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Identification of semiochemicals from four major insect pests of *Eucalyptus* and *Pinus* species in South Africa

by

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Submitted in partial fulfillment for the degