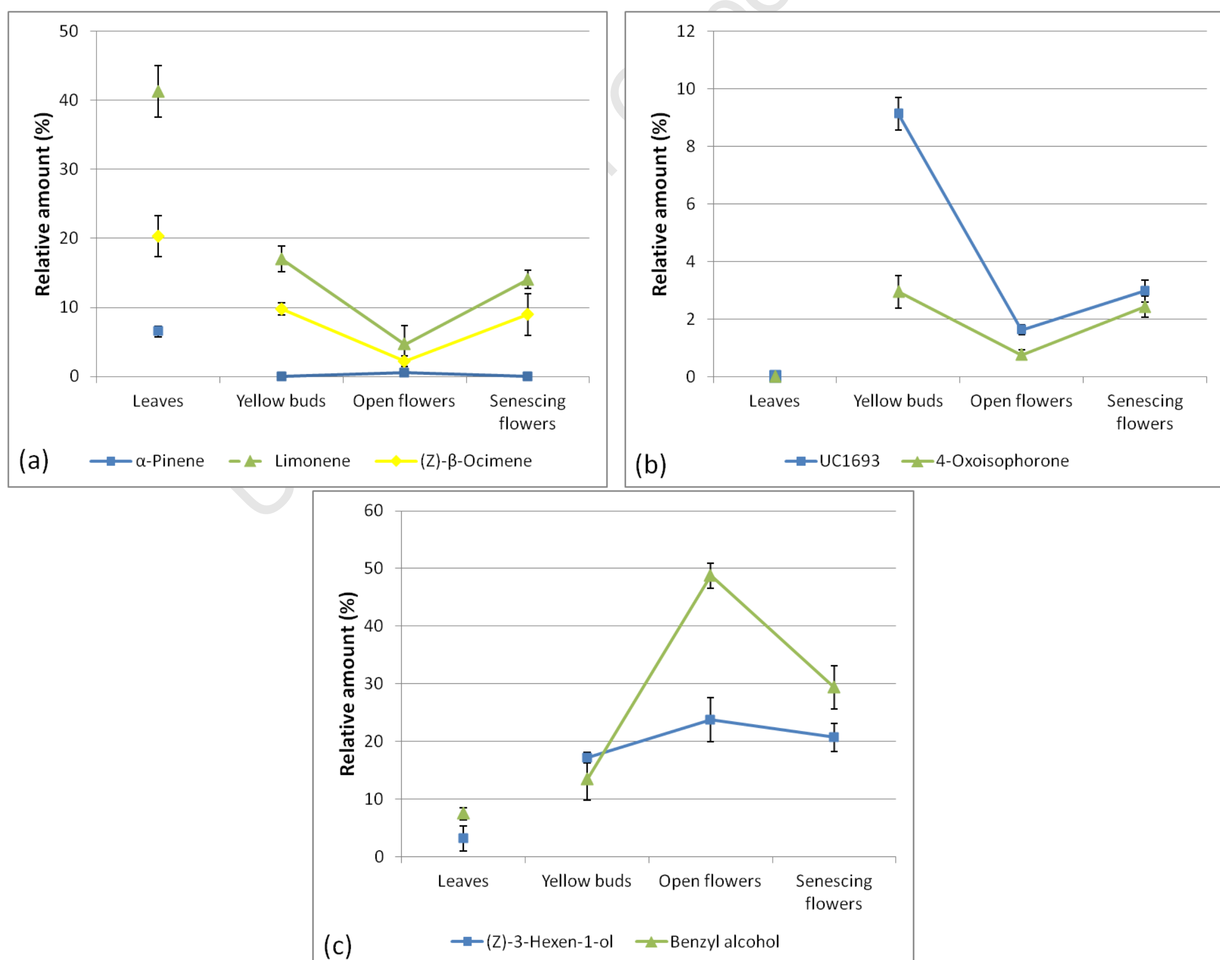


Fig. 2.13. Patterns of relative amounts (mean ± SE) of seven volatile compounds found in leaves and floral stages of *A. saligna*.

Compound classes - The volatile compound classes showed great variation between the different sample types analysed (Fig. 2.14). Monoterpenes clearly dominated in the leaf samples (74.4%), but were only minor compounds in open flowers (9.5%). In contrast, benzenoids dominated in the open flower volatile profile (55.9%), but had their lowest relative amounts in leaves (12.3%). Aliphatic compounds contributed even less to the leaf scent (9.8%), but were important in all floral stages, decreasing from 36% in buds to 30.9% in senescing flowers. Irregular terpenes played no role in leaf scent, and were dominant in the profile of yellow buds (6.9%) and senescing flowers (5.4%). Sesquiterpenes had their maximum relative amount in leaf scent blends (3.5%) as compared to floral stages. No nitrogen compounds were recorded in any of the four sample types. Compared to *A. melanoxylon* and *A. longifolia* where the compound class composition across the different sample types was very similar, the compound class composion of *A. saligna*, like *A. cyclops,* showed more variation across the different sample types.

Total volatile emission - As for *A. longifolia*, and unlike *A. cyclops* and *A. melanoxylon*, the open flowers had the highest volatile emission rate of the floral stages, while the yellow bud stage had the lowest rate (Fig. 2.15). There were significant differences in emission rates for the sample types.

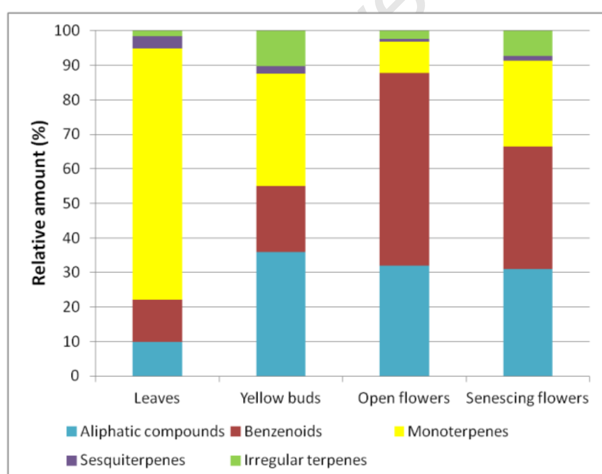


Fig. 2.14. Mean proportions of compound classes in headspace samples of leaves and different floral stages of *A. saligna*.

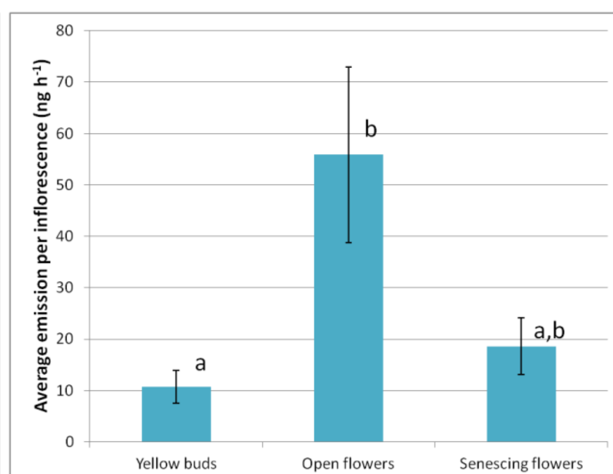
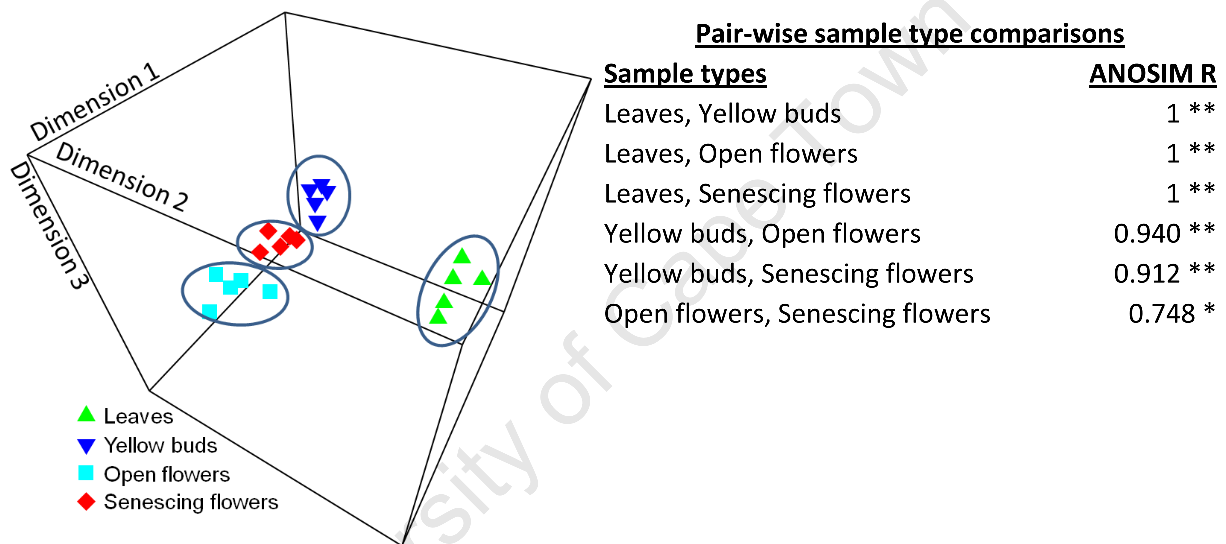


Fig. 2.15. Volatile emission rates (ng h^{-1}) per inflorescence (mean ± SE) of the different floral stages of *A. saligna*. Different letters indicate significant differences at $p < 0.05$. (Tukey HSD, $F_{(2,12)} = 6.727$; $p = 0.011$; $n = 5$).

University of Cape Town

Statistical comparison of volatile profiles of different sample types - The overall separation of the plant volatile profiles, based on Bray-Curtis similarities of the leaves and different floral stages was highly significant (3D stress value = 0.04. ANOSIM Global R = 0.886, p < 0.001) (Fig. 2.16). Pair-wise comparisons between sample types (Fig. 2.16) revealed highly significant differences between leaves and the three floral stages (all R-values = 1), and between yellow buds and the open and senescing flowers (range of R-values: 0.912 - 9.40), and between open flowers and senescing flowers (R-value = 0.748).



Pair-wise sample type comparisons

Sample types	ANOSIM R
Leaves, Yellow buds	1 **
Leaves, Open flowers	1 **
Leaves, Senescing flowers	1 **
Yellow buds, Open flowers	0.940 **
Yellow buds, Senescing flowers	0.912 **
Open flowers, Senescing flowers	0.748 *

University of Cape Town

Fig. 2.16. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarities of the odour composition (87 compounds) of the leaves and different floral stages of *A. saligna*. 3D stress value = 0.04. ANOSIM Global R = 0.886, p < 0.001. Pair-wise group comparisons shown with * indicate significance at p < 0.05, and ** indicates p < 0.01.

The dissimilarity indices calculated by the SIMPER method (Table 2.8) calculated an index of dissimilarity of greater than 50 only between leaves and open flowers. Leaves had a higher dissimilarity to the floral stages (range: 47.0 - 60.0) than the floral stages amongst themselves (range: 24.9 - 39.4).

Table 2.8. Mean dissimilarity indices based on SIMPER procedure (factor: plant parts) (Clarke & Warwick, 2001) between chemical profiles of leaves and different floral stages of *A. saligna*.

Plant part	Leaves	Yellow buds	Open flowers	Senescing flowers
Leaves	-			
Yellow buds	47.0	-		
Open flowers	60.0	39.4	-	
Senescing flowers	49.7	24.9	30.1	-

2.4. Discussion

2.4.1. Scent variation of leaves and different floral parts

Differences in the scent composition have been reported between vegetative and floral plant parts (Flamini et al., 2003; Georgieva et al., 2005), floral life cycle stages (Flamini et al., 2003; Tasin et al., 2005), different stages of the ripeness of fruits (Robertson et al., 1995; Jiang & Kubota, 2004), in different stages of maturity of leaves (Brouat et al., 2000) and different floral parts (Dötterl & Jürgens, 2005; Underwood et al., 2005). In line with these reports, differences were found in the plant volatiles emitted from the leaves and various floral stages of *A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. saligna*. The differences between the leaf volatile profile and the floral volatile profile of both *A. cyclops* and *A. saligna* were more pronounced than that of the other two species investigated and could be attributed to the very high proportions of monoterpenes in the leaf volatile profile. *Acacia cyclops* also showed high proportions of monoterpenes in the leaf volatile profile. The least differences between the leaf and the different floral volatile profiles were observed in *A. longifolia*. For each of the four *Acacia* species, the differences in the chemical profiles of the different plant parts investigated were quantitative (relative amount of compounds in relation to total amount) rather than qualitative (presence or absence of compounds), as the total relative amount of unique compounds was too small to have a significant impact on the variability of the profiles, and also as the total relative amount of the compounds common across all sample types was high.

University of Cape Town

The differences among the volatile profiles of the various floral stages were more pronounced in *A. longifolia* and *A. saligna* than in *A. cyclops* where no statistically clear differences between the volatile profiles of the yellow buds and the open flowers were found. The similarity of the scent profiles of the yellow buds and the open flowers of *A. cyclops* was surprising, and the functional significance thereof, is unclear. The functional significance of the differences between chemical profiles of the leaves and the different floral stages, may relate to pollinator attraction and the defence mechanisms of the plants against herbivores (Pichersky & Gershenzon, 2002; Lucas-Barbosa et al., 2011; Schiestl, 2010).

University of Cape Town

2.4.2. Floral scent in the context of pollination biology

Little is known or confirmed about the pollinators of *Acacia* species (Turnbull, 1997). Insect visitors to *Acacia* flowers are largely determined by the local pool of potential visitors and the floral resources on offer (Stone et al., 2003). *Acacia* flower visitors comprise of three trophic groups namely specialist pollen and flower feeders (beetles, bees and flies), specialist nectar feeders (birds, butterflies and bee flies) and opportunist foragers (flies, ants and wasps) (Stone et al., 2003). Only a subset of these visitors, however act as effective pollinators (Stone et al., 2003; Gibson et al., 2011). *A. cyclops*, *A. melanoxylon, A. longifolia* and *A. saligna* do not produce nectar (Milton & Moll, 1982; Stone et al., 2003; Gibson et al., 2011), and therefore nectar feeders are excluded from the pollinator assemblages of the *Acacia* species. The open, accessible structure of the *Acacia* flower heads, whether spherical or elongated, makes them accessible to a wide variety of insect visitors (Stone et al., 2003) and it is therefore generally accepted that a generalist pollinator spectrum could be expected (Stone et al., 2003; Gibson et al., 2011). In their native range the most important pollinators of Australian acacias are bees and wasps, followed by flies, beetles and birds, whereas in South Africa, the native honeybees (*Apis mellifera capensis* and *A. mellifera scutellata*) are the main pollinators of the Australian *Acacia* spp. (Gibson et al., 2011 and references therein).

Gibson et al. (2011) compiled a list of insect visitors to some invasive Australian *Acacia* species from both Australia and South Africa, with an indication of the pollinator status of the insect species, from published and previously unpublished data. For *A. cyclops*, no specific information is available about insect visitors or the exact pollinator assemblage of the plant neither in its Australian origin nor in South Africa, whereas in the case of *A. melanoxylon*, four insect visitor species were recorded in Australia, two (Diptera: Cecidomyiidae and Diptera: Chironomidae) merely as visitors, and two (Diptera: Lauxaniidae and Coleoptera: Chrysomelidae) of which the pollinator status is uncertain (Gibson et al., 2011). In the case of *A. longifolia* however, of the eleven insect visitor species to *A. longifolia* recorded in Australia, ten species belonged to the Hymenoptera and seven of those, four bee species and three wasp species, have been indicated as possible pollinators (Gibson et al., 2011). Similarly for *A. saligna*, 29 insect species (19 in Australia and 10 in South Africa) have been recorded as flower visitors to *A. saligna* with seventeen species including twelve coleopterans, eight dipterans, one hemipteran and three hymenopterans (including the native honey bees) possibly having a pollination role (Gibson et al., 2011).

University of Cape Town

Although floral scents are complex blends of compounds with various functions and roles, current knowledge of scent chemistry has increased to such an extent that testable predictions can be made in a comparative context regarding possible pollinator guilds of flowers (Dobson, 2006; Jürgens et al., 2006; Goodrich & Raguso, 2009). Even at the compound class level it is sometimes possible to predict the pollinator guild of the plant, e.g. sulphur containing compounds are typically found in plants that are either pollinated by bats or carrion flies (Knudsen et al., 2006).

Observations of flowers with scent containing mostly aliphatic compounds, have shown they were mostly pollinated by beetles, e.g. curculionid beetles pollinating *Eupomatia* species in the Eupomatiaceae family, weevils pollinating *Zygogynum* and *Exospermum* in the Winteraceae family,

nitidulid beetles pollinating *Wettinia* palms (Arecaceae) and chrysomelid beetles pollinating *Ceroxylon*, *Mauritia* and *Geonoma* species of the Arecaceae family (Dobson, 2006) as well as *Phyllophaga* beetles (Scarabaeidae, Melolonthinae) pollinating *Cyathostegia mathewsii* (Leguminosae) (Lewis et al., 2003). Fatty acid derivatives or aliphatic compounds were also associated with pollination by midges in species of *Theobroma* (Sterculiaceae) (Dobson, 2006). Floral scents dominated by terpenoids are common among bee-pollinated plants and these generally have low amounts of benzenoids and fatty acid derivatives (Dobson, 2006). Cecidomyiid midge pollinators seem to be attracted to plant species with scents dominated by terpenoids (Dobson, 2006). Monoterpenes seem to have evolved primarily for defence against herbivores and ants; however, monoterpenes are also important for pollinator attraction, especially in bees (Jürgens, 2004; Dötterl & Vereecken, 2010; Schiestl, 2010). Irregular terpenes have also been associated both with pollinator attraction and indirect plant defence (Tholl et al., 2011). Pollination case studies in plant species where benzenoids are the prominent compound class in the floral scent are scarce (Dobson, 2006). It seems that benzenoids evolved signalling functions in angiosperms, primarily for pollinator attraction, and particularly for attraction of butterflies and moths (Schiestl, 2010). Nevertheless, benzenoids were also found to be the most abundant compounds in two species of Papaveraceae that are mainly pollinated by beetles (Dobson, 2006).

University of Cape Town

Acacia cyclops flower scent predominantly contained aliphatic compounds, which is supportive of beetle pollination (Lewis et al., 2003; Dobson, 2006), and a large proportion of irregular terpenes, which is supportive of pollination by bees and butterflies (Andersson, 2003; Dötterl et al., 2005a; Guédot et al., 2008). Butterflies however, are obligate nectar feeders (Stone et al., 2003) and, since *A. cyclops* does not produce nectar (Milton & Moll, 1982; Stone et al., 2003; Gibson et al., 2011), it can be assumed that the Lepidoptera would not be part of the pollinator guild. The open flower volatile profiles of both *A. melanoxylon* and *A. longifolia* had the monoterpenes as dominant compound class, followed by aliphatic compounds and benzenoids compounds. The dominant

monoterpenes compound class is supportive of attracting bees as pollinators (Jürgens, 2004; Schiestl, 2010). The benzenoids compound class was dominant in *A. saligna* which is presumably attracting for butterflies and moths (Schiestl, 2010). Again, since *A. saligna* also does not produce nectar, and butterflies are obligate nectar feeders, it is unlikely that they are part of the *A. saligna* – pollinator assemblage (Milton & Moll, 1982; Stone et al., 2003; Gibson et al., 2011). Accordingly, neither moths nor butterflies have been recorded as flower visitors on *A. saligna* (Gibson et al., 2011). Aliphatic compounds, a secondary compound class in the open flower profile of *A. saligna*, are supportive of beetles as pollinators (Lewis et al., 2003; Dobson, 2006).

University of Cape Town

Three individual compounds identified in the floral volatile profiles of *A. cyclops*, *A. melanoxylon* and *A. saligna* have high potential to be pollinator attractants. These compounds are 4-Oxoisophorone in *A. cyclops*, p-Anisaldehyde in *A. melanoxylon* and Benzyl alcohol in *A. saligna*. 4-Oxoisophorone is known from several plant species and families (Knudsen et al., 2006) and to my knowledge this is the first time that it has been recorded from an *Acacia* species. It occurred in all sample types of *A. cyclops* and had its highest abundance in the scent of open flowers. Furthermore, it occurred in *A. saligna* with highest relative amount in the yellow bud volatile profile. 4-Oxoisophorone is a flavour compound of carotenoid origin (Goff & Klee, 2006). In plants, the presence of carotenoids is revealed by the rich colour of flowers, fruits and storage organs in the yellow-to-red part of the visual spectrum (Farré et al., 2010). Carotenoids accumulate in different plant tissues and help to attract pollinators to flowers and aid in seed dispersion through the strong red, orange and yellow colours they impart and which attract frugivores to ripe fruits (Goff & Klee, 2006; Simkin et al., 2010). It is known that 4-Oxoisophorone stimulates electroantennal responses in moths (Guédot et al., 2008), butterflies (Andersson, 2003) and bees (Dötterl et al., 2005a). Bees are common pollinators of many *Acacia* species (Stone et al., 2003) and 4-Oxoisophorone might therefore play a role as bee attractant in *A. cyclops* due to the high abundance of the compound in the scent of the open flowers. However, in the scent of *A. melanoxylon* and *A. saligna*, it only occurred in very small

amounts in the open flower scent profiles, and it is unlikely that it will play a similar pollinator attractant role in *A. melanoxylon* and *A. saligna* than in *A. cyclops*.

p-Anisaldehyde (alternative name, 4-Methoxybenzaldehyde) in high concentrations in the volatile blend of Canada thistle scent was shown to be an attractant to different families of pollinating bees (Theis, 2006). *p*-Anisaldehyde was found in the yellow bud, open flower and senescing flower scent profiles of *A. melanoxylon* with the highest relative amounts in the open flowers (9%) where it could be a pollinator attractant.

Benzyl alcohol is also a very common compound (Knudsen et al., 2006). In the scent of Canada thistle Benzyl alcohol was an attractant to florivores (Theis, 2006) and in the scent of Oilseed Rape, Benzyl alcohol was an attractant to cabbage seed weevil (Smart & Blight, 1997). This compound is also an attractant to honey bees (Wadhams et al., 1994). *Arum creticum* (Araceae) attracts a number of beetle families and, being pollinated by bruchid beetles, has Benzyl alcohol as dominant compound (Dobson, 2006). Butterfly-pollinators often show scent preferences that are characterised by aromatics including Benzyl alcohol (Dobson, 2006; Schiestl, 2010). Benzyl alcohol is also found in plants species that attract nectar feeding insects (Jürgens et al., 2009). None of the four *Acacia* species however produce nectar (Gibson et al., 2011). Benzyl alcohol occurred in all of the four sample types from *A. saligna,* where it peaked with almost 50% of the open flower volatile profile as well as in all four sample types of *A. longifolia*. In *A. longifolia* little variation in relative amounts was observed, although the highest amount was recorded in the open flower profile. Due to the high relative amount of the compound in the scent of the open flowers, and despite it also occurring in the leaf volatile profiles of *A. saligna* and *A. longifolia*, it possibly functions as pollinator attractant in *A. saligna* and *A. longifolia*. A number of bees and wasps were observed in the suite of flower visitors of *A. longifolia*, whereas beetles, flies, bees, ants and wasps were recorded as flower visitors

University of Cape Town

to *A. saligna* (Gibson et al., 2011). Benzyl alcohol also occurred in the scent profiles of *A. cyclops* and *A. melanoxylon*, however, it was in very low proportions.

In summary, despite the lack of detailed knowledge about the pollinator assemblage of *A. cyclops*, the volatile profile of its open flowers was supportive of a bee and beetle pollinator syndrome. Bees and wasps are the main flower visitors for *A. longifolia* (Gibson et al., 2011) with clear support for bees as pollinator in the chemical volatile profile of the open flowers of *A. longifolia*. The currently known flower visitor assemblage of *A. melanoxylon* on the other hand, includes only flies and one beetle species (Gibson et al., 2011), while the volatile profile seemed supportive of bees and beetles as pollinators. The possible discrepancy lies in not enough information being available on the pollination biology of *A. melanoxylon*. The flower visitors to *A. saligna* include many beetle species, flies, bees and ants with evidence for beetles and bees to be pollinators (Gibson et al., 2011). The volatile profile of the open flowers of *A. saligna*, was supportive of beetles and bees as pollinators, but also lent support for moths and butterflies as pollinators. *A. saligna* is a non-nectar producing plant, and therefore excludes butterflies as obligate nectar feeders from the pollinator assemblage (Milton & Moll, 1982; Stone et al., 2003; Gibson et al., 2011). Besides, no lepidopterans were recorded as visiting the *A. saligna* flowers (Gibson et al., 2011). Each of *A. cyclops*, *A. melanoxylon, A. longifolia* and *A. saligna*, with different volatile profiles particularly pertaining to the concentrations of the compounds, and despite many of the same compounds being present in their floral scents, seems to employ a different chemical strategy to attract the same set of pollinators. Further investigation is required to confirm pollinator assemblages of these species and to elucidate on these different chemical strategies.

2.4.3. Leaf and floral scent in the context of chemical defence

Plants have developed many strategies to defend themselves against herbivorous arthropods and often use the emission of volatile compounds in these defence strategies (Büchel et al., 2011).

University of Cape Town

Terpenoids and green leaf volatiles (GLVs) represent the major classes of the plant volatiles used in these defence strategies (Arimura et al., 2009) with terpenoids being the most abundant and structurally diverse group of plant secondary metabolites and released by many higher plants (Dudareva et al., 2004; Cheng et al., 2007). Terpenoids are usually produced both in the leaves and the flowers (Dudareva et al., 2004). GLVs are aliphatic 6-carbon primary alcohols, aldehydes and acetates that are ubiquitous throughout the plant world (Mumm & Dicke, 2010). Two compounds, (*Z*)-3-Hexenyl acetate, a GLV, and (*Z*)-*β*-Ocimene, a monoterpene occurred in high proportions throughout the volatile profiles of each of *A. cyclops*, *A. melanoxylon, A. longifolia* and *A. saligna* and their putative functions in the context of plant defence are discussed.

(*Z*)-3-Hexenyl acetate is a well-known green leaf volatile (Knudsen et al., 2006). GLVs occur naturally in vegetative and floral scents of intact, healthy and uninfested plant tissue (Knudsen & Gershenzon, 2006; Matsui, 2006), but are also known to increase in response to wounding or damage (Azuma et al., 1997; Holopainen, 2004; Mumm & Dicke, 2010) and are often characteristic of wounded tissue (Grison et al., 1999; Arimura et al., 2001). (*Z*)-3-Hexenyl acetate mostly plays a role in plant defence (Knudsen & Gershenzon, 2006) and it has been shown to attract egg parasitoids (Büchel et al., 2011) and carnivorous arthropods (Mumm & Dicke, 2010) and to deter insects from laying eggs on injured plants (Pichersky & Gershenzon, 2002).

In the scent profiles of *A. cyclops*, the relative amount of (*Z*)-3-Hexenyl acetate was low in the leaf scent bouquet comparing to that of the floral stages, especially the green bud stage. The relative amount decreased from the green to the yellow buds stage, but remained constant thereafter until floral senescence. Surprisingly, the proportions of GLVs in the leaf profiles in *A. melanoxylon*, *A. longifolia* and *A. saligna*, were much lower than in the floral volatile profiles of these species. Similar to *A. cyclops*, there was a decreasing trend from the yellow buds to the senescing flowers in the relative amounts of the compound. The decreasing trend from bud to flower stage had also been

University of Cape Town

recorded for a cultivar of raspberry (Robertson et al., 1995). The distribution pattern of (*Z*)-3-Hexenyl acetate in the leaf profiles compared to the floral profiles, may indicate that it occurs naturally in the intact plants, and that its abundance, especially in the case of *A. cyclops*, is not due to damage induced to the plant by the sampling method used. The plants were either not that sensitive to mechanical damage (due to the sample preparation method) or that the effect of the damaged induced GLVs had dissipated by the time the headspace samples were taken (a few hours after collection of the plant material) (see also Holopainen, 2004).

(*Z*)-*β*-Ocimene, is a ubiquitous floral terpenoid volatile (Knudsen et al., 2006) and emission thereof often follows herbivore feeding (i.e. induced defence) (Bouvier et al., 2005). It also plays a role in resistance to pathogens and tolerance to high temperature (Bouvier et al., 2005), and it can play a role in deterring ovipositing insects (Castells & Berenbaum, 2008). (*Z*)-*β*-Ocimene occurred in all four of the *Acacia* species discussed here. In *A. cyclops* the relative amounts of (*Z*)-*β*-Ocimene were highest in leaves and decreased from high relative amounts in green buds (which may have been highly sensitive to the mechanical damage during sample preparation) through floral development reaching the lowest proportions in the open flower stage. Actual emission of the scent showed the lowest emission rates in the senescing flower stage. Although (*Z*)-*β*-Ocimene occurred in high, almost equal relative amounts through all four sample types in the volatile profiles of *A. melanoxylon*, the actual emission rate also decreased from the yellow bud scent to almost half the amount in the senescing flower scent. The decreasing trend in relative amount from bud to flower stage has also been recorded for garland (Flamini et al., 2003). Similar to *A. melanoxylon*, (*Z*)-*β*-Ocimene also occurred in high, almost equal relative amounts through all four sample types in the volatile profiles of *A. longifolia*, however, the actual emission rate in the floral profiles peaked in the open flower profile. The lowest proportions of (*Z*)-*β*-Ocimene have been recorded in *A. saligna* where the relative amount in leaf emissions was 20.4%, and in floral volatiles less than half of this amount. The actual emission rates in the floral profiles were almost similar. Generally, in all four

University of Cape Town

plant species, the proportion of (*Z*)-*β*-Ocimene was higher in the leaf volatile profiles than in the floral volatile profiles. This pattern seems to support the assumed defence role of this compound in leaves. In *A. longifolia*, where there was a much higher emission rate of (*Z*)-*β*-Ocimene in the open flower scent than in the yellow bud and senescing flower scent, this compound may play a role in the defence against florivores (Theis, 2006). The generally high abundance levels of this compound may however also be a cumulative effect of several influences to which the sampling preparation method may have contributed. It has been reported that damage-induced compounds, mainly terpenes, are synthesized a few hours after the initial damage took place, as opposed to GLVs which are released within a few minutes (Holopainen, 2004).

Monoterpenes in particular seem to have evolved primarily for defence against herbivores and ants e.g. by acting as toxins, feeding deterrents, and oviposition deterrents to a large variety of insects (Jürgens, 2004; Jürgens et al., 2006; Schiestl, 2010). However, monoterpenes are also important for pollinator attraction, especially in bees (Jürgens, 2004; Schiestl, 2010). The function of such compounds is therefore likely to be context dependent. The importance of understanding the context in which plant-insect interactions takes place, before predictions can be made about the putative functions of compounds, can be demonstrated with the monoterpene, Limonene, which occurred in all volatile profiles in all four species. Limonene is possibly the single most common compound in floral volatiles (Dudareva & Pichersky, 2000; Knudsen et al., 2006). It is both a floral and vegetative volatile (Azuma et al., 1999; Bruce & Cork, 2001). Bees are presumably attracted to Limonene (Williams & Whitten, 1983; Jürgens, 2004; Dobson, 2006), which together with visual cues, may attract bees over short distances (Levin et al., 2001; Jürgens, 2004). However, Limonene also acts as an oviposition deterrent e.g. in some species of *Macaranga* (Lindgren et al., 1996). Except for *A. cyclops*, the relative amounts of Limonene in the leaf profiles of *A. melanoxylon* and *A. saligna* were much higher than in the floral profiles, indicating a defence mechanism emitted by leaves. In *A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. saligna*, Limonene had the lowest relative amounts in

University of Cape Town

the open flower profiles, which are meant to attract insect visitors for pollination. The steep increase in the relative amounts in the senescing flower scent profile might be due to other signals for pollinator attraction being "switched off" again in the senescing floral stages (Moraga et al., 2009) thereby increasing the relative amounts of Limonene in the senescing flower volatile profile. Limonene, which was present in all volatile profiles of open flowers in relatively high amounts (except in the case of *A. cyclops*), may both deter florivorous insects (Goodrich & Raguso, 2009) while at the same time attract pollinating bees which are assumed to be pollinators of almost all *Acacia* species (Stone et al., 2003; Gibson et al., 2011). In *A. longifolia*, Limonene was present in almost similar proportions in the leaf profile and all floral stages, except for the much-reduced relative amount in the open flower profile. This pattern also concurs with the probable trade-off between resource allocations to defence as opposed to reproduction, in order to produce other pollinator attractant scent in the open flowers (Arimura et al., 2005; Hanley et al., 2007).

In summary, there has been little research on the plant volatiles of *Acacia* species in general, and studies that have been done have concentrated more on the identification of compounds, than on evaluation of their particular role or function (Flath et al., 1983; Zygadlo et al., 1996; Kaiser, 1997; Lamarque et al., 1998; Willmer et al., 2009). To my knowledge, this study is the first report on the plant volatiles of invasive *Acacia* species. The putative functions of the prominent compounds in the context of chemical defence as discussed above, need to be confirmed with further investigations. Of particular interest would be the role of monoterpenes such as Limonene, *β*-Linalool, *α*-Pinene and *β*-Pinene as well as the role of other GLVs and other aliphatic compounds such as the long chain *n*-Alkanes found in the headspace of these *Acacia* species, as these compounds were also prominent compounds in the scent of these *Acacia* species.

University of Cape Town

2.4.4. Scent variation of senescing flowers

A wide spectrum of volatiles from all compound classes was detected in the senescing flower volatile profiles of *A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. saligna*. Decreased levels of emitted volatiles are characteristic of flower senescence (Verdonk et al., 2003) and have also been shown in *Vachellia* (*Acacia*) *seyal seyal* and *Vachellia* (*Acacia*) *seyal fistula* (Willmer et al., 2009) as well as in other plants (Dötterl et al., 2005b; Theis & Raguso, 2005). However, decreased emission rates of plant volatiles in senescing flowers as opposed to open flowers, were only observed for *A. cyclops* and *A. longifolia*.

University of Cape Town

The volatile profile of the senescing flowers of *A. cyclops* was characterised by a higher evenness index in compound distribution than in the yellow bud and open flowers profiles, and especially than in the leaf and green bud profiles, as it lacked the dominance of certain compounds e.g. (*Z)*-3-Hexenyl acetate and (*Z*)-*β*-Ocimene found in the other sample types of *A. cyclops*. It seems that no obvious function can be ascribed to the emitted scent of senescing flowers, and the lack of dominant compounds seemed to be congruent with the position of senescing flowers in the floral life cycle where e.g. pollinator attraction does not play a role. Senescence of leaves and flowers is an active process and requires particular gene expression and synthesis of new proteins to enable the transition of attractive flowers to seed producing units, and might result in the release of different or new ratios of volatile compounds (Wang et al., 2001). Thus, compounds functioning as pollinator attractants in open flowers may be "switched off" in the senescing flowers in order to direct pollinators to open flowers to optimise pollination (Moraga et al., 2009). "Switching off" may occur by the increased production of certain compounds, which results in a change to the ratio of other compounds including pollinator attracting compounds, in the total volatile bouquet (Wang et al., 2001). In *A. cyclops,* the relative amounts of Hexan-1-ol, Benzaldehyde, Benzyl alcohol, Limonene and *β*-Linalool increased from the open flower scent to the senescing flower scent. In the volatile profiles of *A. melanoxylon*, *A. longifolia* and *A. saligna*, a similar increase in relative amounts

however, was only observed with respect to Hexan-1-ol and Limonene. A different set of compounds showed increased relative amounts in the senescing flower volatile profiles of the other species. In addition to Hexan-1-ol and Limonene, in *A. melanoxylon* these included (*Z*)-3-Hexen-1-ol, *α*- and *β*-Pinene, and *β*-Caryophyllene; in *A. longifolia* these included (*Z*)-3-Hexen-1-ol and Benzaldehyde; and in *A. saligna*, these also included Benzaldehyde. The observation of the increasing evenness index in compound distribution towards the end of the floral life cycle cannot be made for the other three species. In both *A. melanoxylon* and *A. longifolia* the evenness index of compound distribution throughout all the sample types was relatively high and was at similar levels for all the sample types. In *A. saligna* the compound distribution in the volatile profile of the yellow buds had the highest evenness index, and the highest level of dominance of compounds was found in the volatile profile of the open flowers, with the level of evenness in the senescing flower profile still high.

University of Cape Town

CHAPTER 3: VOLATILE CHARACTERISATION OF *A. KARROO*, *A. SIEBERIANA* var. *WOODII* AND *A. XANTHOPHLOEA*

3.1. Introduction

Dasineura dielsi (Diptera: Cecidomyiidae), a galling midge from Australia, was being assessed as a possible biological control agent for *Acacia cyclops* in South Africa in 2001, and the host specificity tests included some African *Acacia* species along with a number of phyllodineous Australian acacias (Adair, 2005). This was to assure that native *Acacia* species would not become hosts should the gall midge be released (Zimmermann et al., 2004). Of the five African acacias that were assessed (Adair, 2005), two, namely *A. karroo* and *A. sieberiana* var. *woodii*, were selected for this study to investigate their volatile profiles. An additional species, *A. xanthophloea,* was also included.

According to the controversial retypification of the genus *Acacia* in 2005 (Willmer et al., 2009; Miller et al., 2011), these three species from the subgenus *Acacia* should be called *Vachellia karroo*, *Vachellia sieberiana* var. *woodii* and *Vachellia xanthophloea*. However, to retain continuity with the work that motivated the inclusion of these species in this project (Adair, 2004, 2005) and in the context of the biological control programmes of the invasive Australian acacias in South Africa (Dennill & Donnelly, 1991; Hoffmann et al., 2002; Donnelly & Hoffmann, 2004; Impson et al., 2004, 2008, 2011; Moseley et al., 2009; Wood & Morris, 2007; Post et al., 2010), it was decided to still refer to these species as *Acacia* species.

Acacia karroo Hayne is highly polymorphic and variable in height (Ward, 2011), usually with a dense, rounded crown (Coates Palgrave, 2005; Smit, 2008). *Acacia sieberiana* var. *woodii* DC. is one of the most common of the flat-topped acacias and *Acacia xanthophloea* Benth. is a tall, well-shaped tree with characteristic greenish-yellow powdery bark (Coates Palgrave, 2005; Smit, 2008). The leaves of all three species consist of several pairs of pinnae (Coates Palgrave, 2005; Smit, 2008). *Acacia karroo*

University of Cape Town

has golden yellow and *A. xanthophloea* has bright yellow globular flowerheads, and *A. sieberiana* var. *woodii* has strong scented creamish-coloured globose inflorescences (Coates Palgrave, 2005; Smit, 2008). Kaiser (1997) describes the fragrance of *A. karroo* as distinctly sweet scented. *Acacia karroo* has commercial and agricultural (Mkhabela, 2003; Coates Palgrave, 2005), as well as medicinal (Nyila et al., 2012; Van Wyk, 2011) uses. *Acacia karroo* is also known as an encroacher (Ward, 2011) and is invasive in Australia (Csurches & Edwards, 1998) and other countries (Daehler, 1998).

Plant volatile characterisation has been done for only a few African *Acacia* species i.e. *A. karroo* (Kaiser, 1997), *Vachellia seyal seyal*, *V. seyal fistula, V. etbaica, V. brevispica, V. drepanplobium* and *Senegalia mellifera* (Willmer et al., 2009). Unfortunately, Kaiser (1997) did not describe methods in detail, and direct comparison between that study and this is not possible and it was therefore considered worthwhile to redo the volatile characterisation of *A. karroo*.

University of Cape Town

The objective of this study was to characterise the volatile profiles of the three African acacias namely *A. karroo*, *A. sieberiana* var. *woodii* and *A. xanthophloea* in order to compare and contrast the vegetative and floral profiles of each species and to compare these to the invasive Australian acacias. Prominent compounds found in the profiles are discussed in the context of their putative functions.

3.2. Methods and Materials

In order to maintain full comparability with samples from *A. cyclops*, *A. melanoxylon*, *A, longifolia* and *A. saligna* (Chapter 2), flowers and leaves of *A. karroo*, *A.sieberiana* var. *woodii* and *A. xanthophloea* were harvested and sampled following the same method as used for *A. cyclops* and the other Australian invasive species described in Chapter 2.

For each species, shoots comprising leaves and flowers were collected from randomly-selected roadside trees in residential areas in the Western Cape, South Africa. *Acacia karroo* material was collected from 15 trees on 5 December 2009 and 21 January 2010 in Kuils River (S 33° 55' 24.2" E 18° 41' 49.4"), Bellville (S 33° 53' 37.9" E 18° 36' 47.1"), Brackenfell (S 33° 54' 08.0" E 18° 41' 48.3") and Durbanville (S 33° 50' 30.4" E 18° 39' 59.3"). *Acacia sieberiana* var. *woodii* material was collected from 15 trees on 21 January 2010 and 8 December 2010 in Kuils River (S 33° 55' 24.3" E 18° 41' 46.7'') and Bellville (S 33° 52' 24.4" E 18° 37' 56.1" and S 33° 51' 44.4" E 18° 37' 51.0"). *Acacia xanthophloea* material was collected from five trees on 5 December 2009 in Kuils River (S 33° 55' 24.0" E 18° 41' 45.4") and Bellville (S 33° 52' 24.4" E 18° 37' 56.1"). *Acacia karroo* and *A. sieberiana* var. *woodii* collections produced three samples from five trees each while *A. xanthophloea* collections produced one sample from five trees.

The plant cuttings were transported to a laboratory where the leaves and open flowers were carefully removed from the shoots with a pair of forceps. Flowers and leaves were pooled to create three samples of the leaves and three samples of the open flowers, thus giving three replicates of each of the required plant parts. Each sample contained material from five individual trees. Control samples were prepared to distinguish between plant volatiles and ambient contaminants by collecting surrounding air separately after taking the leaf and flower scent samples of the pooled material of each group of five trees. The flowers used in the samples were counted to quantify the emission rate per flower per hour. Trapping of the headspace volatiles, GC-MS analyses of the scent samples, determining the emission rate of the flowers and the statistical analyses was performed in the same manner as described in Chapter 2.

University of Cape Town

3.3. Results

3.3.1. Volatile profile of *Acacia karroo*

The chemical composition of the leaf and flower volatiles of *A. karroo* is summarised in Table 3.1. In total 68 compounds were found belonging to six different chemical classes, namely aliphatic compounds (30), benzenoids (10), monoterpenoids (17), sesquiterpenoids (5), irregular terpenes (4) and nitrogen containing compounds (2).

Leaf and flower scent differed qualitatively. Nearly twice as many compounds were found in flowers as compared to leaves (61 vs. 34). Out of these, only seven were unique for leaves, and 34 were found only in floral scent. The total relative amount of unique compounds in the leaf profile, however, was low (2.7%) with only one compound, (*E*)-*β*-Ocimene, contributing more than 1% (Table 3.1). In contrast, in open flowers, unique compounds added up to a total of 26.9%. Six compounds contributed more than 1% to the constituent, namely Methyl (*E*)-2-hexenoate (2.8%), 6-Methyl-5-hepten-2-one (3.1%), Benzene propanol (3.6%), Cinnamaldehyde (1%), Cinnamyl alcohol (8.5%) and Pentacosane (1.5%) (Table 3.1).

University of Cape Town

Quantitative differences between the sample types are demonstrated by the 27 compounds (or 39% of the total number of compounds) that were common across both sample types. All common compounds summed up to a total of 97.3% for leaves, and 73.1% for open flowers. However, typically only a few common compounds dominated the scent profile, e.g. considering only compounds contributing >5%, in the leaf headspace these were (*Z*)-*β*-Ocimene (62.4%) and (*Z*)-3-Hexenyl acetate (21.7%). Correspondingly, the Shannon evenness index is low with 0.39 (Table 3.1). In the floral scent bouquet, which contained nearly twice as many individual compounds as the leaf scent, four compounds contributed >5%, thus dominating the profile: Benzyl alcohol (31.4%), *α*-Farnesene (10%), (*Z*)-*β*-Ocimene (7.8%), and Benzaldehyde (5.9%). The other compounds common to

both leaves and flowers added up to 13.3% for leaves and 18% for flowers. Consequently, scent from open flower samples had a higher Shannon evenness index than scent from leaves (Table 3.1).

3.3.2. Volatile profile of *Acacia sieberiana* var. *woodii*

The chemical composition of the leaves and flowers of *A. sieberiana* var. *woodii* is summarised in Table 3.1. In total 75 compounds belonging to six different chemical classes, namely aliphatic compounds (34), benzenoids (8), monoterpenoids (15), sesquiterpenoids (14), irregular terpenes (2) and nitrogen containing compounds (2) were detected.

Flower samples contained more compounds than leaf samples (63 vs. 46). Qualitative differences among the leaf and flowers samples are further demonstrated by 41 of the 75 compounds (55%) occurring in only one sample type; their total relative amount however was extremely low in both the leaf (1.1%) and flower (3.9%) samples. In leaves none of the unique compounds exceeded 1% of the total relative amount (Table 3.1). In open flowers only two flower-specific compounds contributed (just) more than 1% to the total scent emission, namely 4-Penten-1-yl acetate (1.2%) and 2,2,6-Trimethyl-6-vinyldihydro-2H-pyran-3(4H)-one (1.7%) (Table 3.1).

University of Cape Town

Quantitative differences between leaves and flowers are demonstrated by the 34 compounds (45% of the total number of compounds) that were found in both sample types. The scent of common compounds added up to a total relative amount of 98.9% for leaves, and 96.1% for open flowers. Four compounds contributed more than 5% to the scent of leaves and flowers. In leaves, these were (*Z*)-3-Hexenyl acetate (57%), (*E*)-Linalool oxide (furanoid) (15%), (*Z*)-Linalool oxide (pyranoid) (7.2%) and (*Z*)-*β*-Ocimene (7.6%). In floral scent the major common compounds were again (*E*)-Linalool oxide (furanoid) (51.9%), and (*Z*)-Linalool oxide (pyranoid) (13.4%), but here accompanied by Indole (7.5%) and (*Z*)-Linalool oxide (furanoid) (6.7%). All less dominant common compounds added up to a

total of 12% in leaf and 16.6% in flower samples. The Shannon evenness indices indicate a low to medium evenness in scent composition in leaf (0.42) as well as flower (0.45) bouquets (Table 3.1).

3.3.3. Volatile profile of *Acacia xanthophloea*

The composition of volatiles from leaves and flowers of *A. xanthophloea* is summarised in Table 3.1. A total of 60 compounds, 34 in the leaf sample type and 47 in the floral sample type, belonging to six different chemical classes, namely aliphatic compounds (29), benzenoids (9), monoterpenoids (14), sesquiterpenes (4), irregular terpenes (3) and nitrogen containing compounds (1) were found.

Qualitative differences between the leaf and flowers sample types are also demonstrated by the high number of compounds (39 out of 60 = 65%) uniquely found in leaf or flower samples only. In the leaf bouquet, the unique compounds contributed a total of 23.9%, mostly due to the high proportion of Hexyl acetate (18%), followed by (*E*)-3-Hexenyl butyrate (1.7%), whereas in open flowers Hexyl acetate was lacking and the contribution of the unique compounds to the floral bouquet was very low (6.4%) (Table 3.1). In open flowers, only three unique compounds contributed >1% to the total scent emission, namely *α*-Pinene (1.1%), *β*-Pinene (1.2%) and (*E,E*)-2,6-dimethyl-1,3,5,7-octatetraene (1%) (Table 3.1).

Quantitative differences between the sample types are demonstrated by the 21 compounds (35% of all compounds) that were found in both flowers and leaves. Their total contribution added up to 76.1% in leaf samples and 93.6% in flower samples. In both leaf and flower samples, three compounds contributed each more than 5% to the total scent. In leaves, these were (*Z*)-*β*-Ocimene (32.4%), (*Z*)-3-Hexenyl acetate (17%) and Hexan-1-ol (10.2%). In flower samples, (*Z*)-*β*-Ocimene was more dominant (63.6%), and accompanied by *β*-Linalool (5.4%) and Methyl salicylate (7.8%). The stronger predominance of (*Z*)-*β*-Ocimene results in lower evenness index for flowers (0.44) as

University of Cape Town

compared to leaves (0.62) (Table 3.1). The summarised relative amounts of the other common compounds reached about 17% in both leaves and flowers.

Table 3.1. Mean relative amounts (%) of compounds identified by GC-MS from headspace samples of leaves and open flowers of *A. karroo, A. sieberiana* var. *woodii* and *A. xanthophloea*. The number of samples in which a compound occurred is given in brackets. Scent compounds are listed according to compound class and Kovats retention index (KRI). CAS = Chemical Abstracts Service Registry Number. tr = trace amount (<0.1 % of total sample). Compound identification criteria: a = comparison of MS with published data (e.g. NIST library); b = comparison of MS and retention time with published data; c = comparison of MS and retention time with published data and authentic standard. Unknowns that did not reach at least 1% of relative amount in any sample were pooled with the superscript digit indicating the number of pooled compounds. Mass fragments for unknowns are listed with the base peak first, followed by the other fragments in order of decreasing abundance.

Compound	**KRI**	**CAS**	***A. karroo***		***A. sieberiana* var. *woodii***		***A. xanthophloea***	
			Leaves	**Flowers**	**Leaves**	**Flowers**	**Leaves**	**Flowers**
Number of samples			3	3	3	3	1	1
Number of plants used per sample			5	5	5	5	5	5
Mean scent emission per inflorescence (ng h^{-1})			-	105.1	-	924.3	-	139.7
Total number of compounds			68		75		60	
Compounds per sample type			34	61	46	63	34	47
Evenness index			0.39	0.66	0.42	0.45	0.62	0.44
Aliphatic compounds								
Aliphatic aldehydes								
(*E*)-2-Hexenal[b]	1242	6728-26-3	0.8 (2)	1.2 (3)	-	0.1 (3)	0.1 (1)	2.2 (1)
(*E,Z*)-2,6-Nonadienal[b]	1608	557-48-2	-	-	-	-	0.5 (1)	1.6 (1)
Cinnamaldehyde[a]	2043	104-55-2	-	1 (3)	-	-	-	tr
Aliphatic esters								
4-Penten-1-yl acetate[c]	1204	1576-85-8	-	-	-	1.2 (2)	-	-
Hexyl acetate[b]	1288	142-92-7	1.2 (1)	0.2 (1)	0.3 (3)	0.6 (3)	18 (1)	-
Methyl (*E*)-2-hexenoate[b]	1308	13894-63-8	-	2.8 (3)	-	-	2.5 (1)	2.3 (1)
(*Z*)-3-Hexenyl acetate[b]	1333	3681-71-8	21.7 (3)	4.2 (3)	57.0 (3)	0.2 (3)	17.0 (1)	tr
(*E*)-2-Hexenyl acetate[b]	1348	2497-18-9	0.3 (3)	0.2 (2)	-	tr	0.3 (1)	-
Methyl 3-hydroxy-3-methylbutanoate[b]	1366	6149-45-7	-	-	-	tr	-	-
Hexyl butanoate[b]	1419	2639-63-6	-	-	-	-	0.8 (1)	-
Hexyl 2-methylbutyrate[b]	1431	10032-15-2	-	-	-	-	0.3 (1)	-
Hexyl 3-methylbutanoate[b]	1446	10032-13-0	-	-	-	-	0.8 (1)	-
(*E*)-3-Hexenyl butyrate[b]	1474	53398-84-8	0.2 (3)	tr	-	-	1.7 (1)	-

University of Cape Town

Compound	KRI	CAS	*A. karroo*		*A. sieberiana* var. *woodii*		*A. xanthophloea*	
			Leaves	Flowers	Leaves	Flowers	Leaves	Flowers
(*Z*)-3-Hexenyl isovalerate[b]	1480	35154-45-1	0.7 (3)	-	-	-	0.9 (1)	-
(3*Z*)-3-Hexenyl (2*Z*)-2-butenoate[a]	1591	65405-80-3	-	-	-	-	0.4 (1)	-
Methyl 3-hydroxy-2-methylpropanoate[c]	1597	42998-03-8	-	-	-	tr	-	-
Methyl jasmonate[b]	2345	1211-29-6	-	-	-	tr	-	-
Unidentified aliphatic ester			-	-	-	-	0.7 [2]	-
Aliphatic alcohols								
Isobutanol[b]	1114	78-83-1	-	-	-	0.1 (3)	-	-
1-Penten-3-ol[b]	1165	616-25-1	-	-	-	tr	-	-
3-Methyl-3-buten-1-ol[b]	1274	763-32-6	-	-	-	0.1 (2)	-	-
Hexan-1-ol[c]	1352	111-27-3	2.3 (2)	0.8 (3)	0.2 (1)	0.2 (3)	10.2 (1)	1.3 (1)
(*E*)-3-Hexen-1-ol[b]	1374	928-97-2	tr	0.1 (3)	-	tr	-	tr
(*Z*)-3-Hexen-1-ol[b]	1387	928-96-1	1.8 (3)	3.7 (3)	3.1 (3)	0.2 (3)	2.4 (1)	0.4 (1)
(*Z*)-2-Hexen-1-ol[b]	1420	928-94-9	1.1 (2)	1.5 (2)	tr	tr	0.8 (1)	1.0 (1)
6-Methyl-1-heptanol[a]	1531	1653-40-3	tr	0.1 (2)	-	-	-	-
n-Octanol[b]	1565	111-87-5	-	-	0.2 (3)	-	-	-
(*Z*)-3-Nonen-1-ol[b]	1683	10340-23-5	-	-	-	-	-	tr
(*E*)-2-Nonen-1-ol[b]	1714	31502-14-4	-	tr	-	-	0.2 (1)	0.4 (1)
Cucumber alcohol[b]	1784	28069-72-9	-	tr	-	-	0.2 (1)	0.3 (1)
Aliphatic alkanes								
Dodecane[c]	1200	112-40-3	-	-	0.6 (1)	-	-	-
Tridecane[c]	1300	629-50-5	-	tr	0.7 (2)	tr	-	-
Tetradecane[c]	1400	629-59-4	-	-	tr	-	-	-
Pentadecane[c]	1500	629-62-9	-	-	tr	-	-	-
Heptadecane[c]	1700	629-78-7	-	-	tr	-	-	-
Nonadecane[c]	1900	629-92-5	-	-	-	tr	-	-
Eicosane[c]	2000	112-95-8	-	-	-	tr	-	-
Heneicosane[c]	2100	629-94-7	-	0.7 (3)	-	tr	-	-
Tricosane[c]	2300	638-67-5	-	0.4 (3)	tr	tr	-	-
Tetracosane[c]	2400	646-31-1	-	0.1 (1)	-	tr	-	-
Pentacosane[c]	2500	629-99-2	-	1.5 (3)	tr	0.2 (3)	tr	-
Hexacosane[c]	2600	630-01-3	tr	0.2 (2)	-	0.2 (3)	-	-
Heptacosane[c]	2700	593-49-7	tr	1.1 (3)	tr	tr	tr	-
Octacosane[c]	2800	630-02-4	-	-	-	tr	-	-
Nonacosane[c]	2900	630-03-5	tr	0.6 (3)	0.1 (3)	tr	tr	-
Aliphatic acids								
(*E*)-3-Methyl-4-decenoic acid[c]	1669	22882-86-6	-	0.3 (3)	-	-	-	-
Aliphatic ketones								
Acetoin[b]	1287	513-86-0	-	-	-	tr	-	-
(*Z*)-Cinerone[c]	1579	17190-71-5	0.1 (3)	-	-	-	-	tr

University of Cape Town

Compound	KRI	CAS	*A. karroo*		*A. sieberiana* var. *woodii*		*A. xanthophloea*	
			Leaves	Flowers	Leaves	Flowers	Leaves	Flowers
5-Methyl-5-vinyldihydro-2(3H)-furanone[b]	1683	1073-11-6	-	0.2 (3)	tr	tr	-	-
Unidentified aliphatic compounds								
m/z: 57, 82, 67, 41, 55, 39, 71, 43, 44, 69[a]	1427		-	tr	-	-	-	tr
m/z: 79, 81, 77, 41, 93, 43, 39, 91, 193, 72[a]	1506		-	-	-	-	-	0.2 (1)
Benzenoids								
Benzaldehyde[c]	1551	100-52-7	0.2 (3)	5.9 (3)	0.2 (3)	2.2 (3)	0.1 (1)	0.1 (1)
Methyl benzoate[c]	1650	93-58-3	-	0.1 (3)	tr	-	tr	0.1 (1)
Benzyl acetate[b]	1755	140-11-4	-	0.7 (3)	-	-	-	-
Methyl salicylate[c]	1806	119-36-8	0.2 (2)	0.3 (3)	0.3 (3)	tr	3.7 (1)	7.8 (1)
Ethyl salicylate[b]	1837	118-61-6	-	-	-	-	-	0.3 (1)
Benzyl alcohol[c]	1902	100-51-6	0.2 (3)	31.4 (3)	0.2 (3)	0.3 (3)	0.2 (1)	0.5 (1)
2-Phenylethyl alcohol[c]	1938	60-12-8	-	0.1 (3)	tr	tr	0.1 (1)	tr
Benzenepropyl acetate[b]	1971	122-72-5	-	0.2 (3)	-	-	-	-
p-Anisaldehyde[a]	2015	123-11-5	-	-	-	-	-	tr
Benzenepropanol[a]	2045	122-97-4	-	3.6 (3)	-	tr	-	0.2 (1)
Dimethyl salicylate[b]	2093	606-45-1	-	tr	-	tr	-	-
Cinnamyl acetate[a]	2180	103-54-8	-	0.4 (2)	-	-	-	-
Eugenol[b]	2197	97-53-0	-	tr	-	-	-	tr
Cinnamyl alcohol[b]	2300	104-54-1	-	8.5 (3)	-	-	-	0.2 (1)
Benzyl benzoate[b]	2655	120-51-4	-	-	-	tr	-	-
Monoterpenoids								
α-Pinene[c]	1087	80-56-8	-	-	-	-	-	1.1 (1)
β-Pinene[c]	1137	127-91-3	-	-	-	-	-	1.2 (1)
Limonene[c]	1225	138-86-3	-	0.4 (3)	0.6 (3)	tr	-	-
(*Z*)-*β*-Ocimene[b]	1252	3338-55-4	62.4 (3)	7.8 (3)	7.6 (3)	2.5 (3)	32.4 (1)	63.6 (1)
(*E*)-*β*-Ocimene[b]	1253	3779-61-1	1.7 (3)	-	-	-	1.0 (1)	0.6 (1)
1,3,8-p-Menthatriene[b]	1385	21195-59-5	tr	-	-	-	-	tr
(*E*)-Linalool oxide (furanoid)[c]	1454	34995-77-2	0.3 (3)	0.9 (3)	15.0 (3)	51.9 (3)	-	0.2 (1)
(*E,E*)-2,6-Dimethyl-1,3,5,7-octatetraene[a]	1466	460-01-5	1.0 (3)	0.3 (3)	-	-	-	1.0 (1)
(*Z*)-Linalool oxide (furanoid)[c]	1486	5989-33-3	-	0.6 (3)	1.9 (3)	6.7 (3)	-	0.3 (1)
2,2,6-Trimethyl-6-vinyldihydro-2H-pyran-3(4H)-one[c]	1491	33933-72-1	-	-	-	1.7 (3)	-	-
β-Linalool[c]	1556	78-70-6	0.8 (3)	1.6 (3)	0.5 (3)	0.2 (3)	2.3 (1)	5.4 (1)
Isobornyl acetate[a]	1610	125-12-2	-	tr	-	-	-	-
Hotrienol[b]	1623	29957-43-5	-	-	-	-	-	0.1 (1)
1-Menthol[b]	1651	2216-51-5	-	tr	-	-	-	-
α-Terpineol[b]	1708	98-55-5	-	tr	tr	tr	-	-

University of Cape Town

Compound	KRI	CAS	*A. karroo*		*A. sieberiana* var. *woodii*		*A. xanthophloea*	
			Leaves	Flowers	Leaves	Flowers	Leaves	Flowers
(*E*)-Linalool oxide (pyranoid)[c]	1720	39028-58-5	tr	tr	7.2 (3)	13.4 (3)	-	tr
Borneol[b]	1725	507-70-0	-	-	tr	-	-	-
6,6-Dimethyl-2-methylene-Bicyclo[3.1.1]heptan-3-ol[a]	1731	5947-36-4	tr	-	-	-	-	-
Verbenone[a]	1740	18309-32-5	tr	-	tr	-	-	-
(*Z*)-Linalool oxide (pyranoid)[a]	1775	14049-11-7	-	-	2.3 (3)	4.6 (3)	-	0.4 (1)
Nerol[b]	1810	106-25-2	-	-	-	tr	-	-
(*Z,Z*)-2,6-Dimethyl-3,5,7-octatriene-2-ol[a]	1824	1174030-42-2	tr	-	-	-	-	-
(*E,E*)-2,6-Dimethyl-3,5,7-octatriene-2-ol[a]	1836	206115-88-0	0.5 (3)	0.2 (3)	tr	-	0.6 (1)	0.5 (1)
2,6-Dimethyl-3,7-Octadiene-2,6-diol[b]	1945	13741-21-4	-	tr	-	tr	-	0.1 (1)
(*Z*)-Jasmone[b]	1963	488-10-8	-	-	tr	0.1 (3)	-	-
2,6-Dimethyl-1,7-octadiene-3,6-diol[b]	2135	51276-33-6	-	-	-	tr	-	-
Sesquiterpenoids								
(*E*)-*α*-Bergamotene[c]	1584	13474-59-4	-	-	-	tr	-	-
Bergamotene[c]	1593	17699-05-7	-	-	tr	0.2 (3)	-	-
Longifolene[a]	1600	475-20-7	0.2 (3)	tr	0.1 (3)	-	-	-
α-Bergamotene[c]	1610	17699-05-7	-	-	0.3 (3)	1.4 (3)	-	-
β-Caryophyllene[c]	1625	87-44-5	0.2 (3)	0.2 (2)	tr	tr	-	-
(*Z*)-*β*-Farnesene[a]	1665	28973-97-9	-	-	tr	0.4 (3)	-	-
Germacrene D[b]	1724	23986-74-5	-	-	tr	-	-	-
(*Z,E*)-*α*-Farnesene[b]	1726	26560-14-5	tr	0.1 (3)	-	-	-	0.2 (1)
β-Sesquiphellandrene[b]	1727	20307-83-9	-	-	-	tr	-	-
α-Farnesene[b]	1765	502-61-4	1.6 (3)	10.0 (3)	-	tr	1.3 (1)	3.9 (1)
β-Bisabolene[b]	1786	495-61-4	-	-	-	tr	-	-
(*Z*)-Nerolidol[b]	2034	142-50-7	-	-	tr	tr	-	-
2,3-Dihydrofarnesol[c]	2177	51411-24-6	-	0.3 (1)	-	-	-	-
Farnesyl acetate[b]	2283	4128-17-0	-	0.1 (3)	0.1 (3)	1.7 (3)	-	tr
Farnesol[b]	2354	4602-84-0	-	-	tr	tr	-	-
Unidentified sesquiterpenes	1652		-	-	tr [1]	0.9 [1]	-	tr [1]
Irregular terpenes								
6-Methyl-5-hepten-2-one[b]	1354	110-93-0	-	3.1 (3)	0.3 (3)	0.4 (3)	0.3 (1)	0.6 (1)
2,6-Dimethyl-6-octanol[a]	1437	78-69-3	-	tr	-	-	-	-
2,6-Dimethyl-7-octen-2-ol[a]	1474	18479-58-8	-	tr	tr	-	-	-
Dihydro-*β*-ionone[b]	1842	17283-81-7	-	-	-	-	-	tr
(*E*)-*α*-Ionone[b]	1879	127-41-3	-	0.3 (3)	-	-	-	-
β-Ionone[b]	1986	14901-07-6	-	-	-	-	-	tr

University of Cape Town

Compound	KRI	CAS	*A. karroo*		*A. sieberiana* var. *woodii*		*A. xanthophloea*	
			Leaves	Flowers	Leaves	Flowers	Leaves	Flowers
Nitrogen containing compounds								
Benzyl nitrile[b]	1939	140-29-4	-	-	-	tr	-	-
2-Piperidinone[c]	2156	675-20-7	-	1.0 (1)	-	-	-	-
Indole[c]	2478	120-72-9	0.1 (3)	0.3 (3)	0.7 (3)	7.5 (3)	tr	0.8 (1)

3.3.4. Scent variation between species

Despite an apparent higher scent emission rate of *A. sieberiana* var. *woodii* compared to that of *A. karroo* and *A. xanthophloea*, and possibly due to a small sample size, the statistical methods used did not detect a significant difference in the mean scent emission rates from flowers of *A. karroo*, *A.sieberiana* var. *woodii* and *A. xanthophloea* ($F_{(2, 4)} = 2.047$, $p = 0.244$; $n = 3$ for *A. karroo* and *A.sieberiana* var. *woodii*; $n = 1$ for *A. xanthophloea*) (Fig. 3.1). Large differences between species as well as between leaf and flower samples within species were however, evident when comparing the relative amounts of compound classes that reflect different biosynthetic pathways (Fig. 3.2). In *A. karroo* the relative amounts of benzenoids were much higher in flowers than in leaves (51.2% and 0.6% respectively). Sesquiterpenoids followed a similar pattern (10.8% vs. 2%), whereas monoterpenoids were more dominant in leaf emissions (66.8%) than in flower scent (12.1%). In *A. sieberiana* var. *woodii* main differences between leaf and floral volatile profiles were mainly due to aliphatic compounds (62.4% in leaf scent vs. 3.7% in floral scent), followed by monoterpenes (35.1% vs. 81.2%) and nitrogen containing compounds (0.7% vs. 7.5%). Also in *A. xanthophloea,* differences between the leaf and floral volatile profiles were mainly due to aliphatic compounds (57.8% for leaves vs. 10.2% for flowers) complemented by differences of monoterpenoids (36.3% vs. 74.7%).

The overall separation of the plant volatile profiles, based on Bray-Curtis similarities of the leaves and flowers for *A. karroo*, *A.sieberiana* var. *woodii* and *A. xanthophloea* was highly significant (in all three cases: 2D-NMDS stress value = 0; ANOSIM Global $R = 1$, $p < 0.01$.). The dissimilarity indices calculated by the SIMPER method revealed a dissimilarity index of 65.3 between the leaf and floral

University of Cape Town

profiles of *A. karroo*, 36.2 between the leaf and floral profiles of *A.sieberiana* var. *woodii*, and 48.4 between the leaf and floral profiles of *A. xanthophloea.* These results correlate well with the distribution of compounds per compound class as seen in Fig. 3.2.

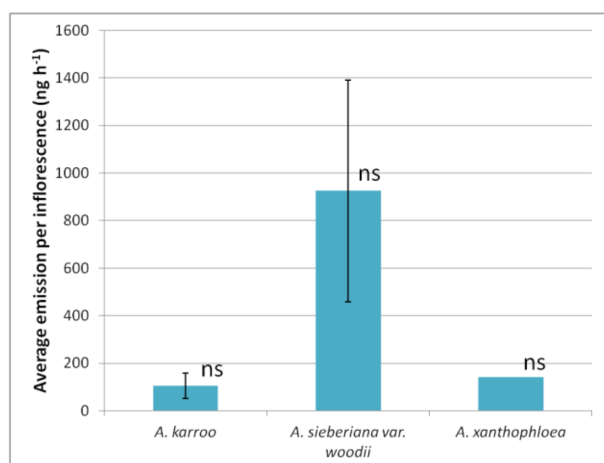


Fig. 3.1. Volatile emission rates (ng h^{-1}) per inflorescence (mean ± SE) of open flowers of *A. karroo*, *A.sieberiana* var. *woodii* and *A. xanthophloea*. ns = no significant differences between emission rates.

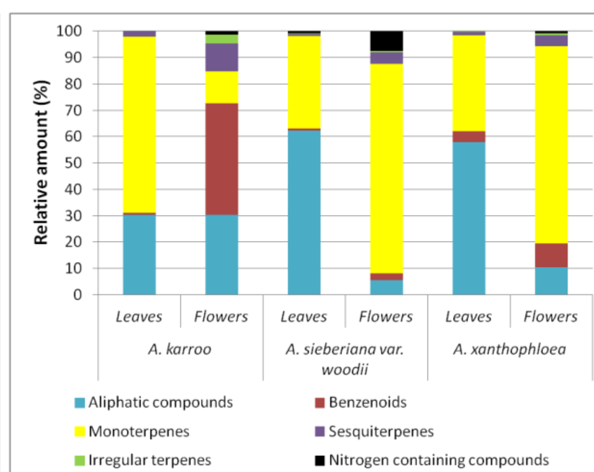


Fig. 3.2. Mean proportions of compound classes in headspace samples of leaves and flowers of *A. karroo*, *A.sieberiana* var. *woodii* and *A. xanthophloea*.

University of Cape

3.4. Discussion

Kaiser (1997) identified 48 compounds (compared to the 62 in this study) in the floral scent of *A. karroo* from one sample taken in Nelspruit, Mpumalanga, South Africa. Twenty-two compounds were common between the results of his study and the present study. Kaiser (1997) highlighted four compounds as being of particular interest namely (*Z*)-3-Methyl-3-decenoic acid, Methyl (*E*)-3-methyl-4-deconoate, Methyl (*Z*)-3-methyl-4-deconoate and Methyl (*Z*)-cascarillate. The first compound was identified many years before in the absolute oil of *A. farnesiana*, and the latter three compounds were never before described as occurring naturally (Kaiser, 1997). These four compounds were not found in the floral profile identified during this study. There are a number of possible reasons to explain the differences found in the results of the two studies. Firstly, differences may be due to geographical origin (population differences) (Jhumur et al., 2008) as Kaiser used a

tree from the Botanical Garden in Nelspruit, Mpumalanga (the eastern most province in South Africa), whereas in this study trees were sampled much further South and West. *Acacia karroo* has a large variety of growth forms and other morphological traits which have been shown to have a genetic basis (Archibald & Bond, 2003). The chemical composition of *A. karroo* has been demonstrated to differ in populations in different geographical regions (Malan & Swartz, 1995) and *A. karroo* has been divided into a number of subspecies or even species (Ward, 2011). It is therefore possible that genetic variation (Tollsten & Bergström, 1993) might also have contributed to the different volatile profiles. A seasonal effect (Pecetti et al., 2004) is also possible because Kaiser's single sample was taken in November, the summer rainfall season of the Mpumalanga province, compared to December/January, which is the dry summer season in the Western Cape. Finally, different methods for scent sampling and analyses may have contributed to the differences. Unfortunately Kaiser (1997) did not describe his method in all detail, particularly with respect to the duration of sampling, analysis using GC-MS, and the type of chromatography column used. In this study three samples from five trees each, thus representing 15 trees in total, were analysed which may explain the much higher number of compounds found as compared to Kaiser (1997).

University of Cape Town

3.4.1. Leaf and flower volatiles in plant defence

The functional significance of the chemical profiles of leaves may relate to the defence mechanisms of the plants against herbivores (Pichersky & Gershenzon, 2002; Lucas-Barbosa et al., 2011; Schiestl, 2010; Büchel et al., 2011). Despite the leaf volatile profile of *A. karroo* differing from that of *A. sieberiana* var. *woodii* and *A. xanthoephloea* in the composition of the component classes, the components contributing to the differences, were the same compounds, albeit present in different ratios. (*Z*)-*β*-Ocimene, a monoterpene dominated the scent profile of the leaves of *A. karroo* whereas (*Z*)-3-Hexenyl acetate, an aliphatic compound dominated the scent profile of the leaves in *A. sieberiana* var. *woodii*. Both (*Z*)-*β*-Ocimene and (*Z*)-3-Hexenyl acetate are commonly occurring plant volatiles (Knudsen et al., 2006), and since they occurred at very high relative amounts in the

leaves of *A. karroo*, *A. sieberiana* var. *woodii* and *A. xanthoephloea*, it is suggested that they play a role in plant defence in these three species, in line with their commonly-accepted role in plant defence (Pichersky & Gershenzon, 2002; Bouvier et al., 2005; Knudsen & Gershenzon, 2006; Castells & Berenbaum, 2008; Mumm & Dicke, 2010; Büchel et al., 2011). In addition to (*Z*)-*β*-Ocimene and (*Z*)-3-Hexenyl acetate, Hexyl acetate occurred in a high relative amount in the leaf volatile profile of *A. xanthophloea*. This compound had been shown to be induced by pathenogenic attack (Piesik et al., 2011) and to stimulate antennal responses in the vetch aphid (Visser & Piron, 1995). Since Hexyl acetate was not detected in the floral volatile profile of *A. xanthophloea*, a role in plant defence is suggested for this compound.

University of Cape Town

(*Z*)-*β*-Ocimene was found in a high relative amount in the floral scent of *A. xanthophloea,* being almost twice that of the leaf profile. Although (*Z*)-*β*-Ocimene is often associated with plant damage (Bouvier et al., 2005), Pecetti et al. (2004) reported on some attractive effect of Ocimene in lucerne (*Medicago sativa* L.) on honey bees. The role of (*Z*)-*β*-Ocimene in such high relative amount in the flower scent of *A. xanthophloea*, compared to the leaf profile, requires further investigation.

3.4.2. Flower volatiles in pollinator attraction

Benzyl alcohol, a common floral compound (Knudsen et al., 2006) occurring in the floral scent of *A. karroo* also occurred in the floral scent of *A. saligna* in high relative amounts. This compound often occurs in plants that are butterfly pollinated (Dobson, 2006; Jürgens et al., 2009; Schiestl, 2010). Cinnamyl alcohol is another compound found in a high relative amount in the floral scent of *A. karroo*, but not in the leaf profile. The odour of Cinnamyl alcohol (alternative name: 3-Phenyl-2-propen-1-ol) was historically linked with plants producing ‘aromatic’ scents that are sweet and spicy (Griffiths et al., 1999). Kaiser (1997) describes the scent of *A. karroo* as “a rich aromatic-floral fragrance rounded up by Ionone-floral, green and waxy notes”. The scent of Cinnamyl alcohol is an attractant to various coleopteran species and often used as bait in traps in an agricultural setup

(Ventura et al., 2000; Plepys et al., 2002; Tóth et al., 2004). Cinnamyl alcohol may also play a role in attracting pollinating honey bees and bumble bees of blueberry flowers (Rodriguez-Saona et al., 2011). α-Farnesene, a sesquiterpene which also occurred in the floral scent of *A. karroo*, typically occurs widely in floral volatiles (Knudsen et al., 2006) and is among the most typical inducible sesquiterpenes emitted by herbivore-damaged plants (Rodriquez-Saona et al., 2001; Holopainen, 2004; Effmert et al., 2005). Codling moth females use various isomers of α-Farnesene to locate suitable oviposition sites and the larvae also follow these compounds to locate the fruit they feed on (Thompson & Pellmyr, 1991; Landolt et al., 2000). In aphids and ants α-Farnesene is a known compound of alarm pheromones (Willmer et al., 2009). α-Farnesene is also attractive to bees, including *Apis mellifera* (Blight et al., 1997) that are flower visitor of all acacias (Stone et al., 2003). It is suggested that all three these compounds, Benzyl alcohol, Cinnamyl alcohol and α-Farnesene to a greater or lesser extent, play a role in pollinator attraction in *A. karroo*.

Unlike any of *A. karroo* and *A. xanthophloea* or any of the four Australian *Acacia* species evaluated (Chapter 2), a large proportion (76.7%) of the floral volatile profile of *A. sieberiana* var. *woodii* is made up of β-Linalool and (*E*)-Linalool oxide (furanoid), (*Z*)-Linalool oxide (furanoid), (*E*)-Linalool oxide (pyranoid) and (*Z*)-Linalool oxide (pyranoid), which are the Linalool derivatives (Raguso & Pichersky, 1999; Matich et al., 2003). The Linalool derivatives are flavour compounds found in papaya, grapes, wine and tea leaves and are used in perfumery and enology (Raguso & Pichersky, 1999). The scent of linalool has been described as "light and refreshing, floral-woody, with a faint citrusy note" (Kamatou & Viljoen, 2008). To the human nose, the scent of *A. sieberiana* var. *woodii* is pleasant and reminescent of the odour of a mixture of tropical fruits such as kiwi, guava, pineapple and papaya (Personal observation) which can probably be ascribed to the Linalool oxides. Both furanoid and pyranoid forms of Linalool oxide are widespread in floral volatiles (Knudsen et al., 2006) and the (*Z*)- and (*E*)-forms of both furanoid and pyranoid Linalool oxides are often found co-occurring in plant scent (Lewis et al., 2003; Jürgens & Dötterl, 2004; Johnson et al., 2007; Jürgens et

University of Cape Town

al., 2008; and several more). The furanoid : pyranoid ratio in *A. sieberiana* var. *woodii* was 1.8:1 for the leaf volatile profile and 3.3:1 for the floral volatile profile. High amounts of Linalool and the Linalool oxides were also found in the floral scent of *Vachellia seyal seyal, V. seyal fistula, V. etbaica, V. brevispica* and *Senegalia mellifera* (Willmer et al., 2009). Furanoid and pyranoid Linalool oxides produce electroantennal responses in moths and butterflies (Raguso & Light, 1998; Andersson & Dobson, 2003). Also, Linalool from which the Linalool oxides derives, is often found in the scent of flowers that are pollinated by moths, bees, beetles and butterflies (Jürgens et al., 2003; Dobson, 2006; Dötterl et al., 2006; Theis, 2006; Jürgens et al., 2009). Generally, fruity odours attract beetles as pollinators (Harborne, 2001). These compounds together with their very high emission rate may reflect plant adaptations to pollinators (Andersson & Dobson, 2003).

There is however no specific information available about the pollinators of *A. sieberiana* var. *woodii* and it is therefore uncertain what the nature of the pollinator assemblage of this species is. Abdullahi et al. (2011) reported honeybees as foraging for nectar on, amongst others, *A. sieberiana* var. *woodii* trees, and, by implication, honeybees might therefore also be pollinators of *A. sieberiana* var. *woodii*. High levels of emission of volatiles may increase the attractiveness of the plant to its pollinators and thereby increase reproductive success (Dötterl et al., 2006).

University of Cape Town

CHAPTER 4: ANTENNAL RESPONSE OF *DASINEURA DIELSI* TO FLORAL SCENT COMPOUNDS OF ITS HOST

4.1. Introduction

Considering that adult midges are weak flyers (Kolesik, 2000), and must find a suitable host within the short period of their ephemeral life span, they need clear cues to indicate the presence of the correct host plant species and, at least in the case of *D. dielsi*, with suitable flowers. The inflorescences of *A. cyclops* are bright yellow and large enough to be visually apparent, but they are largely visually-indistinguishable from many other co-occurring *Acacia* species and are generally obscure among the foliage of the plants on which they are borne. These features emphasize that olfactory cues are likely to play a central role in the interaction between *D. dielsi* and *A. cyclops*. Nothing is however known about the olfactory responses of *D. dielsi* to volatile chemicals emitted by the flowers of *A. cyclops* and their role for host finding.

University of Cape Town

In order to test whether olfactory cues play a role in the ability of *D. dielsi* to locate suitable oviposition sites, and thereafter to identify which compounds in the scent of *A. cyclops* may be attractants to the midge, it was decided to employ electrophysiological experiments to screen for the volatile compounds that the insects can detect (Schoonhoven et al., 2005). Many electrophysiological studies have been done on midges; however, most of them have focused on the identification of the chemical compounds of sex pheromones (Amarawardana, 2009 and many references therein). In contrast, not many electrophysiological studies have been done on the responses of female midges to host plant volatiles, the exceptions being Anfora et al. (2005) on *D. mali* and Birkett et al. (2004b) on the orange wheat blossom midge.

For *D. dielsi*, the first objective was to use electroantennography (EAG) to test the antennal responses of *D. dielsi* to a broad spectrum of the most prominent compounds (>5% relative amount)

of the six compound classes in the floral scent of *A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. saligna* (Chapter 2), along with compounds Anfora et al. (2005) tested on *D. mali*, the Apple leaf curling midge. Although the focus of the project was on the olfactory responses of *D. dielsi* females to host plant volatiles, male midges were included in this experiment to assess whether there may be gender related differences in olfactory responses to host plant volatiles. The second objective was to screen for those compounds in the scent of *A. cyclops* that *D. dielsi* is responsive to on an electrophysiological level using gaschromatography coupled with electroantennogram detection (GC-EAD).

University of Cape Town

4.2. Methods and Materials

Dasineura dielsi galls were collected on 10 January 2011 and 27 January 2011 from *A. cyclops* trees at the Koeberg Nature Reserve (S 33° 39' 14.8", E 18° 26' 00.4") near Melkbosstrand in the Western Cape, South Africa. The galls were placed in lightweight, transparent, fabric, emergence bags and kept in the refrigerator at 7°C until a day before insects were needed (Adair, 2004). Galls were then moved to the laboratory under room temperature (summer) to allow the developing midges to complete their development and to emerge from the galls as adults thereby providing a constant supply. Midges were removed from the emergence bags by coaxing them into a glass tube from where they were transferred to small Eppendorf vials and held until needed for the electrophysiological studies.

4.2.1. Electroantennograms (EAG)

Seven male and seven female adult *D. dielsi* midges were used for the EAG experiments which were conducted between 12 January 2011 and 21 January 2011. For measurements, the excised head of a midge with both antennae (Fig. 4.1) was mounted between glass micropipette electrodes filled with insect ringer solution (8.0 g/l NaCl, 0.4 g/l KCl, 0.4 g/l $CaCl_2$) (Fig. 4.1). The head was inserted in the

indifferent electrode and the tips of both antennae were inserted in the different (recording) electrode. Signals were interfaced with a 2-channel USB acquisition controller) provided by Syntech (Hilversum, The Netherlands) to a personal computer.

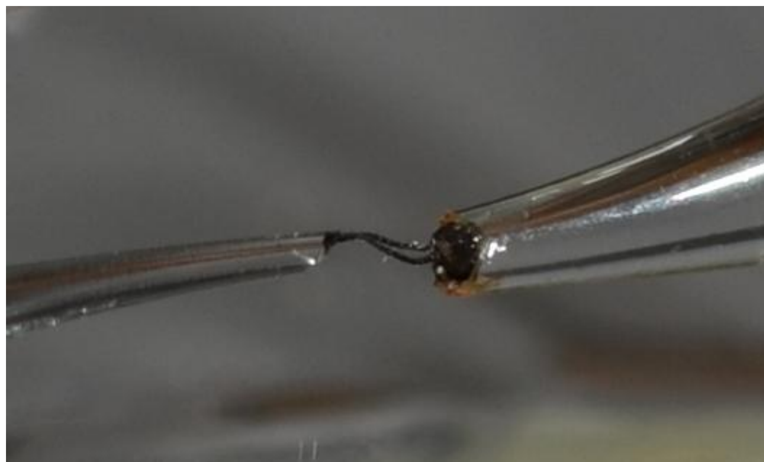


Fig. 4.1. Excised head with antennae of *D. dielsi* mounted between the EAG micro capillary electrodes. Antennal size from scape to tip is approximately 725 µm in length. (Photo: S.D. Johnson)

University of Cape Town

Compounds belonging to the six compound classes were identified from the floral scent profiles of *A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. saligna* (Chapter 2) and from Anfora et al. (2005) and were purchased as authentic standards from commercial suppliers (Table 4.1). Dilutions of 1% were prepared in paraffin oil (alpha Pharm).

A test suite of 16 individual compounds (Table 4.1) was compiled with a reference standard compound, Methyl benzoate and two controls, paraffin oil and air. The reference standard was used for normalization of the results. Methyl benzoate was selected from the compounds identified in the floral scent of *A. cyclops*. The sequence of compounds in the test suite started with air, paraffin oil and Methyl benzoate (reference standard). The other sixteen compounds were included in batches of four compounds with Methyl benzoate being included again after each batch. The sequence was ended with paraffin oil and air as controls. The reference standard was included to control for the potential decrease in antennal sensitivity during the measurements and differences in overall sensitivity between individual antennae. The sequence of the sixteen compounds was randomised

for each new test to eliminate the effect of possible physiological bias towards a compound. The whole test suite was tested on each antennal preparation.

Table 4.1. Compounds with relative amounts >5% in the floral scent profiles of *A. cyclops* (CY), *A. melanoxylon* (ME), *A. longifolia* (LO), *A. saligna* (SA) were purchased as authentic standards from commercial suppliers and were compiled into a test suite for EAG experimentation. Class denotes the compound class for the compound (AC = Aliphatic compound, BC = Benzenoid compound; MT = Monoterpene, ST = Sesquiterpene; IT = Irregular terpene; NCC = Nitrogen containing compound). Source indicates the volatile profile the compound was selected from or the study it was selected from (DM = *Dasineura mali*, Anfora et al., 2005).

Compound	Class	Source	Purity	Supplier
(*Z*)-3-Hexenyl acetate	AC	CY, ME, LO, DM	≥98%	SAFC, supply Solutions, Japan
(*Z*)-3-Hexen-1-ol	AC	ME, LO, SA, DM	≥98%	Sigma-Aldrich, Steinheim, Germany
C_8-C_{20} Alkane standard solution	AC	CY, ME, LO, SA, DM	-	Fluka-Sigma-Aldrich
C_{21}-C_{40} Alkane standard solution	AC	CY, ME, LO, SA, DM	-	Fluka-Sigma-Aldrich
p-Anisaldehyde	BC	ME	≥99%	Fluka-Sigma-Aldrich, Steinheim, Germany
Benzaldehyde	BC	ME, DM	≥99%	Merck Schuchardt, Hohenbrunn, Germany
Benzyl alcohol	BC	LO, SA, DM	≥99%	Sigma-Aldrich
Methyl salicylate	BC	DM	≥98%	Sigma-Aldrich
Methyl benzoate[1]	BC	CY	≥99.5%	Fluka-Sigma-Aldrich, Steinheim, Germany
Limonene	MT	ME, LO, SA, DM	≥95%	Fluka
Ocimene mixture of isomers	MT	CY, ME, LO, SA, DM	≥90%	SAFC, supply Solutions, Sigma-Aldrich, Steinheim, Germany
α-Pinene	MT	SA	≥99%	Fluka-Sigma-Aldrich
β-Linalool	MT	ME, LO, SA, DM	≥95%	Fluka-Sigma-Aldrich, Steinheim, Germany
β-Pinene	MT	ME, DM	≥99%	Fluka-Sigma-Aldrich
4-Oxoisophorone	IT	CY, SA	98%	Sigma-Aldrich, Steinheim, Germany
β-Caryophyllene	ST	ME, DM		Sigma-Aldrich
Indole[2]	NCC	-	≥99%	Sigma-Aldrich, Steinheim, Germany
Paraffin Oil[3]	-	-	-	alpha Pharm

Notes:
[1]: Reference compound
[2]: Compound selected for inclusion as compound class representative
[3]: Dilutant

Two microliters of a 1% dilution (in paraffin oil) of each test compound were placed onto a piece of chromatography paper (approx. 75mm x 3 mm) and inserted in a Pasteur pipette that served as

University of Cape Town

holder for the odour source. Separate pipettes were used for each compound. Stimuli were released into a continuous flow of humidified air that passed over the antenna with pulse duration of 0.5 sec, and a flow of 10 ml/sec regulated by a CS-05 Stimulus Controller (Syntech, Hilversum, Netherlands). In all EAG tests, antennae were stimulated at about 20 - 30 sec intervals.

4.2.2. Gas chromatography coupled to electroantennographic detection (GC-EAD)

Plant material was collected on 25, 26 and 27 January 2011 from randomly selected *A. cyclops* trees at the Koeberg Nature Reserve (S 33° 39' 14.8", E 18° 26' 00.4") near Melkbosstrand in the Western Cape, South Africa. Despite the risk of obtaining potentially high levels of green leaf volatiles due to wounded tissue (Grison et al., 1999; Arimura et al., 2001), open flowers were picked from the plant material in order to eliminate the effect of leaf and other flower stage volatiles. The flowers removed from the plant cuttings were enclosed within a polyacetate oven bag (25 cm × 30 cm; Kalle Bratschlauch, Wiesbaden, Germany). Volatiles were trapped in a large adsorbent tube which was filled with 120 mg of Tenax-TA® (mesh 60–80, Supelco, Bellefonte, USA) by drawing air through the bag with a membrane pump (Spectrex PAS-500, Redwood City, California, USA) with a flow rate of 80 ml min^{-1} for 45 hours and 30 min. After each 15 hour period, the plant material was replaced. Volatiles were eluted with 200 µl of Acetone (SupraSolv, Merck, Germany) and stored in a freezer at −80°C for later use in the electrophysiological analyses.

University of Cape Town

The GC-EAD experiments were done between 31 January 2011 and 10 February 2011. The GC-EAD system consisted of a gas chromatograph (Varian CP-3800 GC (Varian, Palo Alto, California) equipped with a flame ionization detector (FID), and an EAD setup (heated transfer line, 2-channel USB acquisition controller) provided by Syntech (Hilversum, Netherlands). An aliquot (2 µl) of the floral scent sample (headspace samples in Acetone as a solvent) were injected into a quartz vial. The vial with the scent sample was loaded into the injector of the gas chromatograph (Varian CP-3800 gas

chromatograph, Palo Alto, California) by means of a ChromatoProbe device (Amirav & Dagan, 1997). The scent sample was injected splitless at 60 °C, followed by opening the split vent after 1 min and heating the oven at a rate of 10 °C min^{-1} to 200 °C. The end temperature was held for 5 min. A Varian FactorFour capillary column (DB5-column) was used for the analyses (length 30 m, inner diameter 0.25 mm, film thickness 0.25 µm). The column was split at the end by the four arm flow splitter GRAPHPACK 3D/2 (Gerstel, Mülheim, Germany) into two pieces of deactivated capillary (length 50 cm, inner diameter 0.25 mm) leading to the FID and EAD setup. Makeup gas (He, 16 ml min^{-1}) was introduced through the fourth arm of the splitter. For measurements, the excised head of a midge with both antennae (Fig. 4.1) was mounted between glass micropipette electrodes filled with insect ringer solution (8.0 g/l NaCl, 0.4 g/l KCl, 0.4 g/l $CaCl_2$) for the EAG experiments. The electrodes were held with micromanipulators (Syntech MP15) and connected to a high impedance input AC/DC amplifier (model UN-06; Syntech, Hilversum, The Netherlands). Effluent from the EAD port was eluted into a purified, humidified air stream that passed over the excised head with antennae. Amplified EAD responses were digitized and shown and processed using the AutoSpike software (Syntech). Due to the small size of the antennae and the difficulties in connecting the antennae between the electrodes, the results obtained were not tested for repeatability and a compound was treated as EAD active on achieving the first clear result.

University of Cape Town

4.2.3. Gas Chromatography-Mass Spectrometry (GC-MS) analyses

To identify the compounds eliciting signals in the insect antennae, 2 µl of the floral scent sample (headspace samples in Acetone as a solvent) were injected into a quartz vial. The vial with the scent sample was loaded into the injector of the gas chromatograph (Varian CP-3800 gas chromatograph, Palo Alto, California) by means of a ChromatoProbe device (Amirav & Dagan, 1997). The GC was equipped with a 30 m x 0.25 mm internal diameter (film thickness 0.25 µm) Alltech DB5 column coupled to a Varian 1200 quadrupole mass spectrometer in electron-impact ionization mode at 70eV (Shuttleworth & Johnson, 2010a). The flow of helium carrier gas was 1 ml min^{-1}. The injector was

held at 40 °C for 2 min with a 20:1 split and then increased to 200 °C at 200 °C min^{-1} in split-less mode for thermal desorption. The temperature of the GC oven was held for 3 min at 40 °C, whereafter it was ramped up to 240 °C at 10 °C min^{-1} and held there for 12 min. Identification of compounds included the use of the Varian Workstation software with the NIST05 mass spectral library (NIST/EPA/NIH Mass Spectral Library (data version: NIST 05); MS search software version 2.0 d) as well as verification by using retention times of authentic standards and published Kovats indices (references in the NIST 05 library; El-Sayed, 2011) wherever possible.

4.2.4. Statistical analyses

EAG amplitudes in response to the compounds were expressed in relation to the responses to the reference standard (Methyl benzoate) because of the differences in overall sensitivity between individual antennae, and to compensate for the decline in antennal sensitivity during a measuring session. In this normalization procedure, responses to the reference compound were defined as 100%. Response values obtained between two references were corrected by linear interpolation. To determine if there were significant differences in the mean responses to the different compounds, one-way ANOVA, followed by the Tukey multiple range test ($\alpha = 0.05$) was used after the data was square root-transformed to improve normality and homescedasticity of the data. Student t-tests ($\alpha = 0.05$) were applied to compare the responses of male and female insects to each individual compound.

University of Cape Town

4.3. Results

4.3.1. Electroantennograms (EAG)

Electroantennogram (EAG) responses to female antennae (Fig. 4.2) showed significantly different responses to *β*-Linalool, 4-Oxoisophorone, Limonene and (*Z*)-3-Hexenyl acetate compared to the air control, and showed significantly different responses to these four compounds and Methyl salicylate

compared to the paraffin oil control ($F_{(18, 114)} = 13.107$, $p < 0.001$; n = 7). The responses of the male antennae (Fig. 4.3) showed significantly different responses in a different set of compounds than those shown in the female responses.

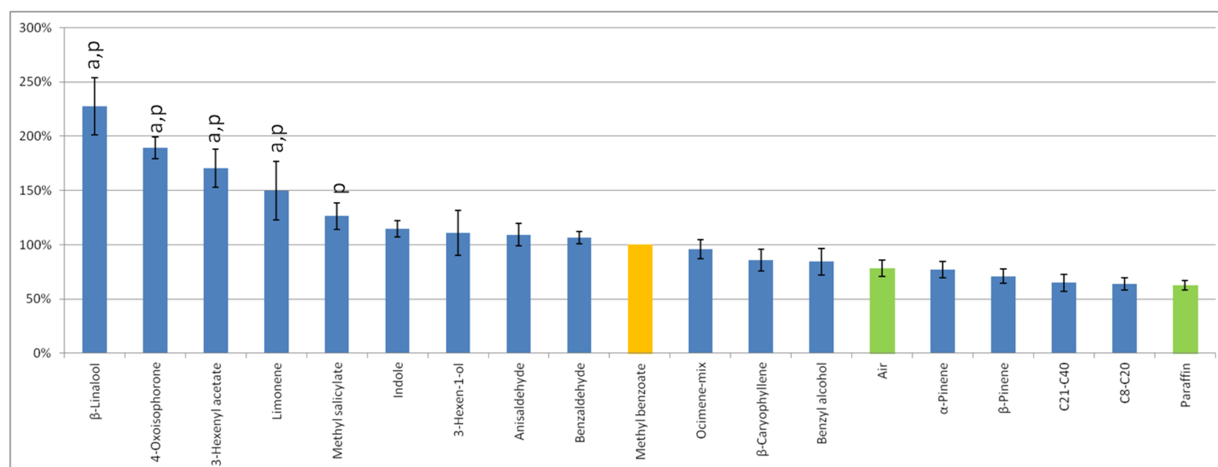


Fig. 4.2. Order of response magnitude (mean ± SE) to authentic standards in EAG experiments with female *D. dielsi* midges. The yellow bar indicates the reference compound, and the green bars indicate the controls. Compounds marked with **a** are significantly different from the air control at $p < 0.001$ except for Limonene with $p = 0.012$. Compounds marked with **p** are significantly different from the paraffin control at $p < 0.001$ except for Methyl salicylate with $p = 0.007$. (Tukey HSD, $F_{(18, 114)} = 13.107$, $p < 0.001$; n = 7).

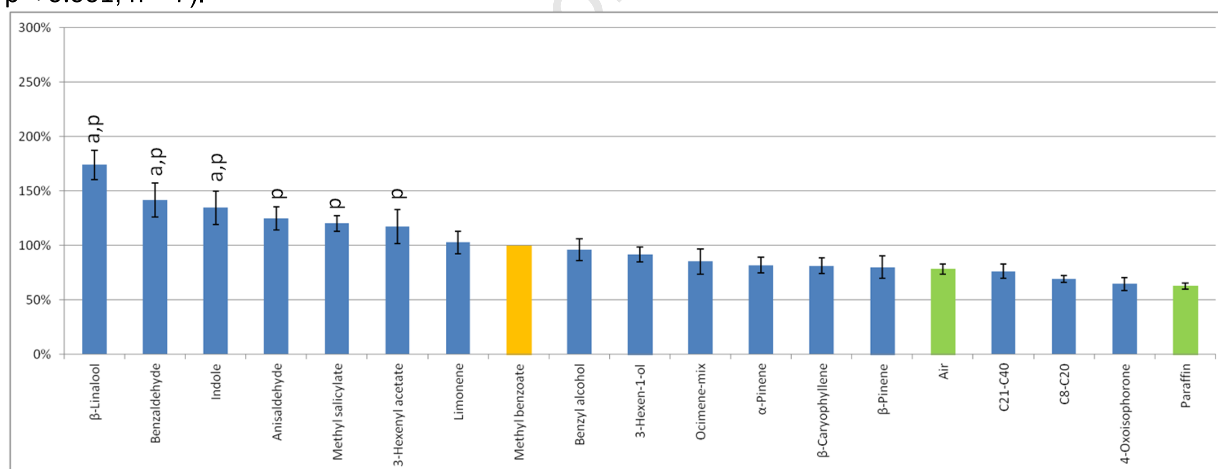


Fig. 4.3. Order of response magnitude (mean ± SE) to authentic standards in EAG experiments with male *D. dielsi* midges. The yellow bar indicates the reference compound, and the green bars indicate the controls. Compounds marked with **a** are significantly different from the air control at $p < 0.01$ except for Indole with $p = 0.016$. Compounds marked with **p** are significantly different from the paraffin control at $p < 0.01$. (Tukey HSD, $F_{(18, 114)} = 8.974$, $p < 0.001$, n = 7)

The responses that were different to the air control were to compounds *β*-Linalool, Benzaldehyde and Indole, and in addition to these three compounds the responses different to paraffin oil included

(*Z*)-3-Hexenyl acetate, Methyl salicylate and *p*-Anisaldehyde ($F_{(18, 114)} = 8.974$, $p < 0.001$, $n = 7$). For the compounds that elicited positive antennal responses, the magnitude of the male responses was generally lower than that of the female responses. Comparing the responses of male and female antennae for each individual compound showed no significant differences for any of the compounds except for 4-Oxoisophorone ($p < 0.05$).

4.3.2. Gas Chromatography-Mass Spectrometry (GC-MS)

The chemical composition of the solvent samples prepared in Acetone for GC-EAD experimentation is summarised in Table 4.2. Forty-four compounds were identified which belonged to five compounds classes, namely Aliphatic compounds (27), Benzenoid compounds (1), Monoterpenes (7), Sesquiterpenes (4) and Irregular terpenes (5). Aliphatic compounds made up 92.3% of the total relative amounts of volatiles emitted by the flowers. The n-Alkanes, being part of that group, made up 89.4% of the total relative amounts of volatiles. Only 28 of the 53 floral scent compounds of *A. cyclops,* as reported on in Chapter 2 (Table 2.1) have been identified in the solvent sample used in the GC-EAD experiments; however, these 28 are the prominent compounds making up 90.5% of the total relative volatile amount reported there. The pseudonym UC1693 assigned to the unidentified compound (KRI = 1693) found in the headspace scent of *A. cyclops*, *A. melanoxylon* and *A. saligna* (Chapter 2) has also been assigned to the unidentified compound (KRI = 1143) preceeding 4-Oxoisophorone on the GC-column (Table 4.2). Evidence that the two compounds detected with different methods were the same, was given by their mass spectra. The volatile profiles listed in Table 2.1 and Table 4.2 were both from the floral scent of *A. cyclops*, but based on different methods of sample collection and analysis.

University of Cape Town

Table 4.2. Mean relative amounts (%) of compounds identified by GC-MS from headspace volatiles of *Acacia cyclops* flowers. Scent compounds are listed according to compound class and Kovats retention index (KRI). CAS = Chemical Abstracts Service Registry Number. tr = trace amount (<0.1 % of total sample). Compound identification criteria: a = comparison of MS with published data (e.g. NIST library); b = comparison of MS and retention time with published data; c = comparison of MS and retention time with published data and authentic standard. Mass fragments for unknowns are listed with the base peak first, followed by the other fragments in order of decreasing abundance.

Compound	KRI	CAS	*Acacia cyclops* flower scent
Number of Compounds			44
Total relative amount (max. 100%)			100
Aliphatic compounds			
Aliphatic esters			
(*Z*)-3-Hexenyl acetate[b]	1009	3681-71-8	0.8
(*Z*)-3-Hexenyl isovalerate[b]	1235	35154-45-1	tr
Unidentified aliphatic ester[a]	1441		tr
Methyl hexadecanoate[b]	1921	112-39-0	tr
Aliphatic alkanes			
Decane[c]	1000	124-18-5	tr
Undecane[c]	1100	1120-21-4	tr
Dodecane[c]	1200	112-40-3	tr
Tridecane[c]	1300	629-50-5	0.4
Tetradecane[c]	1400	629-59-4	0.6
Pentadecane[c]	1500	629-62-9	4.7
Hexadecane[c]	1600	544-76-3	1.7
Heptadecane[c]	1700	629-78-7	41.2
Octadecane[c]	1800	593-45-3	1.8
Nonadecane[c]	1900	629-92-5	36.1
Eicosane[c]	2000	112-95-8	0.30
Heneicosane[c]	2100	629-94-7	1.1
Docosane[c]	2200	629-97-0	tr
Tricosane[c]	2300	638-67-5	0.1
Tetracosane[c]	2400	646-31-1	tr
Pentacosane[c]	2500	629-99-2	tr
Hexacosane[c]	2600	630-01-3	0.1
Heptacosane[c]	2700	593-49-7	0.2
Octacosane[c]	2800	630-02-4	0.3
Nonacosane[c]	2900	630-03-5	0.3
Triacontane[c]	3000	638-68-6	0.2
Hentriacontane[c]	3100	630-04-6	tr
Benzenoids			
Benzyl tiglate[a]	1498	37526-88-8	0.2
Monoterpenoids			
3-Carene[b]	988	13466-78-9	tr
β-Pinene[c]	991	127-91-3	tr

University of Cape Town

Compound	KRI	CAS	*Acacia cyclops* flower scent
Limonene[c]	1033	138-86-3	tr
(*Z*)-*β*-Ocimene[b]	1043	3338-55-4	0.2
(*E*)-*β*-Ocimene[b]	1050	3779-61-1	0.4
β-Linalool[c]	1107	78-70-6	tr
(*E,E*)-2,6-Dimethyl-3,5,7-octatriene-2-ol[a]	1208	206115-88-0	tr
Sesquiterpenoids			
β-Caryophyllene[c]	1440	87-44-5	1.8
α-Humulene[a]	1473	6753-98-6	0.1
β-Sesquiphellandrene[b]	1523	20307-83-9	tr
Caryophyllene oxide[b]	1609	1139-30-6	0.6
Irregular terpenes			
6-Methyl-5-hepten-2-one[b]	1006	110-93-0	tr
4-Oxoisophorone[b]	1142	1125-21-9	3.6
2,2,6-Trimethyl-1,4-cyclohexanedione[a]	1168	20547-99-3	0.3
4-Methoxy-2,2,6-trimethyl-cyclohexanone[a]	1203	17429-03-7	tr
(*E*)-*α*-Ionone[b]	1429	127-41-3	tr
Unidentified compound			
m/z: 56,85,125,43,41,69,153,55,83,39[a] (=UC1693)	1143		2.0

University of Cape Town

4.3.3. Gas chromatography coupled to electroantennographic detection (GC-EAD)

The antennal response of a *D. dielsi* female to the scent of *A. cyclops*, using GC-EAD, is shown in Fig. 4.4. Acetone was used as a solvent, and presented in the GC-EAD and GC-MS as two broad solvent peaks (not shown in Fig. 4.4). The antennal baseline was stable for about 4 minutes; thereafter there was some fluctuation which started even before the acetone solvent peak, and lasted until the end of the solvent peak.

The size of the antennal responses at (g) and (A) (Fig. 4.4) compared to the other fluctuations while the antennal response was recovering to the baseline, warrants acceptance of those responses as valid antennal responses. The responses to (*Z*)-3-Hexenyl acetate (A), Limonene (B) and 4-Oxoisophorone (D) are in accordance with patterns detected from the EAG responses (see Fig. 4.2). *β*-Linalool which occurred in the floral scent of *A. cyclops* (Chapter 2), and to which antennae

showed a significant response in the EAG experiments, was not detected in the GC-EAD experiment possibly due to the fact that it occurred only in trace amounts in the scent (Table 4.2). There were positive antennal responses to Heptadecane (E) and Tricosane (F), and possibly to Heinocosane (*).

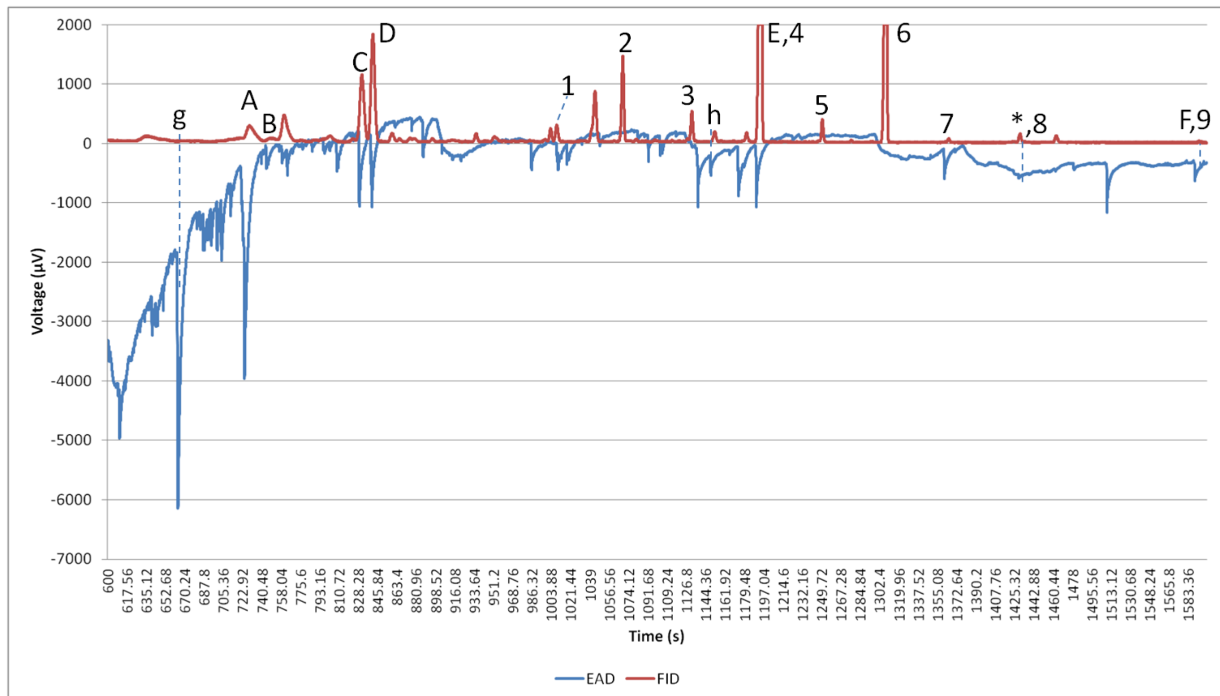


Fig. 4.4. GC-EAD results (after the solvent peaks) of the antennal response of a female *D. dielsi* midge to the scent of *A. cyclops* flowers. Uppercase letters show the compounds to which a positive antennal response was registered: A = (*Z*)-3-Hexenyl acetate, B = Limonene, C = UC1693, D = 4-Oxoisophorone, E = Heptadecane, F = Tricosane). Lower case letters show antennal responses to unidentified compounds (g: KRI = 976, h: KRI = 1632). * shows a compound that might have a positive response, but there is uncertainty due to the baseline drift (* = Heinocosane). Numerals show the alkanes identified in the sample (1 = Tetradecane, 2 = Pentadecane, 3 = Hexadecane, 4 = Heptadecane, 5 = Octadecane, 6 = Nonadecane, 7 = Eicosane, 8 = Heinocosane, 9 = Tricosane). (KRI = Kovat's retention index; DB5-column)

4.4. Discussion

Both *Dasineura dielsi* adult females and males, like most species from the family Cecidomyiidae, are short-lived with life spans of one to two days (Adair, 2005; Rosenheim et al., 2007) and adult midges presumably do not feed (Harris & Rose, 1989; Yukawa, 2000; Rosenheim et al., 2007). In such an ephemeral life time, males need to find females quickly to reproduce (Baer, 2003) and the females

after mating, need to find suitable oviposition sites quickly to achieve their maximum fitness (Yukawa, 2000).

The presence of female sex pheromones, and the attraction thereto in males, have been demonstrated for a number of cecidomyiids e.g. *D. mali* (Suckling et al., 2007), *D. tetensi* (Crook & Mordue (Luntz), 1999), *Contarinia nasturtii* (Boddum et al., 2010) and *Mayetiola destructor* (Boddum et al., 2010). Suckling et al. (2007) reported swarms of midges visibly following field staff placing female sex pheromone baited mass traps during trapping experimentation with *D. mali*. Also, during field experimentation with *D. dielsi* on *A. cyclops* male midges were observed swarming around the galls which were bagged against parasitoid interference, presumably responding to pheromones emitted by entrapped females (J.H. Hoffmann, Personal communication). Males of *D. tetensi*, the blackcurrant leaf midge, showed no response when exposed to plant volatiles emitted from leaf shoots (Crook & Mordue (Luntz), 1999). Cecidomyiid males therefore presumably do not need plant volatiles to locate females for reproduction and this observation might explain the antennal responses to different compounds in *D. dielsi* males and females.

Gender-based differences to certain compounds were also observed in the damson-hop aphid, *Phorodon humuli,* and the bird cherry-oat aphid, *Rhopalosiphum padi* (Pope et al., 2004).The antennal responses of male and female *D. dielsi* differed both in the nature of the compound they responded to and the rank order in which the response was recorded. Two noteworthy gender-based differences that have been observed between male and female responses to the test compounds were the positive response shown by males, but not by females to Benzaldehyde, a compound commonly found in many flowers (Knudsen et al., 2006) and the positive antennal response shown by females, but not by males to 4-Oxoisophorone, a floral volatile typically associated with plants visited by butterflies (Dobson, 2006).

University of Cape Town

4-Oxoisophorone has previously been shown to stimulate antennal responses in bees (Dötterl et al., 2005a), butterflies (Andersson, 2003) and moths (Guédot et al., 2008) and in this study, 4-Oxoisophorone elicited a strong response in the female antennae in both the EAG and GC-EAD experiments. To my knowledge, this report is the first of a dipteran species showing antennal sensitivity to 4-Oxoisophorone. Since 4-Oxoisophorone is an uncommon floral compound (Knudsen et al., 2006), the significant difference in the responses between male and female antennae, together with the size of the antennal responses recorded, suggests a particular function of this compound for females. 4-Oxoisophorone had been detected in the volatile profiles of the leaf as well as the different floral stages of *A. cyclops* with the highest relative amount in the headspace of the open flowers (Chapter 2). *Dasineura dielsi* females are known to oviposit in the open flowers of *A. cyclops* (Adair, 2005), and it is suggested that this compound plays a role in the selection of the right flower stage for oviposition. Further behavioural studies on the attractiveness of this compound to the midges, are however required. Compound UC1693 preceded 4-Oxoisophorone on the GC-column and it can only be speculated that UC1693 might play a role for host finding of *D. dielsi* since it is emitted in considerable relative amounts from open flowers (Chapter 2). For the identification and verification of its biological relevance further studies are needed by using techniques such as preparative GC, to sample enough pure substance, and NMR for final identification.

University of Cape Town

In addition to 4-Oxoisophorone, an irregular terpene, two monoterpenes were also antennally active in *D. dielsi* females. Monoterpenes at low concentrations are often prominent floral odours (Bernays & Chapman, 1994) and Limonene and *β*-Linalool, which occurred in relative amounts of less than 0.1% in the scent sample of *A. cyclops* (Table 4.2), elicited electroantennal responses in *D. dielsi* females in the EAG. However, only Limonene yielded a response in the GC-EAD. This discrepancy is likely due to the differences in concentration: While the authentic standard was employed in relatively high concentration in the EAG, the concentration in the floral scent sample (GC-EAD) was

much lower. Both Limonene and *β*-Linalool have been used as insecticides, being reported to act as neurotoxins, insect growth regulators, repellents and fumigants (Regnault-Roger et al., 1993; Weinzierl, 2000). Limonene affects the sensory nerves of the peripheral nervous system of insects, whilst the exact mode of action of *β*-Linalool is still unclear (Weinzierl, 2000). *β*-Linalool as electrophysiological stimulant has been reported as important for host finding for a number of insects e.g. the *Hadena bicruris* moth (Dötterl et al., 2006) as well as in four species of biting flies (Birkett et al., 2004a).

Of the aliphatic compounds tested for antennal activity, a green leaf volatile (GLVs) and two Alkanes, elicited antennal responses. Antennal receptivity for GLVs is a common feature in phytophagous insects (Visser & Piron, 1995) e.g. the vetch aphid *Megoura viciae* (Visser & Piron, 1995), the vine weevil *Otiorhynchus sulcatus* (Van Tol & Visser, 2002), the leaf beetle *Cassida denticollis* (Müller & Hilker, 2000) and the Ethiopian fruit fly, *Dacus ciliatus* (Alagarmalai et al., 2009) and may be used in host plant location (Van Tol & Visser, 2002). Insects such as the fruitfly, *Drosophila melanogaster*, positively use GLVs to ignore unripe fruit as unsuitable food source (Stensmyr, 2004). Hansson et al. (1999) identified green leaf volatile detecting neurons specific to (*E*)-2-Hexenal, (*Z*)-3-Hexenol and (*Z*)-3-Hexenyl acetate in Japanese scarab beetles.

University of Cape Town

Antennal sensitivity to (*Z*)-3-Hexenyl acetate, a typical GLV (Knudsen et al., 2006) in both *D. dielsi* males and females was recorded in the present study. Since the relative amount of (*Z*)-3-Hexenyl acetate decreased from the green bud stage to the open flower stage in the scent profiles of *A. cyclops* (Chapter 2), it is possible that *D. dielsi* uses the occurrence of (*Z*)-3-Hexenyl acetate to assess whether floral parts are in the right stage to be used for oviposition. Two Alkanes, Heptadecane and Tricosane, elicited antennal responses in the GC-EAD experiments, but not Nonadecane, which was the second most prominent compound (36.1%) in the scent profile of *A. cyclops*, after Heptadecane (41.2%), and which peaked in relative amount in the scent profile of the open flower of *A. cyclops*

(Chapter 2). In contrast to Nonadecane, which despite its high relative amounts triggered no antennal response, Tricosane received a strong response considering its low relative amount (0.13%). Altogether, the floral volatile profile of *A. cyclops* showed great variation in the relative amounts of the different Alkanes (Table 4.2).

There are two possible explanations for the discrepancy between antennal responses to the Alkanes in the GC-EAD tests and the lack of antennal responses to the Alkanes of the Alkane standard mixtures used in the EAG experiments. Firstly, the concentration of the standard mixtures for Alkanes used were outside the response range of the antennal receptors (understimulation or overstimulation e.g. Li et al., 1992; Zhu et al., 1999). Secondly, the identification of the Alkanes might have been incorrect (although the standards had the same retention times and very similar mass spectra).

Except for a significant antennal response to Methyl salicylate, a benzenoids compound, as compared to the paraffin control (but not to the air control) by the female midges, only male midges showed significant responses to the benzenoid compounds. Only male midges further showed a significant antennal response to Indole, a nitrogen containing compound.

Insects usually exhibit selectivity to compounds in the mixture of scents they are confronted with and therefore often have a narrowly-tuned olfactory system specifically configured for a small number of compounds (Li et al., 1992; Stensmyr, 2004). The *D. dielsi* midges were presented with a broad spectrum of compounds from six compound classes, and responded selectively to certain individual compounds suggesting that the *D. dielsi* antennae are selectively tuned to a narrow range of compounds.

University of Cape Town

CHAPTER 5: BEHAVIOURAL STUDIES

5.1. Introduction

Host plant attraction of insects is often expressed by different modalities such as visual, tactile and olfactory cues and signals may be exploited alone, or in combination with others (Raguso & Willis, 2005). Olfaction however, is often the primary modality of plant-insect attraction (Schoonhoven et al., 2005; Urru et al., 2011). In the case of herbivorous insects, plant chemicals play an essential role in host plant selection and, in particular, in the oviposition choice of females (Gouinguené & Städler, 2006; Beyaert et al., 2010). The choice of a plant by ovipositing females is essential for survival and fitness of the offspring, especially in those species where the immature stages are immobile (Gouinguené & Städler, 2006) or encapsulated in a gall on the host plant (Castells & Berenbaum, 2008) as in the case of *D. dielsi*,.

University of Cape Town

The acceptance or rejection of a host plant by the insect depends on behavioural responses to plant features such as colour, shape, and particularly odour (Bernays & Chapman, 1994; Gaskett et al., 2005). Females of several midge species have been shown to be attracted to host plant volatiles. The apple leaf curling midge, *D. mali* distinguishes between volatiles from apple and pear (Galanihe & Harris, 1997). The brassica pod midge, *D. brassicae* (Murchie et al., 1997) responds to secondary plant chemicals from cruciferous plants. Mated females of the blackcurrant leaf midge, *D. tetensi* (Crook & Mordue (Luntz), 1999) respond to host plant volatiles in bioassays with a 4-arm olfactometer. The sorghum midge, *Stenodiplosis sorghicola* responds to colour and odour stimuli from the host plant (Sharma & Franzmann, 2001). The orange wheat blossom midge, *Sitodiplosis mosellana* is attracted to volatiles from intact wheat panicles (Birkett et al., 2004b). The raspberry cane midge, *Resseliella theobaldii* is attracted to volatiles from wounded raspberry canes (Amarawardana, 2009).

The results of electrophysiological experiments described in the previous chapter (Chapter 4) showed positive antennal responses in *D. dielsi* to 4-Oxoisophorone, (*Z*)-3-Hexenyl acetate, *β*-Linalool and Limonene, Heptadecane, Tricosane and UC1693 all of which occurred in the scent of *A. cyclops*. Antennal responses of insects occur at the peripheral level only, and do not indicate the nature of the behavioural responses in terms of attraction or repulsion (Bruce et al., 2005). Neither are all electrophysiologically active volatile compounds relevant or detected behaviourly (Mustaparta, 2002; Schoonhoven et al., 2005). These limitations of electrophysiological measurements necessitated the use of behaviour bioassays with *D. dielsi* on fresh plant material of *A. cyclops* to determine how the midges respond to different plant organs, leaves or flowers, and different floral stages, yellow buds, open flowers or senescing flowers. Behavioural assays were also used to ascertain which of the antennal active compounds identified in the EAG and EAD measurements would elicit behavioural responses in the midges. Because *D. dielsi* occasionally uses *A. melanoxylon*, *A. longifolia* and *A. saligna* as hosts (Post et al., 2010), the behavioural response of females to floral scent blends of these non-standard hosts was also investigated. Since fresh floral plant material of these species was not available at the time the bioassay experiments were conducted, the floral scent of these species were simulated by preparing blends of authentic standard compounds in the same ratios as the major compounds were found in the floral scents of these species (compare Chapter 2).

University of Cape Town

5.2. Methods and Materials

5.2.1. Insects

Galls were collected from *A. cyclops* trees at the Koeberg Nature Reserve (S 33° 39' 14.8", E 18° 26' 00.4") near Melkbosstrand in the Western Cape, South Africa. In the laboratory, the galls were retained in cylindrical transparent plastic flasks (200 ml), and covered with plastic, dome-shaped cones onto which small transparent plastic medicine bottles (20 ml) were attached. After

they emerged from the galls the positively phototactic midges moved upwards into the small bottles (Sharma et al., 2002; Roubos, 2009). It was assumed that the ratio of male to female emergence from the galls was 1:1.5 as determined by Adair (2004, 2005). Due to the elevated constant temperature in the controlled temperature room emergence of the midges was rapid and a constant supply of midges was available, frequently with several aggregated in the small bottle at any given time.

5.2.2. Olfactometer setup

The behavioural responses of females of *D. dielsi* to plant volatiles were assessed using a four-arm olfactometer (Fig. 5.1) based on a design by Pettersson (1970), adapted by Vet et al.(1983) and Pettersson (1993). The olfactometer, with dimensions 107 cm x 107 cm x 18 mm, consisted of three layers of Perspex, each layer 6 mm thick. The middle layer forms a four pointed star-shaped chamber with inlets at the points of the star-shape to which airflow tubes are connected. One of the outer layers has a central hole through which exhaust air escapes and through which the insects can be introduced into the chamber. Compressed air was passed from outside the olfactometer system (Fig. 5.1a) through charcoal (Fig. 5.1b), then water (Fig. 5.1c), before it was passed through a 4-way airflow splitter in four separate, but identical silicone tubes (Fig. 5.1d). Each tube was connected to a flow meter (Dwyer Series VFB Visi-Float Flowmeters) (Fig. 5.1f) which was adjusted to a flow rate of 80-100 ml/min. Thereafter air passed through silicone tubes (ø = 6mm) (Fig. 5.1g) connected to the odour source containers (1 l glass jars, 50 ml Erlenmeyer flasks or Pasteur pipettes, depending on the experiments) (Fig. 5.1h), after which the air passed through Teflon tubes (ø = 4mm) (Fig. 5.1i) which were connected to the arms of the olfactometer (Fig. 5.1j). Air left the olfactometer through the central hole which was positioned in the lower outer layer (Francis et al., 2004). In order to ensure uniformity of conditions in the four olfactometer arms, all tubes and containers between the flow meters and the olfactometer were of equal diameter, length and volume. A lightweight transparent woven fabric mesh was positioned at the point where the Teflon tubes entered the

University of Cape Town

olfactometer, to firmly secure the tubes on the olfactometer and to prevent the midges from moving into the tubes.

Fig. 5.1. The olfactometer experimental setup. a = Airflow being pushed into the system; b = Charcoal filter; c = Water; d = 4-Way airflow splitter; e = Emerge bottles; f = Flowmeters; g = Four identical diameter and length airflow tubes; h = Odour source container; i = Black sleeve for emergence bottle; j = olfactometer; k = Shields; l = Digital microscope for video recordings.

The olfactometer system was placed in a controlled temperature room at 28 ± 3 ^{o}C. Light was provided by an overhead florescent light and a gooseneck cold light shining directly on the olfactometer. In order to prevent possible disturbing effects from movement around the apparatus, off-white coloured wooden shields were placed around the olfactometer (Fig. 5.1k). A digital microscope (Dino-Lite Pro2, Model AD413T) (Fig. 5.1l) connected to a laptop computer was installed centrally above the olfactometer in such a way that the whole olfactometer could be seen. All experimental runs (Table 5.1) were video recorded for later analyses.

5.2.3. Odour sources tested

Three sets of experiments (Table 5.1) were completed, namely with fresh plant material, individual authentic chemical standards, and simulations of the scent of *Acacia cyclops, A. melanoxylon, A. longifolia* and *A. saligna* using mixtures of the main compounds identified in their scent. Each bioassay tested for attraction by setting up one treatment arm in the olfactometer and three controls arms (Vet et al., 1983).

Table 5.1. Terminology used in describing the bioassays performed.

Term	Explanation
Sets of experiments	The three groups of odour sources that were used in the bioassays namely: - fresh plant material, - individual authentic chemical standards, and - simulations of the floral scent
Experiments	For each set of experiments a number of experiments were performed. E.g., the fresh plant material experimental set contained experiments performed with leaves, yellow buds, open flowers and senescing flowers.
Experimental runs	Each experiment contained a number of runs that were required to observe the behaviour of 20 female midges in total. A run could have more than one midge.

5.2.3.1. Fresh plant material

The experimental setup was tested with four air controls where four empty 1 litre glass jars were connected to the olfactometer. Thereafter the olfactory responses of *D. dielsi* females to the scent of leaves, yellow buds, open flowers and senescing flowers of *A. cyclops* were tested. For the experiment with the leaves, 1 litre glass jars with tight lids were used. A treatment was prepared by putting four *A. cyclops* shoots with leaves of approximately 30 cm in length in the jar. Three empty jars were used as air controls. For the experiments with the yellow buds, open flowers and the senescing flowers, the treatments were prepared by putting 60 carefully-picked buds or flowers in 50 ml Erlenmeyer flasks with two glass spouts on opposite sides of the flask to which the connecting tubes were attached. The flasks were plugged with cork bungs covered with clean unused aluminium

University of Cape Town

foil. Three empty flasks of similar size, and plugged in a similar manner, were prepared as air controls. In total, five experiments (Table 5.1) were performed namely with air, leaves, yellow buds, open flowers and senescing flowers.

5.2.3.2. Individual authentic standard compounds

The chemical standard compounds that yielded responses in the EAG and/or GC-EAD experiments (Chapter 4) were selected for the bioassays with female midges (Table 5.2). Solutions of each of the selected compounds were prepared by diluting a percentage corresponding to the relative amounts as found in the floral scent of *A. cyclops* (Table 5.2) (Webster et al., 2010) of the compound in a volume of Dichloromethane (≥99%, Merck) to make up a volume of 100%. A treatment was prepared with 1 µl aliquots of the standards dilution, and three controls were prepared with 1 µl of Dichloromethane. Aliquots were dropped on a 75mm x 4 mm (approx.) piece of chromatography paper which was inserted in a Pasteur pipette that served as container for the odour source. Two minutes preparation time was allowed during which the Pasteur pipettes were connected to the olfactometer before the experiments were started. Approximately twenty minutes were set as the maximum duration of a run in order to ensure a continuous odour source of adequate albeit potentially variable strength. As a control test, 1 µl aliquots of dichloromethane were tested in all four olfactometer arms. Dichloromethane was also tested in one treatment arm against three air controls. In total, eight experiments were performed (Table 5.1), namely 4-Oxoisophorone, (*Z*)-3-Hexenyl acetate, Ocimene mixture of isomers, Limonene, *β*-Linalool, C_8-C_{20} alkane mixture, Dichloromethane (4 x controls) and Dichloromethane (1 x treatment, 3 x air controls).

5.2.3.3. Biotests with mixtures of chemicals as found in the scent of four *Acacia* species

Because *D. dielsi* females occasionally use *A. melanoxylon, A. longifolia* and *A. saligna* as a host (Post et al., 2010), these three species were included as part of the investigation. None were flowering when the bioassay experiments were being done, but chemical standards that were available made

up between 76% and 90% (Table 5.2) of the total scent profiles of the flowers of the different species. The floral scent was simulated by preparing solutions of the authentic standards in the same ratios as found in the volatile profiles of the plants (Table 5.2) (Webster et al., 2010) despite possible vapour pressure differences between headspace composition of the natural plants and the prepared simulated scents. The other minor compounds making up the approx. 10%-24% of the headspace floral scent profiles were substituted with Dichloromethane (≥99%, Merck).

Table 5.2. Available authentic standards and their contribution (relative amount in %) to the scent of the open flowers of four *Acacia* species (Chapter 2) with an indication of the purity of the compounds, and whether the compound was used in the single compound bioassays.

Standard/Compound	Purity	Bioassay	*A. cyclops*	*A. melanoxylon*	*A. longifolia*	*A. saligna*
(*Z*)-3-Hexen-1-ol	≥98% [a]	-	0.55	1.89	3.12	23.74
(*Z*)-3-Hexenyl acetate	≥98% [b]	Yes	23.51	1.71	2.57	0.92
Alkanes C_8-C_{20} mixture	- [c]	Yes	16.17	10.61	16.63	3.24
Alkanes C_{21}-C_{40} mixture	- [c]	-	0.73	4.46	1.33	0.21
p-Anisaldehyde	≥99% [d]		-	9.46	-	-
Benzaldehyde	≥99% [e]	-	0.53	6.47	2.46	4.19
Benzyl alcohol	≥99% [f]		0.18	0.92	6.35	48.77
Indole	≥99% [a]	-	-	0.13	-	-
Limonene	≥95% [g]	Yes	1.58	6.83	7.94	4.68
Methyl benzoate	≥99.5% [d]	-	-	0.11	0.05	0.38
Methyl salicylate	≥98% [f]		-	-	-	0.002
Ocimene mixture of isomers	≥90% [h]	Yes	10.29	28.53	33.91	2.18
4-Oxoisophorone	98% [a]	Yes	23.21	1.26	-	0.76
α-Pinene	≥99% [c]		-	1.66	-	0.53
β-Pinene	≥99% [c]		-	2.55	-	0.27
β-Linalool	≥95% [d]	Yes	0.25	5.74	1.49	0.25
β-Caryophyllene	- [f]	-	0.79	2.72	0.31	0.08
Total			**77.79**	**85.05**	**76.16**	**90.20**

<u>Notes</u>: Suppliers of Compound
[a]: Sigma-Aldrich, Steinheim, Germany; [b]: SAFC, Supply Solutions, Japan; [c]: Fluka-Sigma-Aldrich; [d]: Fluka-Sigma-Aldrich, Steinheim, Germany; [e]: Merck Schuchardt, Hohenbrunn, Germany; [f]: Sigma-Aldrich; [g]: Fluka; [h]: SAFC, Supply Solutions, Sigma-Aldrich, Steinheim, Germany

University of Cape Town

As during the bioassays with the individual authentic standard chemical compounds, the treatment arm was prepared with 1 µl aliquots of the standards dilution, and three controls arms were prepared with 1 µl of Dichloromethane. Aliquots were dropped on a 75mm x 4 mm (approx.) piece

of chromatography paper which was inserted in a Pasteur pipette that served as container for the odour source. Two minutes preparation time was allowed during which the Pasteur pipettes were connected to the olfactometer before the experiments were started. In total, four experiments were performed (Table 5.1) namely with the floral scent simulations of *A. cyclops, A. melanoxylon, A. longifolia* and *A. saligna*.

5.2.4. Behavioural observations

For all the experiments, midges were allowed to enter the olfactometer from below on their own accord. A small emergence bottle, containing an undetermined number of recently emerged midges, was capped with a plastic cover which had a hole in the tip with a size similar to the hole in the olfactometer. The bottle was put in a black plastic sleeve to reduce the light in the bottle, and then inserted under the olfactometer with the hole in the bottle cover aligned with the hole in the olfactometer. In this way a lighter area was created directly above the midges in the olfactometer. The midges, positively phototactic moved into the olfactometer. In these three experiments, the midges entered into the olfactometer against the flow of air. Due to the method of inserting the midges into the olfactometer more than one midge could enter the olfactometer during an experimental run. Midges that escaped through the open hole in the olfactometer during an active run were discarded from the analyses. In cases where no midges had entered the olfactometer within the first two minutes, the bioassay was aborted and restarted. During the experiments with the yellow buds and the senescing flowers, insufficient midges entered the olfactometer within the first two minutes and an alternative insertion method had to be applied. The reason the midges did not enter the olfactometer within the allotted time, remains unexplained but, to overcome the problem, the olfactometer was inverted with the hole on the upper surface of the central chamber pointing upwards, and the midges were inserted through the hole with a customised glass tube. In these cases the airflow was disconnected whilst the midges were being inserted.

University of Cape Town

Except for the experiments with the yellow buds and senescing flowers, where only female midges were selected for insertion into the olfactometer, both males and females moved into the olfactometer. The gender of the midges was determined (based on the colour and shape of the abdomen and the format of the antennae) (Kolesik et al., 2005; Scudder & Cannings, 2006) as soon as they entered the olfactometer. Males were excluded from the scent preference analysis along with a few cases where the gender could not be determined reliably. The number of males entering the olfactometer was recorded for comparisons to the male : female adult emergence ratio as determined by Adair (2004, 2005). Each experimental run was video recorded for later analysis. For each experiment, a total of twenty female midges were followed for an observation time of ten minutes each. Several runs with one to eight females were required to reach the total of twenty female midges. A clean olfactometer was used for two runs and the olfactometer was rotated through 90° after each run to prevent any directional bias. When an olfactometer was used for a second run, the odour source was connected to the same arm that was used for the odour source during the previous run. All experiments were conducted between 24 February 2011 and 7 May 2011.

University of Cape Town

5.2.5. Digital record analysis

Video recordings were made with the software accompanying the digital microscope (Dino-Lite Pro2, Model AD413T) and were captured as .AVI files. These files were watched with Windows Media Player which showed the total length of the recording in minutes and seconds, and indicates the progress of the display as part of the total length in minutes and seconds.

Although many studies recognise five areas in a 4-arm olfactometer (Fig. 5.2a) (Pettersson, 1970, 1976, 1993; Vet et al., 1983; Crook & Mordue (Luntz), 1999; Birkett et al., 2004b; Desneux et al., 2004; Faccoli et al., 2008), a “smoke test” revealed only four areas (Fig. 5.2b). The smoke test was

conducted based on the description by Vet et al. (1983) and Suazo et al. (2003). Webster et al. (2010) also recognises four areas.

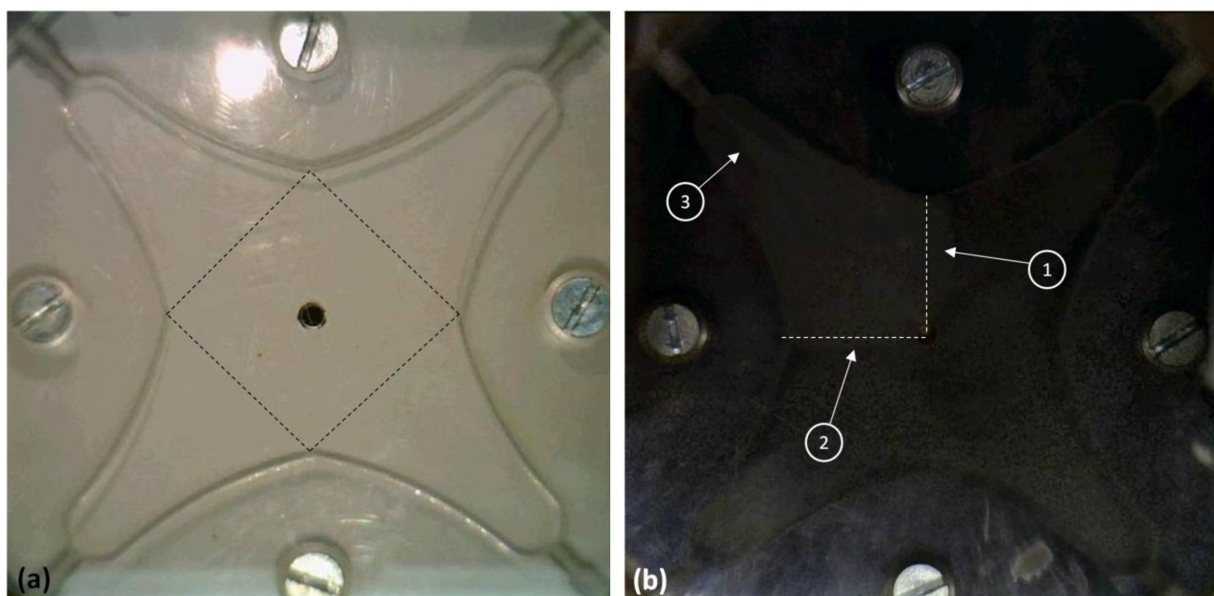


Fig. 5.2. (a) Five logical areas are formed in a 4-arm olfactometer by the four areas in the arms plus a central area as created by the virtual boundaries portrayed by the dashed lines; (b) Four actual areas in a 4-arm olfactometer are formed by the four arms including the area extending to the hole in the centre (see text for description of arrows).

University of Cape

With reference to Fig. 5.2b, air was pushed into the bottom left and bottom right arms of the olfactometer at about 100ml/min and at about 60 ml/min though the top right arm. Smoke that was formed by adding HCl to NH_3OH was pushed into the top left arm also at 100ml/min. Arrow #1 shows that the smoke was spilling over into the top right arm area (with the lower flow rate), but was "stopped" by the air of equal flow rate (arrow #2) as coming in from the bottom left arm. With equal flow rates in each of the arms, four equal sized areas were formed. Analysis of the time a midge spent in the olfactometer was based on the four actual areas thus formed. Arrow #3 shows the stream of smoked air that is being pushed into the olfactometer. It took <2 seconds to fill the whole arm with a uniform amount of smoke indicating that it was not necessary to run the system for any length of time to equilibrate conditions in the system.

The time a midge entered the olfactometer was logged and that time started the ten minute observation period. The quadrant into which the midge entered was logged as the first "choice" the

midge made (Vet et al., 1983). Thereafter the midge was "followed" as it moved through the olfactometer and every time the midge crossed an arm boundary, that time was logged together with the number of the arm the midge crossed into. At the end of the observation period both the total time spent per arm and the number of entries per arm could be determined.

5.2.6. Cleaning procedures

Plant volatiles diffuse easily in all directions and can easily contaminate the system. Each experiment commenced with clean equipment. Olfactometers were cleaned after being used for two runs. Tubes and glassware were cleaned at the end of each day. All equipment was washed and scrubbed in hot water with neutral action detergent (G. Fox & Co., Cape Town, South Africa), rinsed in hot water, dried with paper towels, washed in 99.9% ethanol and then left overnight under a fume hood to make sure they were thoroughly dry and devoid of any residue. Glassware and Teflon tubes were baked for two hours at 200°C before a new set of experiments commenced.

University of Cape Town

5.2.7. Statistical analyses

Statistica (version 10) was used for all statistical analyses. Mean time spent in the treatment arms was tested against a reference value of 2.5 minutes (25% of the 10 minute observation time) in single sample t-tests. Chi-square tests (observed vs expected) were used to analyse the male: female ratios and the number of first choices made. The air and Dichloromethane control experiments were analysed with one-way ANOVA tests.

In order to statistically determine whether the number of midges in the olfactometer had any effect due to interactions between the midges, the following calculation was made for each set of experiments (fresh plant material, individual authentic standards and flower scent simulations). The method is described by using the fresh plant material set of experiments as an example.

The data, time spent in the treatment arm, for all the experiments (air, leaves, yellow buds, open flowers, senescing flowers) in the set was pooled according to the "number of midges" per run in the olfactometer. For the fresh plant material set there were four "number of midges"-categories as the number of midges per run ranged from one to four. The number of runs (or sample size) for each category was counted. To determine a common sample size across the categories, the lowest number was used as sample size. The sample sizes of the other categories were reduced to the common sample size by randomly selecting the runs to be discarded. A one-way ANOVA test was performed on "number of midges" as factor to determine whether there was any effect on the results due to interaction between the midges. The resulting sample sizes per experimental set was n = 18 for fresh plant material, n = 12 for the authentic standard chemical compounds and n = 5 for the floral scent simulations.

The calculation method described above considers only the effect of interaction between the midges and disregards the effect of the scent source in the results. In order to assess the effect of the possible interaction between midges (factor: "number of midges") within the context of the different scent sources (factor: "scent source"), a similar method described above for determining the "number of midges" factor, was applied to determine the "scent source" factor. Not enough data was available for the chemical standards or the flower scent simulation experimental sets, and therefore this assessment could only be applied to the fresh plant material set of experiments. Regrouping the data in a "number of midges" X "scent source" configuration resulted in smaller sample sizes and a minimum sample size of four was used to maintain statistical validity of results. Data points in this configuration less than n = 4 were discarded. A factorial ANOVA test with factors "number of midges" and "scent sources" was performed on four of the five "scent sources" (air, yellow buds, open flowers and senescing flowers) and three of the four "number of midges" categories (one, two and four) in the plant material set of experiments.

University of Cape Town

5.3. Results

5.3.1. Time spent in treatment arm

The four areas of the olfactometer setup were in equilibrium as there were no significant differences for the four arms of the olfactometer when control tests were performed with air ($F_{(3,76)} = 0.992$; $p = 0.4$) and Dichloromethane ($F_{(3,76)} = 0.183$; $p = 0.91$). Dichloromethane as a treatment against air as controls in three arms of the olfactometer is not attractive or repulsive to the midges (t-value = -0.809, $p = 0.43$).

Bioassay experiments with the fresh plant material (Fig. 5.3a), showed a significant difference for the scent of open flowers only (t-value = 3.67, $p < 0.01$). Experiments with the authentic standard chemical compounds (Fig. 5.3b) showed significant differences to the reference value for 4-Oxoisophorone (t-value = 2.75, $p < 0.05$) and *β*-Linalool (t-value = -3.24, $p < 0.01$). For the floral scent simulations there were no significant differences to the reference value (Fig.7.2c).

The mean number of times the midges crossed olfactory arm boundaries was deemed to be indicative of the level of activity of the midges in the olfactometer (Fig. 5.4). Clean air elicited less activity than in the experiment with the Dichloromethane in four treatment arms (Fig. 5.4a). The scent of senescing flowers elicited least activity in the fresh plant material bioassays and activity was highest for the open flowers (Fig. 5.4b); *β*-Linalool elicited most activity in the authentic standard chemical compounds group (Fig. 5.4c); and the *A. saligna* floral scent simulation elicited most activity in the flower scent simulations group of bioassays (Fig. 5.4d).

University of Cape Town

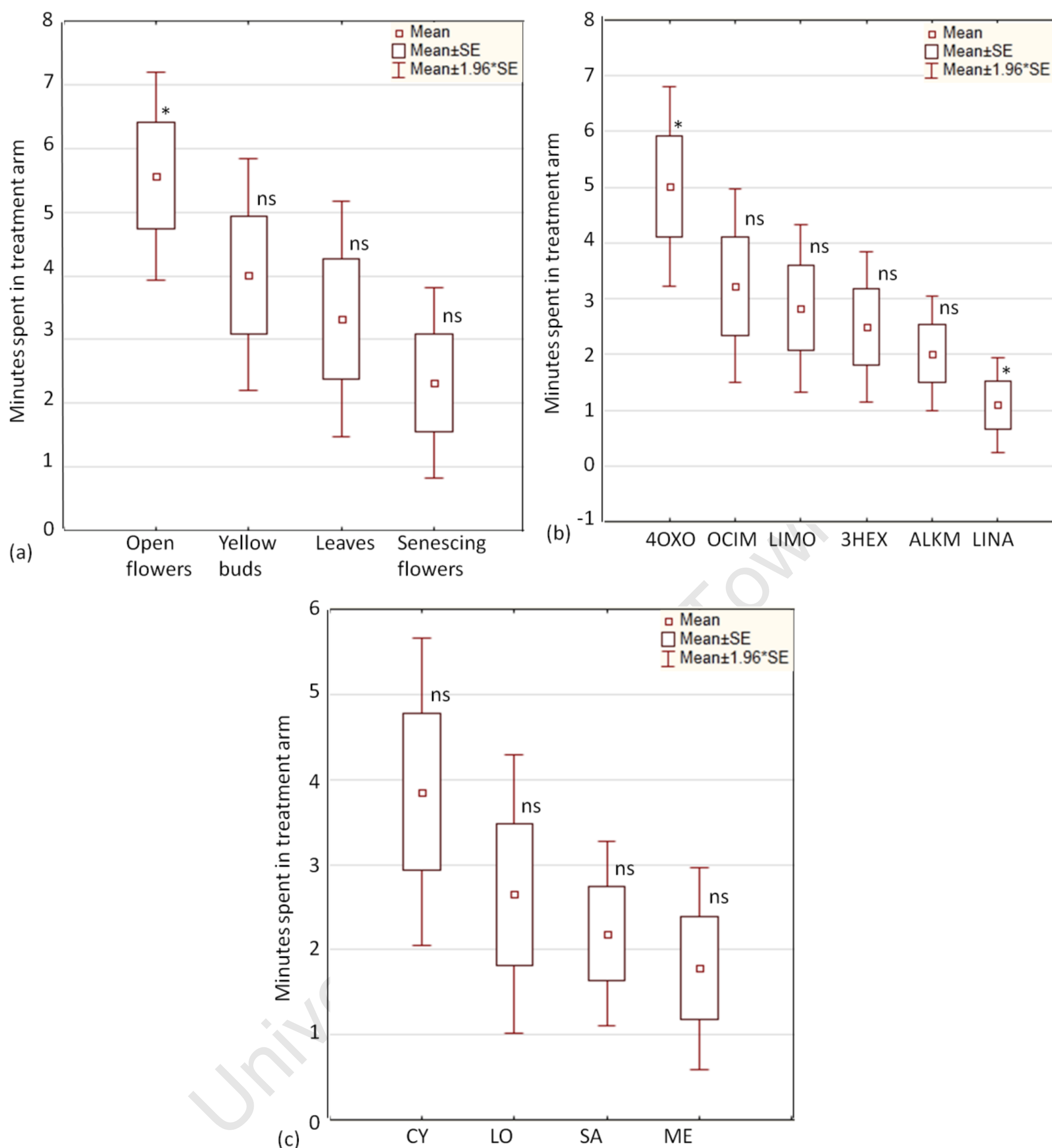


Fig. 5.3. Mean number of minutes spent by *D. dielsi* females in the treatment arm for bioassays with fresh plant material (a), authentic standard chemical compounds, 4OXO = 4-Oxoisophorone, OCIM = Ocimene mixture of isomers, LIMO = Limonene, 3HEX = (*Z*)-3-Hexenyl acetate, ALKM = C_8-C_{20} alkane mixture and LINA = *β*-Linalool (b), and floral scent simulations, CY = *A. cyclops*, ME = *A. melanoxylon*, LO = *A. longifolia* and SA = *A. saligna* (c). * indicates significant differences to the reference value of 2.5 minutes.

The interaction between midges in the olfactometer had no effect on the results obtained in the three experimental sets of bioassays, namely the fresh plant material ($F_{(3, 68)} = 0.371$, $p = 0.78$, $n = 18$), authentic standard chemical compounds ($F_{(5, 66)} = 1.249$, $p = 0.296$, $n = 12$) and floral scent

simulations ($F_{(6, 28)}$ = 1.130, p = 0.371, n = 5) experiments. When the effect of “number of midges” combined with “scent source” on the results was investigated for fresh plant material, only the scent source had an effect on the results (Table 5.3) which confirmed that the interaction between midges in the olfactometer had no effect on the scent preference of the individual midges.

Table 5.3. The effect of scent source (fresh plant material used) and number of midges in the olfactometer on the time spent in the treatment arm; significant difference at p < 0.05 indicated with *.

Effect	Sum of squares	Degrees freedom	Mean squares	F	p
Scent source	17	3	5.666	3.567	0.023*
Number of midges	0.79	2	0.397	0.25	0.78
Scent source X Number of midges	3.13	6	0.522	0.329	0.917

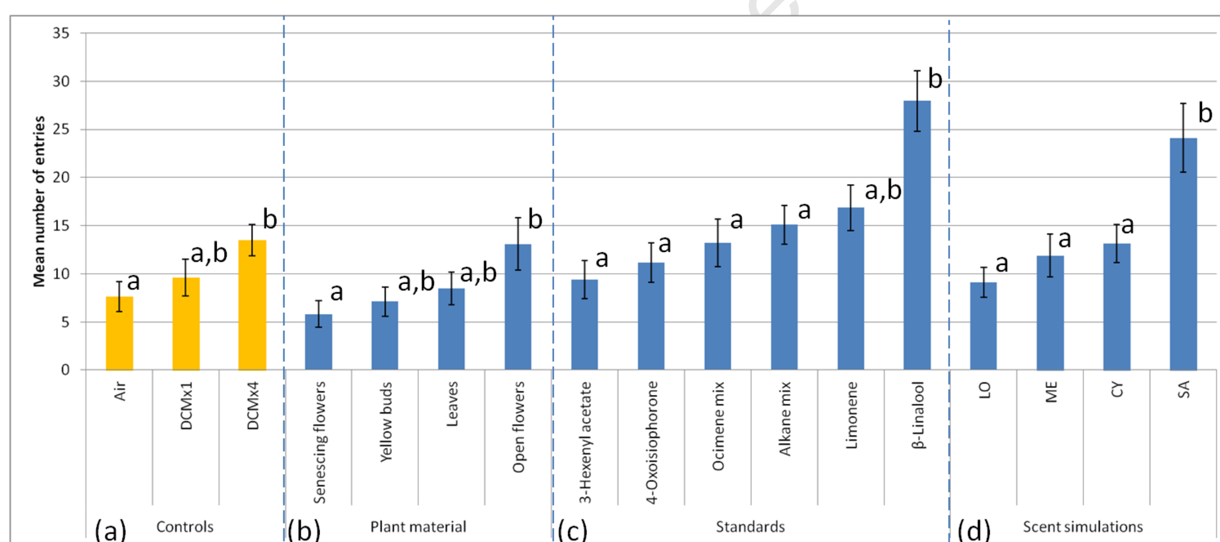


Fig. 5.4. The mean number of times the midges crossed olfactory arm boundaries for the different experiments: (a) controls, (b) fresh plant material, (c) authentic standards chemical compounds, and (d) floral scent simulations. Overall there were significant differences ($F_{(16, 323)}$ = 7.202, p < 0.001, n = 20). Per group of experiments different alphabetic characters indicates significant differences between values. CY = *A. cyclops*, ME = *A. melanoxylon*, LO = *A. longifolia* and SA = *A. saligna.* DCMx1 = one arm with Dichloromethane and three with air; DCMx4 = Dichloromethane in all four arms.

The scent of the authentic standards as an odour source elicited a positive olfactory response for more than twenty minutes, as female midges were still attracted to the treatment arm of the olfactometer after the aliquots of the standards solution which was dripped on chromatography

paper was inserted into the olfactometry system (Fig. 5.5). Arm 4 was the treatment arm with 4-Oxoisophorone diluted in Dichloromethane, and Arms 1-3 containing 100% Dichloromethane as controls. Figure 5.5 shows the location of twenty female midges at 0.5 minute intervals for the duration of ten minutes per midge. The duration of ten minutes per midge corresponds to the chosen observation time per midge for the bioassays. Time of entry into the olfactometer varied as the midges entered the olfactometer on their own accord and therefore at different times.

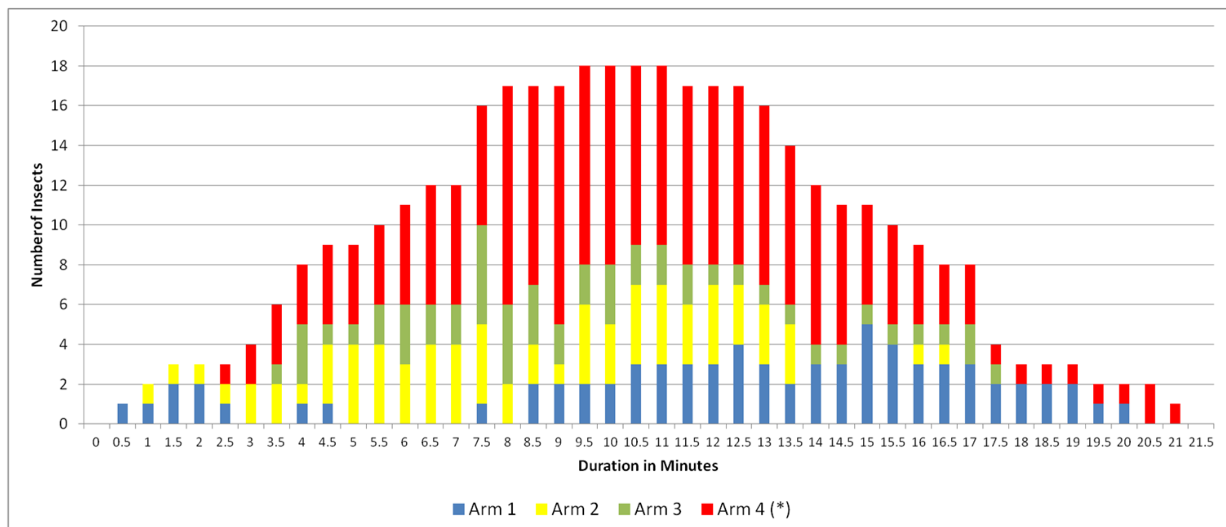


Fig. 5.5. Location of female midges plotted at intervals of 0.5 minutes after start of an experimental run. Arm 4 (*) was the treatment arm with a 23% concentration of 4-Oxoisophorone diluted in Dichloromethane. Arms 1-3 were control arms containing 100% Dichloromethane.

University

5.3.2. Ratio of male to female emergence

The ratio of male to female emergence from the galls is 1:1.5 (Adair, 2004, 2005). Each emergence bottle inserted under the olfactometer contained an undetermined number of males and females, and midges entered the olfactometer on their own accord, with at least two stimuli influencing them, namely a positive phototactic response and an olfactory stimulus response. The ratio of males to females entering the olfactometer may be an early indication of the level of response to an olfactory stimulus (Fig. 5.6). Significant differences were observed between the emergence sex ratio and the olfactometer entry sex ratio for open flowers ($\chi^2 = 19.17$; $p < 0.001$) where the female numbers were more than the male numbers, and for (*Z*)-3-Hexenyl acetate ($\chi^2 = 4.29$; $p < 0.05$)

where the male numbers were more than female numbers. The observed sex ratio for the *A. cyclops* scent simulation matched the expected sex ratio exactly. The data for yellow buds and senescing flowers is not displayed because only female midges were manually inserted into the olfactometer for those experiments.

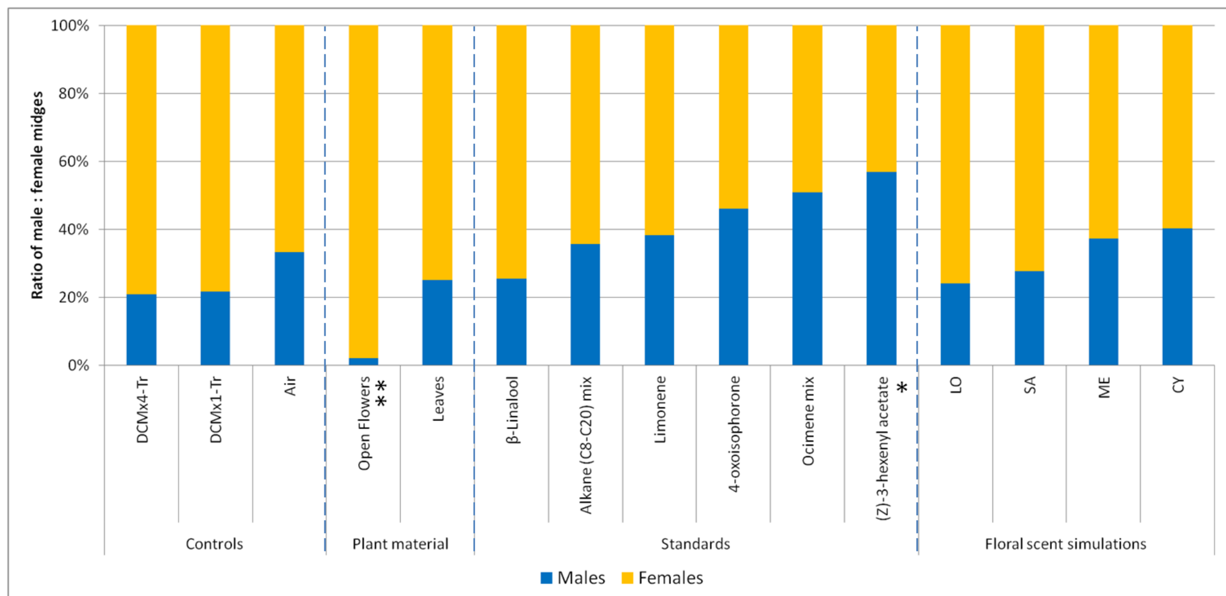


Fig. 5.6. Ratio of male to female midges entering the olfactometer for the different experiments. Significant differences to the 1:1.5 male: female ratio have been indicated with * ($p < 0.05$) and ** ($p < 0.001$).

University

5.4. Discussion

5.4.1. Attraction of *D. dielsi* to plant volatiles of *A. cyclops*

All floral stages, the green buds, yellow buds, open flowers and senescing flowers, are present on the tree at any time during the flowering period of *A. cyclops*. In addition, the flowers, although bright yellow, are rather inconspicuous amongst the foliage of the plant. Insects generally are 'bombarded' with many different volatiles at different concentrations and have to pick out the correct cue to respond to (Bruce & Pickett, 2011). The leaf and different floral scent profiles of *A. cyclops* differed substantially (Chapter 2) and therefore even on the same plant, *D. dielsi* females are immersed in different odours from the host plant and also have to pick out the correct cue to locate a suitable flower for oviposition. *Dasineura dielsi* females were not attracted to the leaf scent, and

neither to the yellow bud and senescing flower scents. The attraction of *D. dielsi* to the scent of the open flowers is supportive of field observations which indicated that the female only oviposits in the open flowers of the host (Adair, 2004, 2005). The finding that *D. dielsi* is not attracted to the scent of the yellow buds despite the fact that its scent profile was similar to the scent profile of the open flowers, correlates well with the inability of the females to oviposit in compact structure of the buds of *A. cyclops* due to its ovipositor which is unsclerotised (Adair, 2004, 2005; Kolesik et al., 2005). The finding that *D. dielsi* is not attracted to the scent of the senescing flowers, fits with the observation that midges have not been observed on senescing flowers in the field (Adair, 2004).

During the bioassay experiment with the open flowers, midges could enter the olfactometer on their own accord. Since the emergence bottles used in the experiments contained both males and females, a few males entered the olfactometer during the experimental runs. There were significantly fewer males than females that entered the olfactometer, the ratio being much lower than that found among adults emerging from galls (Adair, 2004, 2005), indicating that *D. dielsi* males do not respond positively to the scent emitted from open flowers of *A. cyclops*. In their study on the behavioural attraction of host plant volatiles to *D. tetensi*, the blackcurrant leaf midge, Crook & Mordue (Luntz) (1999) noted too that males were not attracted to the host plant volatiles. They also reported that only mated females of *D. tetensi* responded to the host plant volatiles and that the response of virgin females was similar to that of males. Since the mating status of *D. dielsi* females might have influenced their behavioural response to the scent of the open flowers, observations that were made during the experiment with the open flowers, need to be discussed within the context of the mating behaviour of midges in general.

University of Cape Town

In general, Cecidomyiidae mate soon after adult emergence (Barnes, 1935). On emergence, which is mostly in the early morning, females start ‘calling’ (Mólnar et al., 2009; Roubos, 2009; Cook et al., 2011), exhibiting a specific posture where the ovipositor, a telescopic structure which is about

double the length of the female body when prolonged, is extended (Galannihe & Harris, 1997; Van Lenteren et al., 2002; Roubos, 2009). Females maintain this posture until they are mated (Mólnar et al., 2009) after which the ovipositor is withdrawn (Van Lenteren et al., 2002; Mólnar et al., 2009; Roubos, 2009) and sex pheromone production ceases (Van Lenteren et al., 2002). Sex pheromones are presumably produced in secretory cells in the epidermis of the membrane connecting ovipositor segments, and stored in the grooves in the overlying cuticular surface when the ovipositor is retracted (Roubos, 2009). Pheromones are emitted from the grooves when the ovipositor is extended (Dorchin et al., 2007) and pheromone release is regulated by the extension and retraction of the ovipositor (McKay & Hatchett, 1984). Kanno & Harris (2000) showed that mated females can easily be distinguished from virgin females, because mated females no longer exhibit the calling posture. The oviposition posture is assumed when the female midges extend the ovipositor (not as far as when 'calling') and arch the abdomen with the tip of the ovipositor being at right angles with and touching the substrate (Harris & Rose, 1989). Oviposition follows soon after mating (Barnes, 1935).

Dasineura dielsi males and females were not separated after their emergence from the galls in the emergence bottles during the bioassay experiments and it was assumed that most females had mated by the time of the day when the bioassay experiments were conducted (mid-morning to mid-afternoon). 'Calling' by females was never observed whilst the midges were in the emergence bottles or olfactometer, but during the bioassay experiments with the scents of the open flowers and the yellow buds (but never during the bioassays with air controls, leaves or senescing flowers) some female midges were observed to be "calling"and to adopt the "oviposition posture' (Harris & Rose, 1989). Females exhibiting the oviposition posture were presumably depositing (or attempting to deposit) eggs on the side walls of the olfactometer and on the fabric separating the scent tube from the olfactometer of the treatment arm (personal observation). Midges were most active during the bioassays with the open flowers (Fig. 5.4).

University of Cape Town

Further observations were made during the bioassay experiment with the yellow buds. Midges that escaped the emergence bottles were observed hovering and circling above the olfactometer and some attempted to enter the olfactometer through the hole through which the scent-laden air escaped. Some midges inside the olfactometer displayed the ovipositing behaviour described above. The scent composition of the yellow buds was similar to that of the open flowers (Table 2.1, Chapter 2) and the prominent compounds, (*Z*)-3-Ocimene, (*Z*)-3-Hexenyl acetate, UC1693, Heptadecane, 4-Oxoisophorone and Nonadecane, only differed in the ratios (e.g. the ratio between 4-Oxoisophorone and UC1693 in the yellow bud scent comparing to the ratio between 4-Oxoisophorone and UC1693 in the open flower scent) in which they were present in these two scent profiles. Since *D. dielsi* females showed a significantly positive response to the scent of the open flowers and not to the scent of the yellow buds, it is possible that the ratio of the prominent compounds being similar in each of the profiles (and maybe even some of the less prominent compounds) were important in the functional role of the compounds. It is also possible that the volatiles emitted from the yellow buds play a role in long-distance attraction of *D. dielsi* females, and the volatiles emitted from the open flowers play a role in short-distance attraction of the females. This could be a successful strategy as at any given time during the flowering period of *A. cyclops,* all floral stages from early, green buds to senescing flowers are present on the plant. The experimentation was however not designed to test olfaction over various distances and a specific investigation to confirm this observation will be required.

The observation of the “calling posture” and “oviposition posture” exhibited by some of the females during the bioassays with the open flower and yellow bud scents refutes the initial assumption that all the females were mated by the time the experiments were conducted. In fact, during the bioassays with Dichloromethane, a male and female were observed mating. The females that were “calling” (and therefore being unmated) were either too low in numbers to influence the result of

University of Cape Town

the attractiveness of the open flower scent, or the observation about *D. tetensi* unmated females not responding to host plant volatiles does not hold true for *D. dielsi*.

5.4.2. Behavioural response to antennal active compounds

Not all electrophysiologically active volatile compounds are relevant or trigger responses at the behavioural level (Mustaparta, 2002; Schoonhoven et al., 2005) and it is only on the behavioural level where the nature of the response, whether attractive or repulsive, can be established (Bruce et al., 2005). Only two of the six antennally active compounds that were tested in the bioassays, 4-Oxoisophorone and *β*-Linalool, elicited a significant behavioural response.

University of Cape Town

4-Oxoisophorone is known to produce positive antennal responses in butterflies, bees and moths (Andersson, 2003; Dötterl et al., 2005a; Guédot et al., 2008) and in this study, *D. dielsi* showed a behavioural attraction to 4-Oxoisophorone which contributed a large proportion to the total scent composition of open flowers. 4-Oxoisophorone and UC1693 co-occurred in the leaf, green bud, yellow bud, open flower and senescing flower volatile profiles of *A. cyclops*, the yellow bud and open flower volatile profiles of *A. melanoxylon*, and the yellow bud, open flower and senescing flower volatile profiles of *A. saligna* (Chapter 2). These two co-occurring volatiles were also present in scent samples of the *Pachycarpus plicatus* (Apocynaceae: Asclepiadoideae) (Shuttleworth & Johnson, 2010b). As antennal responses in *D. dielsi* were elicited by UC1693 and directly thereafter by 4-Oxosisophorone (following UC1693 on the GC-column), it is suggested that UC1693 also contributes to the attraction of the midges to the open flowers. Since UC1693 remains unidentified, it could however not be tested experimentally. As far as could be determined, this present study is the first report of a dipteran showing a behavioural attraction to 4-Oxoisophorone.

β-Linalool is a common floral volatile (Knudsen et al., 2006) and is an important attractant pheromone for many insects (Borg-Karlson et al., 2003; Georgieva et al., 2005), attracting several

carnivorous arthropods following herbivore damage (Degenhardt et al. 2003; Lücker et al., 2006; Mumm & Dicke, 2010) and is known as a common attractant to cetiniine beetles (Steenhuisen et al., 2010). It often occurs in the floral scent of moth-pollinated taxa (Jürgens et al., 2003; Dobson, 2006; Dötterl et al., 2006) and also in the scent of flowers pollinated by bees, beetles and butterflies (Jürgens et al., 2003; Dötterl et al., 2005a; Theis, 2006; Jürgens et al., 2009). However, despite its seemingly widespread occurrence as an attractant, it is also known to repel some florivores (Theis, 2006) and to repel or kill a variety of arthropods including vertebrate ectoparasites such as lice, fleas and mites and is used as a natural botanical insecticide (Weinzierl, 2000; Trumble, 2002; Weldon & Carroll, 2006). It has been shown to reduce feeding and oviposition of houseflies (Suckling & Karg, 2000) and to be toxic to several Coleoptera that damage post-harvest products (Shaaya & Rafaeli, 2007). Linalool is deemed to act as neurotoxin (Weinzierl, 2000; Trumble, 2002). Although its mode of action is not fully determined, it might be similar to that of Limonene which affects the sensory nerves of the peripheral nervous system and causes spontaneous stimulation of sensory nerves, subsequent signalling to motor nerves resulting in muscle twitching, convulsions, and then paralysis in insects (Weinzierl, 2000). This study has shown that *β*-Linalool, at the low concentration of 0.25%, is repellent to *D. dielsi* females and although paralysis was not observed, the high level of activity observed in the olfactometer in response to the odour of *β*-Linalool (Fig. 5.4c) might be due to aggravated stimulation of the sensory and motor nerves as described above. Insects can detect certain compounds at very low concentrations (Kamatou & Viljoen, 2008) and this seems to be the case for *D. dielsi* with *β*-Linalool. However, although *β*-Linalool as an individual compound acted as a repellent to *D. dielsi*, it seems not to exert the same influence on the midge when blended with the other compounds found in the *A. cyclops* floral scent (Bruce & Pickett, 2011) and a trade-off between attractive and repellent compounds may be displayed in this case.

A number of reasons could explain the lack of a behavioural response to the other four compounds that were tested in the bioassays. Ocimene was included in the electrophysiological and behavioural

University of Cape Town

tests because of the compound's high occurrence in the scent of *A. cyclops* (Table 2.1). In neither of the two test types, were positive responses recorded. This compound might simply not be relevant to the midges at all (Mustaparta, 2002). Behavioural attraction to the C_8-C_{20} Alkane standard solution mixture could not be demonstrated, possibly due to the fact that the concentrations of the individual compounds e.g. Heptadecane, within the mixtures might not have been optimal (Van Tol et al., 2007) or behaviourally relevant (Mustaparta, 2002). Heptadecane elicited a positive electrophysiological response in the GC-EAD experiments, but in the Alkane mixture, Heptadecane was present effectively only as 1.2% instead of the almost 8% in the scent of the open flowers of *A. cyclops*. (*Z*)-3-Hexenyl acetate, a typical green leaf volatile, is possibly also irrelevant to the insect (Mustaparta, 2002) as green leaf volatiles would not necessarily help insects to locate flowers (Honda et al., 1998) and therefore it is unlikely that they play a specific role in the attraction of the midges. However, it might play a role for the midge to distinguish between open and senescing flower stages.

University of Cape Town

5.4.3. Attraction of *D. dielsi* to simulations of the floral scent of four *Acacia* species

Dasineura dielsi females were not attracted or repelled by the simulations of the floral scent of *A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. melanoxylon*. The findings of the experiments with the simulated scent of *A. cyclops* will be contrasted with the findings of the experiments with the simulated scent of the other species.

Despite an apparent preference for the simulated scent of the open flowers of *A. cyclops*, the statistical methods used did not detect a significant behavioural response of *D. dielsi* females to blends of authentic standards. Although midges were attracted to 4-Oxoisophorone when presented as an individual compound, 4-Oxoisophorone did not exert the same effect on the midges when blended into the scent mixture (see also Bruce & Pickett, 2011). This result is not easy to explain and it can only be speculated that maybe some important compound in the scent mixture that attracts

D. dielsi is missing, e.g. the unidentified compound UC1693 that elicited an electrophisiological response and that in addition some compounds in the scent have a repellent effect on the midges.

The lack of olfactory responses of *D. dielsi* to the scent simulations of the non-standard hosts, *A. melanoxylon*, *A. saligna* and *A. longifolia*, may be explained as follows: 4-Oxoisophorone which play important roles in the attraction of *D. dielsi*, and UC1693 which elicited a significant antennal response in *D. dielsi,* were generally present in the scent profiles of these plant in lower relative amounts than in *A. cyclops* while *β*-Linalool, which produced a repellent effect on the midges, was generally present in higher relative amounts than in *A. cyclops*. The scent profile of *A. saligna* is very different from the other three plant species, with almost 50% of the scent profile being made up by Benzyl alcohol. The effect of Benzyl alcohol as an individual compound has not been evaluated, but might contribute to the significant difference in the levels of activity of the midges in the olfactometer. Alternatively, the lack of olfactory responses of *D. dielsi* to the scent simulations of the non-standard hosts may be attributed to the lack of minor compounds that were not included in the prepared scent simulations.

University of Cape Town

CHAPTER 6: GENERAL DISCUSSION

6.1. Introduction

Gall-forming cecidomyiids form part of the insect fauna associated with acacias across the world (Gagné, 2010). The Cecidomyiidae (Order: Diptera) has 6 131 known species in 783 genera of which about 80% of all species described are associated with flowering plants or plant-feeding arthropods (Gagné, 2010). The Cecidomyiinae comprise the largest subfamily of gall midges, with 4763 known species in 595 genera (Gagné, 2010). Gagné (2010) listed hosts for the Cecidomyiinae subfamily in his 2010 Catalogue and indicated that 54 species of midges belonging to 12 genera use 52 *Acacia* species as hosts (Kolesik et al., 2005; Gagné, 2010; Kolesik et al., 2010; Post et al., 2010). All in all, only 101 insect-host associations between midges and *Acacia* species have been recorded (Kolesik et al., 2005, 2010; Gagné, 2010; Post et al., 2010). The interaction between *D. dielsi* and *A. cyclops*, the focus of this study, therefore occurs between members of two very large taxonomic groups with a surprisingly small number of interactions given the sizes of the plant genus and galling insect family and the widespread distribution of both groups.

Despite the large size of the genus *Dasineura*, which contains a number of serious agricultural pests (Gagné, 2010), very little work has been done on olfaction of these insects (Amarawardana, 2009). Currently there have been 16 investigations into mate location by males by means of pheromone cues (Pettersson, 1976; Murchie et al., 1997; Harris et al., 1996; Crook & Mordue (Luntz), 1999; Gries et al., 2000, 2002, 2005; Hillbur et al., 2001, 2005; Birkett et al., 2004b; Heath et al., 2005; Amarawardana, 2009; Andersson et al., 2009; Cross & Hall, 2009; Hall et al., 2009; Liu et al., 2009; Mólnar et al., 2009) and five cases of host plant location by females by means of host plant odour cues (Pettersson, 1976; Murchie et al., 1997; Galanihe & Harris, 1997; Crook & Mordue (Luntz), 1999; Sharma & Franzmann, 2001; Birkett et al., 2004b). Very few studies have been done on the

University of Cape Town

plant volatile characterisation of *Acacia* species with records for only 14 out of more than 1350 species (Flath et al., 1983; Zygadlo et al., 1996; Lamarque et al., 1998; Kaiser, 1997; Willmer et al., 2009). As far as could be established, this study is the first to determine the volatile profiles of invasive *Acacia* species as well as the first to determine whether olfaction plays a role in the host finding of a galling insect on an invasive *Acacia* species.

6.2. Responses of *D. dielsi* females to plant volatiles from *A. cyclops*

In line with other studies showing differences between vegetative and floral plant parts e.g. in *Chrysanthemum coronarium* (Asteraceae) (Flamini et al., 2003) and *Gentiana* (Gentianaceae) species (Georgieva et al., 2005), the chemical composition of the volatile profile of the leaves of *A. cyclops* differed substantially from the yellow coloured floral stages in particular. The difference in compound class profile was also pronounced between the leaf profile and the different floral profiles with a clear dominance of Monoterpenes in the leaf profile. (*Z*)-*β*-Ocimene, a ubiquitous plant volatile compound (Knudsen et al., 2006) which formed a large part of the total leaf volatile complement, is often associated with green leaf volatiles. The tendency that compounds associated with GLVs often decreases as the inflorescence matures (Robertson et al., 1995) was also observed in the case of *A. cyclops*. However, since GLVs are not particularly helpful in distinguishing flowers as oviposition sites from the foliage background in general (Honda et al., 1998), it is not surprising that (*Z*)-*β*-Ocimene did elicit neither an antennal response nor a behavioural response in the midges, despite its relatively high amounts in the floral scent profiles.

The other prominent compound identified in the leaf profile of *A. cyclops*, was the GLV, (*Z*)-3-Hexenyl acetate. Even though a positive antennal response to (*Z*)-3-Hexenyl acetate was recorded by both male and female antennae, *D. dielsi* female midges showed no behavioural attraction or repulsion to the natural leaf scent in the bioassays. Similarly, the moth, *Hadena bicruris* also showed a positive antennal response to (*Z*)-3-Hexenyl acetate in the scent of *Silene latifolia*, but did not show

University of Cape Town

a positive behavioural response in wind tunnel bioassays (Dötterl, 2004). It is known that not all antennal active compounds are relevant at a behavioural level (Mustaparta, 2002). The lack of a positive behavioural response to the GLV, (*Z*)-3-Hexenyl acetate, is congruent with the ubiquitousness of green leaf volatiles in the natural environment, which would not necessarily help insects in accurate location of flowers (Honda et al., 1998).

The discrepancy between the signal detection and signal selection on the behavioural level may lie in differential central nervous system (CNS) processing of chemical stimuli received (Honda et al., 1998). Behavioural responses (e.g. attraction or repulsion) depend on the processing of peripheral inputs within the CNS, whereas electrophysiological responses recorded from insect antennae occur at the peripheral level only (Bruce et al., 2005). Another possible explanation is that (*Z*)-Hexenyl acetate provides information in another behavioural context than that tested here, or only in combination with other compounds. It may be an important synergist (or antagonist) in a blend of behaviourally significant compounds (Schütz et al., 1997; Larsson et al., 2001, 2003; Reddy & Guerrero, 2004) to thereby increase the probability of host finding by the insect (Jhumur et al., 2008). (*Z*)-Hexenyl acetate from *Zea mays* has been indicated as a synergist on *Helicoverpa zea* and *Cydia pomonella* (and other examples from Reddy & Guerrero, 2004). A further explanation may lie in differential gene expression that evolved over time (Wink, 2003) where genes that encoded for the responses to certain volatile compounds, were “switched off” according to ecological needs or the lack thereof, through evolutionary development (Wink, 2003).

University of Cape Town

The distribution of monoterpenes increases as the inflorenscence matures (Robertson et al., 1995). In the floral scent profiles of *A. cyclops*, the monoterpenes that increased towards the senescing flower profile, are Limonene, (*E*)-Linalool oxide (furanoid), *β*-Linalool, *α*-Terpineol and (*Z*)-Linalool oxide (pyranoid). Other patterns that emerged in the floral volatile profiles of *A. cyclops,* were the increase of 4-Oxoisophorone, UC1693 and Nonadecane towards the open flower stage and

Heptadecane towards the yellow bud stage, and a decrease of all of these again in the senescing flowers. Each of these patterns will be discussed in the context of the antennal and behavioural responses of *D. dielsi* to the scent of the flower volatiles.

Dasineura dielsi females were attracted to the natural scent of the open flowers of *A. cyclops*, as well as to the authentic standard of 4-Oxoisophorone. Furthermore, they showed positive antennal responses to 4-Oxoisophorone, UC1693, Heptadecane and Tricosane. 4-Oxoisophorone was considered to be an uncommon plant volatile (Knudsen et al., 2006), however, subsequent to the publication by Knudsen et al. (2006) it has been recorded more often (Raguso et al., 2006; Füssel et al., 2007; Moraga et al., 2009; Dormont et al., 2010; Shuttleworth & Johnson, 2010b), but given the increase in studies in identifying plant volatiles in general, it would probably still be deemed to be relatively uncommon. Previously it has been shown that 4-Oxoisophorone is an electrophysiologically active compound in bees, moths and butterflies (Andersson, 2003; Dötterl et al., 2005a; Guédot et al., 2008) and to my knowledge, this study presents the first record of a dipteran responding electrophysiologically and behaviourally to this compound.

UC1693 was always found in association with and preceding 4-Oxoisophorone, showing up as a "peak pair" with 4-Oxoisophorone in the chromatograms of the samples analysed. This "peak pair" occurred in scent profiles of three of the four Australian *Acacia* (Mimosaceae) species analysed, but were also detected in *Pachycarpus plicatus* (Apocynaceae) (Shuttleworth & Johnson, 2010b; samples run on the same machine under the same conditions as the *Acacia* samples). This is surprising since these two families are not closely related (Mimosaceae belongs to the Subclass Rosidae and Apocynaceae belongs to the Subclass Asteridae) and UC1693 does not seem to be a very common floral volatile, since it has not yet been identified. The "peak pair" and the antennal responses thereto were very evident in the GC-EAD chromatogram. Despite the composition of the yellow bud and open flower scent profiles being very similar, there was a strong difference in the ratio between

University of Cape Town

4-Oxoisophorone and UC1693. In the scent of the open flowers the ratio between 4-Oxoisophorone and UC1693 was 2:1 and in the scent of the yellow buds it was 4:1. Given the regular occurrence of the "peak pair" and the fact that *D. dielsi* showed an attraction to 4-Oxoisophorone, it is suggested that UC1693 also contributed to the attraction of *D. dielsi* to the open flower scent of *A. cyclops*. It is further suggested that *D. dielsi* not responding behaviourally to the scent of the yellow buds, can be explained by the difference in the ratio between 4-Oxoisophorone and UC1693 in the yellow buds.

Heptadecane on the other hand, was the most prominent Alkane in the scent of *A. cyclops* and peaked in relative amount in the yellow bud profile. Tricosane however, occurred in very low relative amounts in the scent of the open flowers of *A. cyclops* and peaked in the senescing flowers. Both these compounds elicited positive antennal responses in the GC-EAD experiments, but not when presented in the Alkane mixtures as was used in the EAG and bioassay experiments. In a natural environment, insects are "bombarded" with a multitude of chemical signals at different concentrations at the same time, and they have to "pick out" the correct signals for their purpose (Van Tol et al., 2007; Bruce & Pickett, 2011). Therefore, presenting the midges with a mixture of Alkanes would simulate the difficulties of scent location in their natural environments closer than presenting them with single compounds only. However, since it is relatively unlikely that the ratios of the Alkanes in the mixture are exactly the same as emitted from natural flowers, it is not surprising that the midges did not respond behaviourally to these two compounds as part of an Alkane mixture.

β-Linalool and Limonene, although not prominent compounds in the scent of any of the *A. cyclops* volatile profiles, elicited positive antennal responses in *D. dielsi* and *β*-Linalool induced a repellent effect on *D. dielsi*. According to Theis and Raguso (2005) and Theis (2006), repellent compounds have infrequently been reported in the literature. As an individual compound, *β*-Linalool had a repelling effect on the midges, however, when *β*-Linalool was presented to the midges within a

University of Cape Town

blend of the open flower scent compounds, it did not exert any effect. Similarly, Webster et al. (2010) have shown aphids to be repelled by certain compounds being presented individually (including *β*-Linalool), but attracted when these same compounds were included in a blend. Interestingly, *β*-Linalool can act as neurotoxin on some insects (Trumble, 2002; Weinzierl, 2000). The reaction includes muscle twitching and convulsions (Weinzierl, 2000) which may explain the high level of activity by the midges observed in the bioassays with *β*-Linalool. So while *β*-Linalool alone is likely to be perceived as repellent, its repelling effects might be overlaid with positive attracting effects by other compounds within a floral scent blend where a reward of a suitable oviposition site is associated with the entire scent bouquet (Theis, 2006). Dose-response experiments may indicate at what concentration the individual repellent compound will become repellent even when in a blend (Webster et al., 2010). Aside from *β*-Linalool being a neurotoxin, monoterpenes at low concentrations in floral odours often have important functions to insects (Bernays & Chapman, 1994), e.g. because of their high volatility, they often play an important role in distance attraction of insect herbivores (Bernays & Chapman, 1994). *β*-Linalool being present at low concentration within the scent of the yellow buds and the scent of the open flowers, might thus play a role in long-distance attraction to the midge (Schütz et al., 1997). Limonene, also a monoterpene, elicited a positive antennal response, but it did not elicit a behavioural response, neither attracting nor repellening midges, although it can act as neurotoxin for some insects, like *β*-Linalool (Weinzierl, 2000). It is assumed that both *β*-linalool and Limonene also affected the neural system in *D. dielsi*, since the level of activity of the midges in the olfactometer during the bioassays with Limonene and *β*-Linalool was similarly and exceptionally high as compared to all other compounds tested. The lack of a negative (repelled) behavioural response to Limonene (as opposed to *β*-Linalool) however, might be due to not enough replicates being tested to ensure greater statistical confidence or due to a concentration being used that was below the toxic threshold levels for *β*-Linalool.

University of Cape Town

Based on the results of this study and the information on the effects of the different compounds presented here, it is suggested that *D. dielsi* in its host finding capability is "selectively tuned" to a small number of compounds (Larsson et al., 2003; Stensmyr, 2004) as i) the majority of floral odorants tested for electrophysiological significance (EAG) were either "ignored" by the olfactory system or below the antennal detection threshold, and ii) only two of these elicited a significant behavioural response. Monophagous or oligophagous insects often use specific key compounds for food or oviposition site location (Larsson et al., 2003) and the attraction to 4-Oxoisophorone and potentially to UC1693, fits this view. The selection of the open flowers, and none of the other floral stages, by *D. dielsi* females as oviposition sites, is olfactory based.

6.3. Volatile profiles of non-standard hosts compared to *A. cyclops*

Variations in volatile composition among species that are closely related are commonly found, e.g. in 8 spp. of *Cypripedium* (Barkman et al., 1997), 13 spp. of *Ficus* (Grison et al., 1999), 13 spp. of *Silene* (Jürgens et al., 2002), 7 spp. of *Dianthus* (Jürgens et al., 2003) and 9 spp. of *Nicotiana* (Raguso et al., 2003). Barkman et al. (1997) studied intra- and interspecific variation of plant volatiles of *Cypripedium* (Orchidaceae) and found that despite substantial qualitative population-level variation in floral scent composition, it was less than interspecific variation and they concluded that every species has a unique floral scent profile. Raguso et al. (2003) also reported intra-specific variation in spp. of *Nicotiana* to be less than interspecific variation. Grison et al. (1999) have found some uncommon compounds in the *Ficus* species they investigation and also suggested uniqueness of floral scent per species. The uniqueness of floral scents often reflects phylogenetic relationships of species and has been used as a tool in chemosystematic studies (Jürgens et al., 2003).

The different hosts used by *D. dielsi*, *A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. saligna*, show interspecific variation in the individual compounds of the volatile profiles of the leaves, the yellow

University of Cape Town

buds, the open flowers and the senescing flowers. Interspecific variation in the volatile profiles of open flowers, the flower stage that *D. dielsi* uses for oviposition, was also evident on the compound class level. In *A. cyclops*, the volatile profile is dominated by aliphatic compounds, whereas in *A. melanoxylon* and *A. longifolia* it is dominated by monoterpenes and in *A. saligna* by benzenoid compounds. The different compound classes denote the different biochemical pathways through which the volatiles are produced (Dudareva & Pichersky, 2006). Patterns emerged in the scent of the open flowers of *A. cyclops* and *A. saligna* which suggest the presence of unique specific compound blends that are characteristic of these species. In the scent of *A. cyclops*, this specific blend consisted of 4-Oxoisophorone and UC1693 co-occuring in a ratio of 2:1, and in the scent of *A. saligna* it is suggested that Benzyl alcohol which occurs in almost 50% of the volatiles in the floral scent of *A. saligna* forms a substantial part of a characteristic blend. Further investigation however will be required to ascertain the nature of all parts of this blend. In the case of *A. melanoxylon* and *A. longifolia* no specific compound or blend could be suggested as being characteristic and unique for these species.

University of Cape Town

Although the volatile profiles of *A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. saligna* contained many similar compounds, the concentrations of these compounds were highly variable, and no similarity in the composition of especially the ten most abundant compounds per profile could be found that would substantiate the hypothesis that similarity in the volatiles profiles would explain the occasional use of *A. melanoxylon*, *A. longifolia* and *A. saligna* as non-standard hosts of *D. dielsi*.

6.4. Volatile profiles of non-host African *Acacia* species compared to the Australian acacias

Intraspecifically, the leaf and floral volatile profiles of each of the African species, *A. karroo*, *A. sieberiana* var. *woodii* and *A.xanthophloea* differed substantially. These results are in line with other studies e.g. Flamini et al. (2003) and Georgieva et al. (2005) and the four Australian species discussed earlier. Interspecifically, the compound class profiles of both the leaf and the flowers volatiles of *A.*

sieberiana var. *woodii* and *A.xanthophloea* showed some similarity due to the very high proportions of aliphatic compounds and monoterpenes in the leaf profiles and high proportions of monoterpenes in the flower volatile profiles. In each case however, the underlying individual compounds, varied qualitatively and quantitatively. The compound class profile of the leaf volatiles of *A. karroo* differed substantially from that of *A. sieberiana* var. *woodii* and *A.xanthophloea*, in the total relative amounts of the monoterpenes, and the compound class profile of the flower volatiles of *A. karroo* different from both *A. sieberiana* var. *woodii* and *A.xanthophloea* in the relative amounts of both the aliphatic compounds and the benzenoids.

University of Cape Town

Generally, there is great dissimilarity between the floral volatile profiles of the African and the Australian acacias. Only in the compound class composition between *A. karroo* and *A. saligna* were similarities detected. However, the underlying individual compounds varied both qualitatively and quantitatively. In the case of *A. karroo*, almost a third of the scent profile comprised of Benzyl alcohol together with *α*-Farnesene and Cinnamyl alcohol (in a ratio of approximately 3:1:1), and it is suggested that this blend is characteristic of *A. karroo*. While it is believed that bees are the main pollinators of *A. sieberiana* var. *woodii* (Harborne, 2001; Dobson, 2006), *A. sieberiana* var. *woodii* had a number of volatiles typically found in plants associated with lepidopteran pollinators (Harborne, 2001; Dobson, 2006). The compounds were (*E*)-Linalool oxide (furanoid), (*Z*)-Linalool oxide (pyranoid), Indole, (*Z*)-Linalool oxide (furanoid) and (*E*)-Linalool oxide (pyranoid) and combined they make up a large part of the total floral volatile profile of this species. These compounds in the flower volatiles of A*. sieberiana* var. *woodii* were differentiated the volatile profile from that of the four Australian species, *A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. saligna*.

In addition to the differences in volatile profiles between species discussed above, some other differences between the Australian acacias (*A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. saligna*) and the African acacias (*A. karroo*, *A. sieberiana* var. *woodii* and *A. xanthophloea*) were observed.

The volatile emission rate (ng per inflorescence per hour) of the African acacias was higher than that of the Australian acacias. The scent of *A. sieberiana* var. *woodii* was very noticeable even on the human nose with strong floral-fruity overtones. Except for potentially attracting the floral visitors, such as pollinators to the plants, the functional significance thereof is unknown. The long-chain *n*-Alkanes (C_{25}-C_{33}) dominated in the floral scent of *A. karroo* and *A. sieberiana* var. *woodii* whereas the short-chain *n*-Alkanes (C_{14}-C_{20}) dominated in *A. cyclops*, *A. melanoxylon*, *A. longifolia* and *A. saligna*; no *n*-Alkanes were detected in *A. xanthophloea*.

6.5. Conclusion

In conclusion, it has been demonstrated that *D. dielsi* females indeed respond to the scent of the open flowers of *A. cyclops* and are likely to use this scent to locate suitable flowers for oviposition. There are no similarities in the scents of the Australian acacias, *A. melanoxylon*, *A. longifolia* and *A. saligna* to the scent of *A. cyclops* that might explain the occasional use of these plants as alternative hosts by *D. dielsi*. Cues other than olfaction should also be investigated to determine whether there are other attractants to *D. dielsi* on the non-standard hosts. Additional olfactory stimuli cannot be ruled out as attractants to *D. dielsi* by these species, as some compounds outside the set tested in this project, may be behaviourally significant to *D. dielsi*. The volatile profiles of the African acacias are suitably different from that of *A. cyclops* not to be attractive to *D. dielsi*, however host plant volatiles are but one of the many aspects that determines host plant suitability.

University of Cape Town

REFERENCES

Abdullahi, G., Sule, H., Chimoya I.A. & Isah, M.D., 2011. Diversity and relative distribution of honeybees foraging plants in some selected reserves in Mubi Region, Sudan Savannah ecological zone of Nigeria. Advances in Applied Science Research 2, 388-395.

Adair, R.J., 2004. Seed reducing Cecidomyiidae as potential biological control agents for invasive Australian wattles in South Africa, particularly *Acacia mearnsii* and *A. cyclops*. Ph.D. Dissertation, University of Cape Town.

Adair, R.J., 2005. The biology of *Dasineura dielsi* Rübsaamen (Diptera: Cecidomyiidae) in relation to the biological control of *Acacia cyclops* (Mimosaceae) in South Africa. Australian Journal of Entomology 44, 446-456.

Adair, R.J., Neser, S. & Kolesik, P., 2000. Australian seed-preventing gall midges (Diptera: Cecidomyiidae) as potential biological control agents for invasive *Acacia* spp. in South Africa. In: Spencer, N.R. (ed), Proceedings of the X International Symposium on Biological Control of Weeds, Bozeman, Montana, 4-14 July 1999, pp. 605-613. Montana State University, Montana, USA.

Alagarmalai, J., Nestel, D., Dragushich, D., Nemny-Lavy, E., Anshelevich, L., Zada, A. & Soroker, V., 2009. Identification of host attractants for the Ethiopian fruit fly, *Dacus ciliatus* Loew. Journal of Chemical Ecology 35, 542-551.

Almeida, F.V.M., Santos, J.C., Silveira, F.A.O. & Fernandes, G.W., 2006. Distribution and frequency of galls induced by *Anisodiplosis waltheriae* Maia (Diptera: Cecidomyiidae) on the invasive plant *Waltheria indica* L. (Sterculiaceae). Neotropical Entomology 35, 435-439.

Amarawardana, L., 2009. The chemical diversity of midge pheromones. Ph.D. Dissertation, University of Greenwich.

Amirav, A. & Dagan, S., 1997. A direct sample introduction device for mass spectrometry studies and GC-MS analysis. European Journal of Mass Spectrometry 3, 105-111.

Andersson, M.N., Haftmann, J., Stuart, J.J., Cambron, S.E., Foster, S.P., Franke, S., Francke, W. & Hillbur, Y., 2009. Identification of sex pheromone components of the Hessian Fly, *Mayetiola destructor*. Journal of Chemical Ecology 35, 81-95.

Andersson, S. & Dobson, H.E.M., 2003. Antennal responses to floral scents in the butterfly *Heliconius melpomene.* Journal of Chemical Ecology 29, 2319-2330.

University of Cape Town

Andersson, S., 2003. Antennal responses to floral scents in the butterflies *Inachis io, Aglais urticae* (Nymphalidae), and *Gonepteryx rhamni* (Pieridae). Chemoecology 13, 13-20.

Andrews, E.S., Theis, N. & Adler, L.S., 2007. Pollinator and herbivore attraction to *Cucurbita* floral volatiles. Journal of Chemical Ecology 33, 1682-1691.

Anfora, G., Ioriatti, C., Moser, S., Germinara, G.S. & De Cristofaro, A., 2005. Electrophysiological responses of two different species of apple gall midges (Diptera: Cecidomyiidae) to host plant volatiles. IOBC/wprs Bulletin 28, 413-417.

Archibald, S. & Bond, W.J., 2003. Growing tall vs. growing wide: tree architecture and allometry of *Acacia karroo* in forest, savanna, and arid environments. Oikos 102, 3-14.

Arimura, G., Kost, C. & Boland, W., 2005. Herbivore-induced, indirect plant defences. Biochimica et Biophysica Acta 1734, 91-111.

Arimura, G., Matsui, K. & Takabayashi, J., 2009. Chemical and molecular ecology of herbivore-induced plant volatiles: Proximate factors and their ultimate functions. Plant and Cell Physiology 50, 911-923.

Arimura, G., Ozawa, R., Horiuchi, J., Nishioka, T. & Takabayashi, J., 2001. Plant-plant interactions mediated by volatiles emitted from plants infested by spider mites. Biochemical Systematics and Ecology 29, 1049-1061.

Ayasse, M., 2006. Floral scent and pollinator attraction in sexually deceptive orchids. In: Dudareva, N. & Pichersky, E. (eds), Biology of floral scent. CRC Press, Boca Raton, Florida, USA.

Azuma, H., Thien, L.B., Kawano, S., 1999. Molecular phylogeny of *Magnolia* (Magnoliaceae) inferred from cpDNA sequences and evolutionary divergence of the floral scents. Journal of Plant Research 112, 291-306.

Azuma, H., Thien, L.B., Toyota, M., Asakawa, Y. & Kawano, S., 1997. Distribution and differential expression of (*E*)-4,8-Dimethyl-1,3,7-nonatriene in leaf and floral volatiles of *Magnolia* and *Liriodendron* taxa. Journal of Chemical Ecology 23, 2467-2478.

Baer, B., 2003. Bumblebees as model organisms to study male sexual selection in social insects. Behavioral Ecology and Sociobiology 54, 521-533.

Barkman, T. J., Beaman, J.H. & Gage, D.A., 1997. Floral fragrance variation in *Cypripedium*: Implications for evolutionary and ecological studies. Phytochemistry 44, 875-882.

University of Cape Town

Barnes, H.F., 1935. Studies of fluctuations in insect populations: VI. Discussion on results of studies I-V. Journal of Animal Ecology 4, 254-263.

Bernays, E.A. & Chapman, R.F., 1994. Host-plant selection by phytophagous Insects. Chapman and Hall, New York, USA.

Beyaert, I., Wäschke, N., Scholz, A., Varama, M., Reinecke, A. & Hilker, M., 2010. Relevance of resource-indicating key volatiles and habitat odour for insect orientation. Animal Behaviour 79, 1077-1086.

Bianchi, F., Careri, M. & Musci, M., 2005. Volatile norisoprenoids as markers of botanical origin of Sardinian strawberry-tree (*Arbutus unedo* L.) honey: Characterisation of aroma compounds by dynamic headspace extraction and gas chromatography–mass spectrometry. Food Chemistry 89, 527-532.

Birkett, M.A., Agelopoulos, N., Jensen, K-M.V., Jespersen, J.B., Pickett, J.A., Prijs, H.J., Thomas, G., Trapman, J.J., Wadhams, L.J. & Woodcock, C.M., 2004a. The role of volatile semiochemicals in mediating host location and selection by nuisance and disease-transmitting cattle flies. Medical and Veterinary Entomology 18, 313-322.

Birkett, M.A., Bruce, T.J.A., Martin, J.L., Smart, L.E., Oakley, J. & Wadhams, L.J., 2004b. Responses of female orange wheat blossom midge, *Sitodiplosis mosellana*, to wheat panicle volatiles. Journal of Chemical Ecology 30, 1319-1328.

Blackmer, J.L., Rodriguez-Saona, C., Byers, J.A., Shope, K.L. & Smith, J.P., 2004. Behavioral response of *Lygus hesperus* to conspecifics and headspace volatiles of alfalfa in a Y-tube olfactometer. Journal of Chemical Ecology 30, 1547-1564.

Blight, M.M., Le Métayer, M., Delègue, M.H.P., Pickett, J.A., Marion-Poll, F. & Wadhams, L.J., 1997. Identification of floral volatiles involved in recognition of oilseed rape flowers (*Brassica napus*) by honeybees (*Apis mellifera*). Journal of Chemical Ecology 23, 1715-1727.

Boddum, T., Skals, N., Hill, S.R., Hansson, B.S. & Hilbur, Y., 2010. Gall midge olfaction: pheromone sensitive olfactory neurons in *Contarinia nasturtii* and *Mayetiola destructor*. Journal of Insect Physiology 56, 1306-1314.

Borg-Karlson, J., Tengo, I., Valterova, C.R., Unelius, T., Taghizadeh, T., Tolasch, T. & Francke, W., 2003. (S)-(+)-Linalool, a mate attractant pheromone component in the bee *Colletes cunicularis*. Journal of Chemical Ecology 29, 1-14.

University of Cape Town

Bouchenak-Khelladi, Y., Maurin, O., Hurter, J. & Van der Bank, M., 2010. The evolutionary history and biogeography of Mimosoideae (Leguminosae): An emphasis on African acacias. Molecular Phylogenetics and Evolution 57, 495-508.

Bouvier, F., Rahier, A. & Camara, B., 2005. Biogenesis, molecular regulation and function of plant isoprenoids. Progress in Lipid Research 44, 357-429.

Brouat, C., McKey, D., Bessière, J., Pascal, L. & Hossaert-McKey, M., 2000. Leaf volatile compounds and the distribution of ant patrolling in an ant-plant protection mutualism: Preliminary results on *Leonardoxa* (Fabaceae: Caesalpinioideae) and *Petalomyrmex* (Formicidae: Formicinae). Acta Oecologica 21, 349-357.

Bruce, T.J. & Cork, A., 2001. Electrophysiological and behavioural responses of female *Helicoverpa armigera* to compounds identified in flowers of African marigold, *Tagetes erecta*. Journal of Chemical Ecology 27, 1119-1131.

Bruce, T.J.A. & Pickett, J.A., 2011. Perception of plant volatile blends by herbivorous insects – Finding the right mix. Phytochemistry 72, 1605-1611.

Bruce, T.J.A., Wadhams, L.J. & Woodcock, C.M., 2005. Insect host location: a volatile situation. Trends in Plant Science 10, 269-274.

Büchel, K., Malskies, S., Mayer, M., Fenning, T.M., Gershenzon, J., Hilker, M. & Meiners, T., 2011. How plants give early herbivore alert: Volatile terpenoids attract parasitoids to egg-infested elms. Basic and Applied Ecology 12, 403-412.

Carruthers, J. & Robin, L., 2010. Taxonomic imperialism in the battles for *Acacia*: Identity and science in South Africa and Australia. Transactions of the Royal Society of South Africa 65, 48-64.

Castells, E. & Berenbaum, M.R., 2008. Host plant selection by a monophagous herbivore is not mediated by quantitative changes in unique plant chemistry: *Agonopterix alstroemeriana* and *Conium maculatum*. Arthropod-Plant Interactions 2, 43-51.

Cheng, A.X., Lou, Y.G., Mao, Y.B., Lu, S., Wang, L.J. & Chen, X.Y., 2007. Plant terpenoids: Biosynthesis and ecological functions. Journal of Integrative Plant Biology 49, 179-186.

Clarke, K.R. & Gorley, R.N., 2006. Primer v5: User Manual/Tutorial. Primer-E Ltd.

Clarke, K.R. & Warwick, R.M., 2001. Change in marine communities: an approach to statistical analysis and interpretation. 2nd ed., Primer-E Ltd, Plymouth, UK.

University of Cape Town

Coates Palgrave, M., 2005. Keith Coates Palgrave Trees of Southern Africa. Edition 3, Imprint 3, Struik Publishers, Cape Town, South Africa.

Cook, M.A., Ozeroff, S.N., Fitzpatrick, S.M. & Roitberg, B.D., 2011. Host-associated differentiation in reproductive behaviour of cecidomyiid midges on cranberry and blueberry. Entomologia Experimentalis et Applicata 141, 8-14.

Crook, D.J. & Mordue (Luntz), A.J., 1999. Olfactory responses and sensilla morphology of the blackcurrant leaf midge *Dasineura tetensi*. Entomologia Experimentalis et Applicata 91, 37-50.

Cross, J.V. & Hall, D.R., 2009. Exploitation of the sex pheromone of apple leaf midge *Dasineura mali* Kieffer (Diptera: Cecidomyiidae): Part 1. Development of lure and trap. Crop Protection 28, 139-144.

Csurches, S. & Edwards, R., 1998. Potential environmental weeds in Australia: Candidate species for preventative control. The Director of the National Parks and Wildlife, Biodiversity Group, Environment Australia (formerly Australian Nature Conservation Agency), Canberra, Australia.

Daehler, C.C., 1998. The taxonomic distribution of invasive angiosperm plants: Ecological insights and comparison to agricultural weeds. Biological Conservation 84, 167-180.

De Moraes, C. M., Lewis, W. J., Pare, P. W., Alborn, H. T. & Tumlinson, J. H., 1998. Herbivore infested plants selectively attract parasitoids. Nature 393:570-573.

Degenhardt, J., Gershenzon, J., Baldwin, I.T. & Kessler, A., 2003. Attracting friends to feast on foes: engineering terpene emission to make crop plants more attractive to herbivore enemies. Current Opinion in Biotechnology 14, 169-176.

Dennill, G.B. & Donnelly, D., 1991. Biological control of *Acacia longifolia* and related weed species (Fabaceae) in South Africa. Agriculture, Ecosystems and Environment 37, 115-135.

Dennill, G.B., Donnelly, D., Stewart, K. & Impson, F.A.C., 1999. Insect agents used for the biological control of Australian *Acacia* species and *A. xanthophloea* (Willd.) Nielsen (Fabaceae) in South Africa. African Entomology Memoir 1, 45-54.

Desneux, N., Rafalimanana, H. & Kaiser, L., 2004. Dose-response relationship in lethal and behavioural effects of different insecticides on the parasitic wasp *Aphidius ervi*. Chemosphere 54, 619-627.

University of Cape Town

Dobson, H.E.M., 2006. Relationship between floral fragrance composition and type of pollinator. In: Dudareva, N. & Pichersky, E. (eds), Biology of floral scent. CRC Press, Boca Raton, Florida, USA.

Donnelly, D. & Hoffmann, J.H. 2004. Utilisation of an unpredictable food source by *Melanterius ventralis* (Coleoptera: Curculionidae), a seed-feeding biological control agent of *Acacia longifolia* (Mimosaceae) in South Africa. Biocontrol 49, 225-235.

Dorchin N., Clarkin C.E., Scott E.R., Luongo M.P. & Abrahamson W.G., 2007. Taxonomy, life history, and population sex ratios of North American *Dasineura* (Diptera: Cecidomyiidae) on goldenrods (Asteraceae). Annals of the Entomological Society of America 100, 539-548.

Dormont, L., Delle-Vedove, R., Bessière, J-M., Hossaert-Mc Key, M. & Schatz, B., 2010. Rare white-flowered morphs increase the reproductive success of common purple morphs in a food-deceptive orchid. New Phytologist 185, 300-310.

Dötterl, S. & Jürgens, A., 2005. Spatial fragrance patterns in flowers of *Silene latifolia*: Lilac compounds as olfactory nectar guides? Plant Systematics and Evolution 255, 99-109.

Dötterl, S. & Vereecken, N.J., 2010. The chemical ecology and evolution of bee–flower interactions: a review and perspectives. Canadian Journal of Zoology 88, 668-697.

Dötterl, S., 2004. Importance of floral scent compounds for the interaction between *Silene latifolia* (Caryophyllaceae) and the nursery pollinator *Hadena bicruris* (Lepidoptera: Noctuidae). Ph.D. Dissertation, University of Bayreuth, Germany.

Dötterl, S., Füssel, U., Jürgens, A. & Aas, G., 2005a. 1,4-Dimethoxybenzene, a floral scent compound in willows that attracts an oligolectic bee. Journal of Chemical Ecology 31, 2993-2998.

Dötterl, S., Jürgens, A., Seifert, K., Laube, T., Weißbecker, B. & Schütz, S., 2006. Nursery pollination by a moth in *Silene latifolia*: The role of odours in eliciting antennal and behavioural responses. New Phytologist 169, 707-718.

Dötterl, S., Jürgens, A., Wolfe, L.M. & Biere, A., 2009. Disease status and population origin effects on floral scent: potential consequences for oviposition and fruit predation in a complex interaction between a plant, fungus, and noctuid moth. Journal of Chemical Ecology 35, 307-319.

Dötterl, S., Wolfe, L.M. & Jürgens, A., 2005b. Qualitative and quantitative analyses of flower scent in *Silene latifolia*. Phytochemistry 66, 203-213.

University of Cape Town

Dudareva, N. & Negre, F., 2005. Practical applications of research into the regulation of plant volatile emission. Currrent Opinion in Plant Biology 8, 113-118.

Dudareva, N. & Pichersky, E., 2000. Biochemical and molecular genetic aspects of floral scents. Plant Physiology 122, 627-633.

Dudareva, N. & Pichersky, E., 2006. Floral scent metabolic pathways: their regulation and evolution. In: Dudareva, N. & Pichersky, E. (eds), Biology of floral scent. CRC Press, Boca Raton, Florida, USA.

Dudareva, N., Pichersky, E. & Gershenzon, J., 2004. Biochemistry of plant volatiles. Plant Physiology 135, 1893-1902.

Effmert, U., Große, J., Röse, U.S.R., Ehrig, F., Kägi, R. & Piechulla, B., 2005. Volatile composition, emission pattern, and localization of floral scent emission in *Mirabilis jalapa* (Nyctaginaceae). American Journal of Botany 92, 2-12.

El-Sayed, A.M., 2011. The Pherobase: Database of Insect Pheromones and Semiochemicals. Available at: http://www.pherobase.com. Accessed: 25 October 2011.

Enright, W.D., 2000. The effect of terrestrial invasive alien plants on water scarcity in South Africa. Physics and Chemistry of the Earth (B) 25, 237-242.

Faccoli, M., Anfora, G. & Tasin, M., 2008. Responses of the Mediterranean pine shoot beetle *Tomicus destruens* (Wollaston) to pine shoot and bark volatiles. Journal of Chemical Ecology 34, 1162-1169.

Farré, G., Sanahuja, G., Naqvi, S., Bai, C., Capell, T., Zhu, C. & Christou, P., 2010. Travel advice on the road to carotenoids in plants. Plant Science 179, 28-48.

Flamini, G., Cioni, P.L. & Morelli, I., 2003. Differences in the fragrances of pollen, leaves, and floral parts of garland (*Chrysanthemum coronarium*) and composition of the essential oils from flowerheads and leaves. Journal of Agricultural and Food Chemistry 51, 2267-2271.

Flath, R.A., Mon, T.R., Lorenz, G., Whitten, C.J. & Mackley, J.W., 1983. Volatile components of *Acacia* sp. blossoms. Journal for Agriculture and Food Chemistry 31, 1167-1170.

Francis, F., Lognay, G. & Haubruge, E., 2004. Olfactory responses to aphid and host plant volatile releases: (*E*)-*β*-Farnesene an effective kairomone for the predator *Adalia bipunctata*. Journal of Chemical Ecology 30, 741-755.

University of Cape Town

Füssel, U., Dötterl, S., Jürgens, A. & Aas, G., 2007. Inter- and intraspecific variation in floral scent in the genus *Salix* and its implication for pollination. Journal of Chemical Ecology 33, 749-765.

Gagné, R.J., 2010. Update for a catalog of the Cecidomyiidae (Diptera) of the world. Digital version 1. http://www.ars.usda.gov/SP2UserFiles/Place/12754100/Gagne_2010_World_Catalog_Cecidomyiidae.pdf

Galanihe, L.D. & Harris, M.O., 1997. Plant volatiles mediate host-finding behaviour of the apple leaf curling midge. Journal of Chemical Ecology 23, 2639-2655.

Gaskett, A.C., Conti, E. & Schiestl, F.P., 2005. Floral odor variation in two heterostylous species of *Primula*. Journal of Chemical Ecology 31, 1223-1228.

Georgieva, E., Handjieva, N., Popov, S. & Evstatieva, L., 2005. Comparative analysis of the volatiles from flowers and leaves of three *Gentiana* species. Biochemical Systematics and Ecology 33, 938-947.

Gibson, M.R., Richardson, D.M., Marchante, E., Marchante, H., Rodger, J.G., Stone, G.N., Byrne, M., Fuentes-Ramírez, A., George, N., Harris, C., Johnson, S.D., Le Roux, J.J., Miller, J.T., Murphy, D.J., Pauw, A., Prescott, M.N., Wandrag, E.M. Wilson, J.R.U., 2011. Reproductive biology of Australian acacias: important mediator of invasiveness? Diversity and Distributions 17, 911-933.

Goff, S.A. & Klee, H.J., 2006. Plant volatile compounds: sensory cues for health and nutritional value? Science 311, 815-819.

Goodrich, K.R. & Raguso, R.A., 2009. The olfactory component of floral display in *Asimina* and *Deeringothamnus* (Annonaceae). New Phytologist 183, 457-469.

Gouinguené, S.P. & Städler, E., 2006. Oviposition in *Delia platura* (Diptera, Anthomyiidae): The role of volatile and contact cues of bean. Journal of Chemical Ecology 32, 1399-1413.

Gries, R., Gries, G., Khaskin, G., King, S., Olfert, O., Kaminski, L., Lamb, R. & Bennett, R., 2000. Sex pheromone of orange wheat blossom midge, *Sitodiplosis mosellana*. Naturwissenschaften 87, 450-454.

Gries, R., Khaskin, G., Bennett, R.G., Miroshnychenko, A., Burden, K. & Gries, G., 2005. (S,S)-2,12-, (S,S)-2,13- and (S,S)-2,14- diacetoxyheptadecanes: Sex pheromone components of red cedar cone midge, *Mayetiola thujae*. Journal of Chemical Ecology 31, 2933-2946.

University of Cape Town

Gries, R., Khaskin, G., Gries, G. Bennett, R.G., King, S.G.G., Morewood, P., Slessor, K.N. & Morewood, W.D., 2002. (Z,Z)-4,7- tridecadien-(S)-2-yl acetate: Sex pheromone of Douglas–fir cone gall midge, *Contarinia oregonensis*. Journal of Chemical Ecology 28, 2283-2297.

Griffiths, D.W., Robertson, G.W., Shepherd, T. & Ramsay, G., 1999. Epicuticular waxes and volatiles from faba bean (*Vicia faba*) flowers. Phytochemistry 52, 607-612.

Grison, L., Edwards, A.A. & Hossaert-McKey, M., 1999. Interspecies variation in floral fragrances emitted by tropical *Ficus* species. Phytochemistry 52, 1293-1299.

Guédot, C., Landolt, P.J. & Smithhisler, C.L., 2008. Odorants of the flowers of butterfly bush, *Buddleja davidii*, as possible attractants of pest species of moths. Florida Entomologist 91, 576-582.

Hall, D.R., Farman, D.I., Cross, J.V., Pope, T.W., Ando, T. & Yamamoto, M., 2009. (S)-2-Acetoxy-5-undecanone, female sex pheromone of raspberry cane midge, *Resseliella theobaldi* (Barnes). Journal of Chemical Ecology 35, 230-242.

Hanley, M.E., Lamont, B.B., Fairbanks, M.M. & Rafferty, C.M., 2007. Plant structural traits and their role in anti-herbivore defence. Perspectives in Plant Ecology, Evolution and Systematics 8, 157-178.

Hansson, B.S., Larsson, M.C. & Leal, W.S., 1999. Green leaf volatile-detecting olfactory receptor neurones display very high sensitivity and specificity in a scarab beetle. Physiological Entomology 24, 121-126.

Harborne, J.B., 2001. Twenty-five years of chemical ecology. Natural Product Reports 18, 361-379.

Harley, K.L.S. & Forno, I.W., 1992. The biological control of weeds – a handbook for practitioners and students. Inkata Press, Melbourne and Sydney, Australia.

Harris, M.O. & Rose, S., 1989. Temporal changes in the egglaying behaviour of the Hessian fly. Entomologia Experimentalis et Applicata 53, 17-29.

Harris, M.O., Foster, S.P., Agee, K. & Dhana, S., 1996. Sex pheromone communication in the apple leafcurling midge (*Dasineura mali*). In: O'Callaghan, M. (ed.) Proceedings of the 49th New Zealand Plant Protection Conference, 13-15 August 1996, Nelson, New Zealand. The New Zealand Plant Protection Society Inc., Auckland, New Zealand.

Harris, P. & Shorthouse, J.D., 1996. Effectiveness of gall inducers in weed biological control. The Canadian Entomologist 128, 1021-1055.

University of Cape Town

Heath, J.J., Zhang, A., Roelofs, W.L. & Smith, R.F., 2005. Flight activity and further evidence for a female-produced sex pheromone of the apple leaf midge, *Dasineura mali,* in Nova Scotia. Northeastern Naturalist 12, 93-102.

Henderson, L., 2001. Alien weeds and invasive plants: A complete guide to declared weeds and invaders in South Africa. Agricultural Research Council, Pretoria, South Africa.

Hillbur, Y., Bengtsson, M., Löfqvist, J., Biddle, A., Pillon, O., Plass, E., Francke, W. & Hallberg, E., 2001. A chiral sex pheromone system in the pea midge, *Contarinia pisi*. Journal of Chemical Ecology 27, 1391-1407.

Hillbur, Y., Celander, M., Baur, R., Rauscher, S., Haftmann, J., Franke, S. & Francke, W., 2005. Identification of the sex pheromone of the swede midge, *Contarinia pasturtii*. Journal of Chemical Ecology 31, 1807-1828.

Hoffmann, J.H., Impson, F.A.C., Moran, V.C. & Donnelly, D. 2002. Biological control of invasive golden wattle trees (*Acacia pycnantha*) by a gall wasp, *Trichilogaster* sp. (Hymenoptera: Pteromalidae) in South Africa. Biological Control 25: 64-73.

Holopainen, J.K., 2004. Multiple functions of inducible plant volatiles. Trends in Plant Science 9, 529-533.

Honda, K., Ômura, H. & Hayashi, N., 1998. Identification of floral volatiles from *Ligustrum japonicum* that stimulate flower visiting by cabbage butterfly, *Pieris rapae*. Journal of Chemical Ecology 24, 2167-2180.

Impson, F.A.C., Kleinjan, C.A., Hoffmann, J.H. & Post, J.A., 2008. *Dasineura rubiformis* (Diptera: Cecidomyiidae), a new biological control agent for *Acacia mearnsii* in South Africa. South African Journal of Science 104, 247-249.

Impson, F.A.C., Kleinjan, C.A., Hoffmann, J.H., Post, J.A. & Wood, A.R., 2011. Biological control of Australian *Acacia* species and *Paraserianthes lophantha* (Willd.) Nielsen (Mimosaceae) in South Africa. African Entomology 19, 186-207.

Impson, F.A.C., Moran, V.C. & Hoffmann, J.H., 2004. Biological control of an alien tree, *Acacia cyclops*, in South Africa: impact and dispersal of a seed-feeding weevil, *Melanterius servulus*. Biological Control 29, 375-381.

Jhumur, U.S., Dötterl, S. & Jürgens, A., 2008. Floral odors of *Silene otites*: Their variability and attractiveness to mosquitoes. Journal of Chemical Ecology 34, 14-25.

University of Cape Town

Jiang, L. & Kubota, K., 2004. Differences in the volatile components and their odor characteristics of green and ripe fruits and dried pericarp of Japanese Pepper (*Xanthoxylum piperitum* DC.). Journal of Agricultural and Food Chemistry 52, 4187-4203.

Jobson, S., 1999. Water stressed regions: the Middle East and Southern Africa – Global solutions. Occasional paper no 16. Water Issues Study Group. School of Oriental and African Studies (SOAS). University of London, United Kingdom. Available at: http://www.soas.ac.uk/water/publications/papers/file38359.pdf. Accessed: 26 July 2011.

Johnson, S.D., Ellis, A. & Dötterl, S., 2007. Specialisation for pollination by beetles and wasps: the role of lollipop hairs and fragrance in *Satyrium microrrhynchum* (Orchidaceae). American Journal of Botany 97, 47-55.

Jordon-Thaden, I.E. & Louda, S.M., 2003. Chemistry of *Cirsium* and *Carduus*: A role in ecological risk assessment for biological control of weeds? Biochemical Systematics and Ecology 31, 1353-1396.

Jürgens, A. & Dötterl, S., 2004. Chemical composition of anther volatiles in Ranunculaceae: Genera-specific profiles in *Anemone*, *Aquilegia, Caltha, Pulsatilla, Ranunculus* and *Trollus* species. American Journal of Botany 91, 1969-1980.

Jürgens, A., 2004. Flower scent composition in diurnal *Silene* species (Caryophyllaceae): phylogenetic constraints or adaptation to flower visitors? Biochemical Systematics and Ecology 32, 841-859.

Jürgens, A., Dötterl, S., Liede-Schumann, S. & Meve, U., 2008. Chemical diversity of floral volatiles in Asclepiadoideae-Asclepiadeae (Apocynaceae). Biochemical Systematics and Ecology 36, 842-852.

Jürgens, A., El-Sayed, A.M. & Suckling, D.M., 2009. Do carnivorous plants use volatiles for attracting prey insects? Functional Ecology 23, 875-887.

Jürgens, A., Feldhaar, H., Feldmeyer, B. & Fiala, B., 2006. Chemical composition of leaf volatiles in *Macaranga* species (Euphorbiaceae) and their potential role as olfactory cues in host-localization of foundress queens of specific ant partners. Biochemical Systematics and Ecology 34, 97-113.

Jürgens, A., Witt, T. & Gottsberger, G., 2003. Flower scent composition in *Dianthus* and *Saponaria* species (Caryophyllaceae) and its relevance for pollination biology and taxonomy. Biochemical Systematics and Ecology 31, 345-357.

University of Cape Town

Jürgens, A., Witt, T., Gottsberger, G., 2002. Flower scent composition in night-flowering *Silene* species (Cayrophyllaceae). Biochemical Systematics and Ecology 30, 383-397.

Junker, R.R. & Blüthgen, N., 2010. Floral scents repel facultative flower visitors, but attract obligate ones. Annals of Botany 105, 777-782.

Kaiser, R., 1997. New or uncommon volatile compounds in the most diverse natural scents. Revista Italiana EPPOS 18, 18-47.

Kamatou, G.P.P. & Viljoen, A.M., 2008. Linalool – A review of a biologically active compound of commercial importance. Natural Product Communications 3, 1183-1192.

Kamatou, G.P.P., Viljoen, A.M., Özek, T. & Baser, K.H.C., 2010. Chemical composition of the wood and leaf oils from the “Clanwilliam Cedar” (*Widdringtonia cedarbergensis* J.A. Marsh): A critically endangered species. South African Journal of Botany 76, 652-654.

Kanno, H. & Harris, M.O., 2000. Physical features of grass leaves influence the placement of eggs within the plant by the Hessian fly. Entomologia Experimentalis et Applicata 96, 69-80.

Klein, H., 2011. A catalogue of the insects, mites and pathogens that have been used or rejected, or are under consideration, for the biological control of invasive alien plants in South Africa. African Entomology 19, 515-549.

Knudsen, J.T. & Gershenzon, J., 2006. The chemical diversity of floral scent. In: Dudareva, N. & Pichersky, E. (eds), Biology of floral scent. CRC Press, Boca Raton, Florida, USA.

Knudsen, J.T., Eriksson, R., Gershenzon, J. & Ståhl, B., 2006. Diversity and distribution of floral scent. The Botanical Review 72, 1-120.

Kolesik, P., 2000. Distribution of infestation by lentil gall midge *Contarinia lentis* (Dipt., Cecidomyiidae) in lentil fields: statistical model. Journal of Applied Entomology 124, 7-10.

Kolesik, P., Adair, R.J. & Eick, G., 2005. Nine new species of *Dasineura* (Diptera: Cecidomyiidae) from flowers of Australian *Acacia* (Mimosaceae). Systematic Entomology 30, 454-479.

Kolesik, P., Adair, R.J. & Eick, G., 2010. Six new species of *Asphondylia* (Diptera: Cecidomyiidae) damaging flower buds and fruit of Australian *Acacia* (Mimosaceae). Systematic Entomology 35, 250-267.

University of Cape Town

Kotze, M.J., Jürgens, A., Johnson, S.D. & Hoffmann, J.H., 2010. Volatiles associated with different flower stages and leaves of *Acacia cyclops* and their potential role as host attractants for *Dasineura dielsi* (Diptera: Cecidomyiidae). South African Journal of Botany 76, 701-709.

Lamarque, A.L., Maestri, D.M., Zygadlo, J.A. & Grosso, N.R ., 1998. Volatile constituents from flowers of *Acacia caven* (Mol.) Mol. var. *caven*, *Acacia aroma* Gill. ex Hook., *Erythrina crista-galli* L. and *Calliandra tweedii* Benth. Flavour and Fragrance Journal 13, 266-268.

Landolt, P.J., Brumley, J.A., Smithhisler, C.L., Biddick, L.L. & Hofstetter, R.W., 2000. Apple fruit infested with codling moth are more attractive to neonate codling moth larvae and possess increased amounts of (*E,E*)-*α*-Farnesene. Journal of Chemical Ecology 26, 1685-1699.

Larsson, M.C., Leal, W.S. & Hansson, B.S., 2001. Olfactory receptor neurons detecting plant odours and male volatiles in *Anomala cuprea* beetles (Coleoptera: Scarabaeidae). Journal of Insect Physiology 47, 1065-1076.

Larsson, M.C., Stensmyr, M.C., Bice, S.B. & Hansson, B.S., 2003. Attractiveness of fruit and flower odorants detected by olfactory receptor neurons in the fruit chafer *Pachnoda marginata*. Journal of Chemical Ecology 29, 1253-1268.

Le Maitre, D.C., Van Wilgen, B.W., Gelderblom, C.M., Bailey, C., Chapman, R.A. & Nel, J.A., 2002. Invasive alien trees and water resources in South Africa: case studies of the costs and benefits of management. Forest Ecology and Management 160, 143-159.

Levin, R.A., McDade, L.A. & Raguso, R.A., 2003. The systematic utility of floral and vegetative fragrance in two genera of Nyctaginaceae. Systematic Biology 52, 334-351.

Levin, R.A., Raguso, R.A. & McDade, L.A., 2001. Fragrance chemistry and pollinator affinities in Nyctaginaceae. Phytochemistry 58, 429-440.

Lewis, G.P., Knudsen, J.T., Klitgaard, B.B. & Pennington, R.T., 2003. The floral scent of *Cyathostegia mathewsii* (Leguminosae, Papilionoideae) and preliminary observations on reproductive biology. Biochemical Systematics and Ecology 31, 951-962.

Li, Y., Dickens, J.C. & Steiner, W.W.M., 1992. Antennal olfactory responsiveness of *Microplitis croceipes* (Hymenoptera: Braconidae) to cotton plant volatiles. Journal of Chemical Ecology 18, 1761-1773.

Lindgren, B.S., Norlander, G. & Birgersson, G., 1996. Feeding deterrence of verbenone to the pine weevil, *Hylobius abietis* (L) (Col, Curculionidae). Journal of Applied Entomology 120, 397-403.

University of Cape Town

Liu, Y., He, X-K., Hall, D., Farman, D., Amarawardana, L., Cross, J. & Liu, Q.R., 2009. (2S,8Z)-2-Butyroxy-8-heptadecene: major component of the sex pheromone of chrysanthemum gall midge, *Rhopalomyia longicauda*. Journal of Chemical Ecology 35, 715-723.

Lucas-Barbosa, D., Van Loon, J.J.A. & Dicke, M., 2011. The effects of herbivore-induced plant volatiles on interactions between plants and flower-visiting insects. Phytochemistry 72, 1647-1654.

Lücker, J., Verhoeven, H.A., Van der Plas, L.H.W. & Bouwmeester, H.J., 2006. Molecular engineering of floral scent. In: Dudareva, N. & Pichersky, E. (eds), Biology of floral scent. CRC Press, Boca Raton, Florida, USA.

Mack, M.C., Simberloff, D., Lonsdale, W.M., Evans, H., Clout, M. & Bazzaz, F.A., 2000. Biotic invasions: causes, epidemiology, global consequences, and control. Ecological Applications 10, 689-710.

Malan, E. & Swartz, P., 1995. A comparative study of the phenolic products in the heartwood of *Acacia karroo* from two different localities. Phytochemistry 39, 791-794.

Maslin, B.R. & McDonald, M.W., 2004. AcaciaSearch- Evaluation of *Acacia* as a woody crop option for southern Australia. Report to the Joint Venture Agroforestry Program, Publication No. 03/017. Rural Industries Research and Development Corporation, Barton, Australia.

Matich, A.J., Young, H., Allen, J.M., Wang, M.Y., Fielder, S., McNeilage, M.A. & MacRae, E.A., 2003. *Actinidia arguta*: volatile compounds in fruit and flowers. Phytochemistry 63, 285-301.

Matsui, K., 2006. Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. Current Opinion in Plant Biology 9, 274-280.

Mckay, P.A. & Hatchett, J.H., 1984. Mating behaviour and evidence of a female sex pheromone in the Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae). Annals of Entomological Society of America 77, 616-620.

Miller, J.T., Murphy, D.J., Brown, G.K., Richardson, D.M. & González-Orozco, C.E., 2011. The evolution and phylogenetic placement of invasive Australian *Acacia* species. Diversity and Distributions 17, 848-860.

Milton, S.J. & Moll, E.J., 1982. Phenology of Australian acacias in the S.W. Cape, South Africa, and its implications for management. Botanical Journal of the Linnean Society, 84: 295-327.

University of Cape Town

Mkhabela, T.S., 2003. The economic value of *Acacia karroo* in small-scale farming systems and game farming in northern KwaZulu-Natal, South Africa. Southern African Forestry Journal 198, 75-78.

Molnár, B., Kárpáti, Z., Szocs, G. & Hall, D.R., 2009. Identification of female-produced sex pheromone of the honey locust gall midge, *Dasineura gleditchiae*, a new urban pest in Europe. Journal of Chemical Ecology 35, 706-714.

Moraga, A.R., Rambla, J.L., Ahrazem, O., Granell, A & Gómez-Gómez, L., 2009. Metabolite and target transcript analyses during *Crocus sativus* stigma development. Phytochemistry 70, 1009-1016.

Moseley, C.T., Cramer, M.D., Kleinjan, C.A. & Hoffmann, J.H., 2009. Why does *Dasineura dielsi* - induced galling of *Acacia cyclops* not impede vegetative growth? Journal of Applied Ecology 46, 214-222.

Müller, C. & Hilker, M., 2000. The effect of a green leaf volatile on host plant finding by larvae of a herbivorous insect. Naturwissenschaften 87, 216-219.

Mumm, R. & Dicke, M., 2010. Variation in natural plant products and the attraction of bodyguards involved in indirect plant defense. Canadian Journal of Zoology, 628-667.

Murchie, A.K., Smart, L.E. & Williams, I.H., 1997. Responses of *Dasineura brassicae* and its parasitoids *Platygaster subuliformis* and *Omphale dypealis* to field traps baited with organic isothiocyanates. Journal of Chemical Ecology 23, 917-926.

Mustaparta, H., 2002. Encoding of plant odour information in insects: peripheral and central mechanisms. Entomologia Experimentalis et Applicata 104**,** 1-13.

Nyila, M.A., Leonard, C.M., Hussein, A.A. & Lall, N., 2012. Activity of South African medicinal plants against *Listeria monocytogenes* biofilms, and isolation of active compounds from *Acacia karroo*. South African Journal of Botany 78, 220-227.

Paré, P.W. & Tumlinson, J.H., 1999. Plant volatiles as a defense against insect herbivores. Plant Physiology 121, 325-331.

Pecetti, L., Tava, A., Felicioli, A., Pinzauti, M. & Piano, E., 2004. Effect of three volatile compounds from lucerne flowers on their attractiveness towards pollinators. Bulletin of Insectology 55, 21-27.

Pellmyr, O. & Thien, L.B., 1986. Insect reproduction and floral fragrances: keys to the evolution of the angiosperms. Taxon 35, 76-85.

University of Cape Town

Pettersson, J., 1970. An aphid sex attractant. Entomologica Scandinavica 1, 63-73.

Pettersson, J., 1976. Ethology of *Dasyneura brassicae* Winn. (Diptera: Cecidomyiidae). I. Laboratory studies of olfactory reactions to the host-plant. Symposium Biologica Hungarica 16, 203-208.

Pettersson, J., 1993. Odour stilmuli affecting autumn migration of *Rhopalosiphum padi* (L.) (Hemiptera: Homoptera). Annals of Applied Biology 122, 417-425.

Pichersky, E. & Gershenzon, J., 2002. The formation and function of plant volatiles: perfumes for pollinator attraction and defense. Current Opinion in Plant Biology 5, 237-243.

Piesik, D., Lemńczyk, G., Skoczek, A., Lamparski, R., Bocianowski, J., Kotwica, K. & Delaney, K.J., 2011. *Fusarium* infection in maize: Volatile induction of infected and neighboring uninfected plants has the potential to attract a pest cereal leaf beetle, *Oulema melanopus*. Journal of Plant Physiology 168, 1534-1542.

Plepys, D., Ibarra, F. & Löfstedt, C., 2002. Volatiles from flowers of *Platanthera bifolia* (Orchidaceae) attractive to the silver Y moth, *Autographa gamma* (Lepidoptera: Noctuidae). Oikos 99, 69-74.

Pope, T.W., Campbell, C.A.M., Hardie, J. & Wadhams, L.J., 2004. Electroantennogram responses of the three migratory forms of the damson-hop aphid, *Phorodon humuli*, to aphid pheromones and plant volatiles. Journal of Insect Physiology 50, 1083-1092.

Post, J.A., Kleinjan, C.A., Hoffmann J.H. & Impson, F.A.C., 2010. Biological control of *Acacia cyclops* in South Africa: The fundamental and realized host range of *Dasineura dielsi* (Diptera: Cecidomyiidae). Biological Control 53, 68-75.

Raguso, R. A. & Pichersky, E., 1999. A day in the life of a linalool molecule: chemical communication in a plant-pollinator system. Part 1: Linalool biosynthesis in flowering plants. Plant Species Biology 14, 95-120.

Raguso, R.A. & Light, D.M., 1998. Electroantennogram responses of male *Sphinx perelegans* hawkmoths to floral and 'greenleaf volatiles'. Entomologia Experimentalis et Applicata 86, 287-293.

Raguso, R.A. & Willis, M.A., 2005. Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta.* Animal Behaviour 69, 407-418.

Raguso, R.A., Levin, R.A., Foose, S.E., Holmberg, M.W. & McDade, L.A., 2003. Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in *Nicotiana*. Phytochemistry 63, 265-284.

University of Cape Town

Raguso, R.A., Schlumpberger, B.O., Kaczorowski, R.L. & Holtsford, T.P., 2006. Phylogenetic fragrance patterns in *Nicotiana* sections Alatae and Suaveolentes. Phytochemistry 67, 1931-1942.

Reddy, G. V. P. & Guerrero, A., 2004. Interactions of insect pheromones and plant semiochemicals. Trends in Plant Science 9, 253-261.

Regnault-Roger, C., Hamraoui, A., Holeman, M., Theron, E. & Pinel, R., 1993. Insecticidal effect of essential oils from Mediterranean plants upon *Acanthoscelides obtectus* Say (Coleoptera, Bruchidae), a pest of kidney Bean (*Phaseolus vulgaris* L.). Journal of Chemical Ecology 19, 1233-1244.

Richardson, D.M. & Kluge, R.L., 2008. Seed banks of invasive Australian *Acacia* species in South Africa: Role in invasiveness and options for management. Perspectives in Plant Ecology, Evolution and Systematics 10, 161-177.

Richardson, D.M. & Van Wilgen, B.W., 2004. Invasive alien plants in South Africa: how well do we understand the ecological impacts? South African Journal of Science 100, 45-52.

Richardson, D.M., Carruthers, J., Hui, C., Impson, F.A.C., Robertson, M.P., Rouget, M., Le Roux, J.J. & Wilson, J.R.U., 2011. Human-mediated introductions of Australian acacias – a global experiment in biogeography. Diversity and Distributions, 17, 771-787.

Robertson, G.W., Griffiths, D.W., Woodford, J.A.T. & Birch, A.N.E., 1995. Changes in the chemical composition of volatiles released by the flowers and fruits of the red raspberry (*Rubus idaeus*) cultivar Glen Prosen. Phytochemistry 38, 1175-1179.

Rodriguez-Saona, C., Crafts-Brandez, S.J., Parc, P.W. & Henneberry, T.J., 2001. Exogenous methyl jasmonate induces volatile emission in cotton plants. Journal of Chemical Ecology, 27, 679-695.

Rodriguez-Saona, C., Parra, L., Quiroz, A. & Isaacs, R., 2011. Variation in highbush blueberry floral volatile profiles as a function of pollination status, cultivar, time of day and flower part: implications for flower visitation by bees. Annals of Botany 107, 1377-1390.

Rosenheim, J.A., Jepson, S.J., Matthews, C.E., Smith, D.S. & Rosenheim, M.R., 2007. Portrait of an ephemeral adult stage: egg maturation, oviposition and longevity of the gall midge *Rhopalomyia californica* (Diptera: Cecidomyiidae). Annals of the Entomological Society of America 100, 449-561.

University of Cape Town

Roubos, C.R., 2009. Monitoring and managing blueberry gall midge (Diptera: Cecidomyiidae) in Rabbiteye Blueberries. Ph.D. Dissertation, University of Florida, USA

Schiestl, F.P., 2010. The evolution of floral scent and insect chemical communication. Ecology Letters 13, 643-656.

Schoonhoven, L.M., Van Loon, J.J.A. & Dicke, M., 2005. Insect-plant biology. 2nd Edition. Oxford University Press Inc., New York.

Schütz, S., Weißbecker, B., Klein, A. & Hummel, H.E., 1997. Host plant selection of the Colorado potato beetle as influenced by damage induced volatiles of the potato plant. Naturwissenschaften 84, 212-217.

Scudder, G.G.E. & Cannings, R.A., 2006. The Diptera families of British Columbia. Forest Investment Account (FIA) - Forest Science Program, University of British Columbia.

Seigler, D.S., 2003. Phytochemistry of *Acacia* - sensu lato. Biochemical Systematics and Ecology 31, 845-873.

Shaaya, E. & Rafaeli, A., 2007. Essential oils as biorational insecticides–potency and mode of action. In: Ishaaya, I., Nauen, R. & Horowitz, A.R. (eds), Insecticides design using advanced technologies. Springer-Verlag, Berlin Heidelberg, Germany.

Shannon, C. & Weaver, W., 1949. The Mathematical Theory of Communication. University of Illinois Press, Urbana.

Sharma, H.C. & Franzmann, B.A., 2001. Orientation of sorghum midge, *Stenodiplosis sorghicola*, females (Diptera: Cecidomyiidae) to color and host-odor stimuli. Journal of Agricultural and Urban Entomology 18, 237-248.

Sharma, H.C., Franzmann, B.A., & Henzell, R.G., 2002. Mechanisms and diversity of resistance to sorghum midge, *Stenodiplosis sorghicola* in *Sorghum bicolour.* Euphytica 124, 1-12.

Shuttleworth, A. & Johnson, S.D., 2010a. The missing stink: sulphur compounds can mediate a shift between fly and wasp pollination systems. Proceedings of the Royal Society B-Biological Sciences 277, 2811-2819.

Shuttleworth, A. & Johnson, S.D., 2010b. Floral scents of chafer-pollinated asclepiads and a potential hybrid. South African Journal of Botany 76, 770-778.

University of Cape Town

Simkin, A.J., Kuntz, M., Moreau, H. & McCarthy, J., 2010. Carotenoid profiling and the expression of carotenoid biosynthetic genes in developing coffee grain. Plant Physiology and Biochemistry 48, 434-442.

Smart, L.E. & Blight, M.M., 1997. Field discrimination of oilseed rape, *Brassica napus* volatiles by cabbage seed weevil, *Ceutorhynchus assimilis*. Journal of Chemical Ecology 23, 2555-2567.

Smit, N., 2008. Field guide to the acacias of South Africa. Briza Publications, Pretoria, South Africa.

Steenhuisen, S-L., Raguso, R.A., Jürgens, A. & Johnson, S.D., 2010. Variation in scent emission among floral parts and inflorescence developmental stages in beetle-pollinated *Protea* species (Proteaceae). South African Journal of Botany 76, 779-787.

Stensmyr, M.C., 2004. The fly nose-function and evolution. Doctoral Thesis, Swedish University of Agricultural Sciences, Alnarp.

Stiling, P. 1999. Ecology theories and application. 3rd Edition. Prentice Hall, New Jersey.

Stone, G.N., Raine, N.E., Prescott, M. & Willmer, P.G., 2003. Pollination ecology of acacias (Fabaceae, Mimosoideae). Australian Systematic Botany, 16, 103-118.

Suazo, A., Torto, B., Teal, P.E.A. & Tumlinson, J.H., 2003. Response of the small hive beetle (*Aethina tumida*) to honey bee (*Apis mellifera*) and beehive-produced volatiles. Apidologie 34, 525-533.

Suckling, D.M. & Karg, G., 2000. Pheromones and other semiochemicals. In: Rechcigl, J.E. & Rechcigl, N.A (eds), Biological and biotechnological control of insect pests. Lewis Publishers, Boca Raton, Florida.

Suckling, D.M., Walker, J.T.S., Shaw, P.W., Manning, L-A., Lo, P., Wallis, R., Bell, V., Sandanayaka, W.R.M., Hall, D.R., Cross, J.V. & El-Sayed, A.M., 2007. Trapping *Dasineura mali* (Diptera: Cecidomyiidae) in apples. Journal of Economic Entomology 100, 745-751.

Tao, Y-S. & Li, H., 2009. Active volatiles of cabernet sauvignon wine from Changli County. Natural Science 1, 176-182.

Tasin, M., Anfora, G., Ioriatti, C., Carlin,S., De Cristofaro, A., Schmidt, S., Bengtsson,M., Versini,G. & Witzgall, P., 2005. Antennal and behavioural responses of grapevine moth, *Lobesia botrana* female to volatiles from grapewine. Journal of Chemical Ecology 31, 77-87.

Theis, N. & Raguso, R.A., 2005. The effect of pollination on floral fragrance in thistles. Journal of Chemical Ecology 31, 2581-2600.

University of Cape Town

Theis, N., 2006. Fragrance of Canada thistle (*Cirsium arvense*) attracts both floral herbivores and pollinators. Journal of Chemical Ecology 32, 917-927.

Tholl, D., Sohrabi, R., Huh, J-H. & Lee, S., 2011. The biochemistry of homoterpenes – Common constituents of floral and herbivore-induced plant volatile bouquets. Phytochemistry 72, 1635-1646.

Thompson, J.N. & Pellmyr, O., 1991. Evolution of oviposition behaviour and host preference in Lepidoptera. Annual Review of Entomology 36, 65-89.

Tollsten, L. & Bergström, G., 1993. Fragrance chemotypes of *Platanthera* (Orchidaceae): The result of adaptation to pollinating moths? Nordic Journal of Botany 13, 607-613.

Tóth, M., Schmera, D. & Imrei, Z., 2004. Optimization of a chemical attractant for *Epicometis* (*Tropinota*) *hirta* Poda. Zeitschrift für Naturforschung C 59, 288-292.

Trumble, J.T., 2002. Caveat Emptor: Safety considerations for natural products used in arthropod control. American Entomologist 48, 7-13.

Turnbull, J.W., 1997. Australian Vegetation. In: Doran, J.C. & Turnbull, J.W. (eds), Australian trees and shrubs: species for land rehabilitation and farm planting in the tropics. Australian Centre for International Agricultural Research, Canberra, Australia.

Turton, A.R., 2001. Hydropolitics and security complex theory: An African perspective. 4th Pan-European International Relations Conference, University of Kent, Canterbury (UK), 8-10 September 2001.

Underwood, B.A., Tieman, D.M., Shibuya, K., Dexter, R.J., Loucas, H.M., Simkin, A.J., Sims, C.A., Schmelz, E.A., Klee, H.J. & Clark, D.G., 2005. Ethylene-regulated floral volatile synthesis in *Petunia* corollas. Plant Physiology 138, 255-266.

Urru, I., Stensmyr, M.C. & Hansson, B.S., 2011. Pollination by brood-site deception. Phytochemistry 72, 1655-1666.

Van Driesche, R. Hoddle, M. & Center, T., 2008. Control of pests and weeds by natural enemies: an introduction to biological control. Blackwell Publishing, Malden, USA.

Van Lenteren, J.C., Schettino, M., Isidoro, N., Romani, R. & Van Schelt, J., 2002. Morphology of putative female sex pheromone glands and mating behavior in *Aphidoletes aphidimyza*. Entomologia Experimentalis et Applicata 102, 199-209.

University of Cape Town

Van Tol, R.W.H.M. & Visser, J.H., 2002. Olfactory antennal responses of the vine weevil *Otiorhynchus sulcatus* to plant volatiles. Entomologia Experimentalis et Applicata 102, 49-64.

Van Tol, R.W.H.M., James, D.E., De Kogel, W.J. & Teulon, D.A.J., 2007. Plant odours with potential for a push–pull strategy to control the onion thrips, *Thrips tabaci*. Entomologia Experimentalis et Applicata 122, 69-76.

Van Wilgen, B.W. & De Lange, W.J., 2011. The costs and benefits of biological control of invasive alien plants in South Africa. African Entomology 19, 504-514.

Van Wilgen, B.W., Reyers, B., Le Maitre, D.C., Richardson, D.M. & Schonegevel, L., 2008. A biome-scale assessment of the impact of invasive alien plants on ecosystem services in South Africa. Journal of Environmental Management 89, 336-349.

Van Wilgen, B.W., Richardson, D.M., Le Maitre, D.C., Marais, C. & Magadlela, D., 2001. The Economic Consequences of Alien Plant Invasions: Examples of Impacts and Approaches to Sustainable Management in South Africa. Environment, Development and Sustainability 3, 145-168.

Van Wyk, B. & Van Wyk, P., 1997. Field guide to trees of Southern Africa. Struik Publishers, Cape Town, South Africa.

Van Wyk, B-E., 2011. The potential of South African plants in the development of new medicinal products, South African Journal of Botany 77, 812-829.

Ventura, M.U., Martins, M.C. & Pasini, A., 2000. Responses of *Diabrotica speciosa* and *Cerotoma arcuata tingomariana* (Coleoptera: Chrysomelidae) to volatile attractants. Florida Entomologist 83, 403-410.

Verdonk, J.C., De Vos, C.H.R., Verhoeven, H.A., Haring, M.A., Van Tunen, A.J. & Schuurink, R.C., 2003. Regulation of floral scent production in *Petunia* revealed by targeted metabolomics. Phytochemistry 62, 997-1008.

Vet, L.E.M., Van Lenteren, J.C., Heymans, M. & Meelis, E., 1983. An airflow olfactometer for measuring olfactory responses of hymenopterous parasitoids and other small insects. Physiological Entomology 8, 97-106.

Visser, J.H. & Piron, R.G.M., 1995. Olfactory antennal responses to plant volatiles in apterous virginoparae of the vetch aphid *Megoura viciae*. Entomologia Experimentalis et Applicata 77, 37-46.

University of Cape Town

Wadhams, L.J., Blight, M.M., Kerguelen, V., Le Métayer, M., Marion-Poll, F., Masson, C., Pham-Delègue, M.H. & Woodcock, C.M., 1994. Discrimination of oilseed rape volatiles by honey bee: novel combined gas chromatographic-electrophysiological behavioural assay. Journal of Chemical Ecology 20, 3221-3231.

Wang, T., Lu, L., Wang, D. & Thompson, J.E., 2001. Isolation and characterization of senescence-induced cDNAs encoding deoxyhypusine synthase and eucaryotic translation initiation factor 5A from tomato. Journal of Biological Chemistry 276, 17541-17549.

Ward, D., 2011. Population differentiation in a purported ring species, *Acacia karroo* (Mimosoideae). Biological Journal of the Linnean Society 104, 748-755.

Webster, B., Bruce, T., Pickett, J. & Hardie, J., 2010. Volatiles functioning as host cues in a blend become non-host cues when presented alone to the black bean aphid. Animal Behaviour 79, 451-457.

Weinzierl, R.A., 2000. Botanical insecticides, soaps, and oils. In: Rechcigl, J.E. & Rechcigl, N.A (eds), Biological and biotechnological control of insect pests, Lewis Publishers, Boca Raton, Florida.

Weldon, P.J. & Carroll, J.F., 2006. Vertebrate chemical defense: secreted and topically acquired deterrents of arthropods. In: Debboun, M., Frances, S.P. & Strickman, D (eds), Insect repellents: principles, methods, and uses. CRC Press, Boca Raton, New York.

Wheeler, G.S., 2006. Chemotype variation of the weed *Melaleuca quinquenervia* influences the biomass and fecundity of the biological control agent *Oxyops vitiosa.* Biological Control 36, 121-128.

Williams, N.H. & Whitten, W.M., 1983. Orchid floral fragrances and male Euglossine bees: methods and advances in the last sesquidecade. The Biological Bulletin 164, 355-395.

Willmer, P.G., Nuttman, C.V., Raine, N.E., Stone, G.N., Pattrick, J.G., Henson, K., Stillman, P., McIlroy, L., Potts, S.G & Knudsen, J.T., 2009. Floral volatiles controlling ant behaviour. Functional Ecology 23, 888-900.

Wink, M., 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry 64, 3-19.

Wood, A.R. & Morris, M.J., 2007. Impact of the gall-forming rust fungus *Uromycladium tepperianum* on the invasive tree *Acacia saligna* in South Africa: 15 years of monitoring. Biological Control 41, 68-77.

University of Cape Town

Yukawa, J., Uechi, N., Tokuda, M. & Sato, S., 2005. Radiation of gall midges (Diptera: Cecidomyiidae) in Japan. Basic and Applied Ecology 6, 453-461.

Yukawa, J. 2000. Synchronization of gallers with host plant phenology. Population Ecology, 42, 105-113.

Zhang, Q. & Schlyter, F., 2004. Olfactory recognition and behavioural avoidance of angiosperm non-host volatiles by conifer-inhabiting bark beetles. Agricultural and Forest Entomology 6, 1-19.

Zhu, J., Cossé, A.A., Obrycki, J.J., Boo, K.S. & Baker, T.C., 1999. Olfactory reactions of the twelve-spotted lady beetle, *Coleomegilla maculata* and the green lacewing, *Chrysoperla carnea* to semiochemicals released from their prey and host plant: electroantennogram and behavioral responses. Journal of Chemical Ecology 25, 1163-1177.

Zimmermann, H.G. & Olckers, T., 2003. Biological control of alien plant invaders in Southern Africa. In: Neuenschwander, P., Borgemeister, C. & Langewald, J. (eds), Biological control in IPM systems in Africa. CAB International 2003.

Zimmermann, H.G., Moran, V.C. & Hoffmann, J.H., 2004. Biological control in the management of invasive alien plants in South Africa, and the role of the Working for Water programme. South African Journal of Science 100, 34-40.

Zygadlo, J.A., Lamarque, A.L., Maestri, D.M., Velasco Negueruela, A., Perez Alonso, M.J. & Garcia Vallejo, M.C., 1996. Volatile constituents from flowers of *Acacia praecox* Gris. Flavour and Fragrance Journal 11, 3-5.

University of Cape Town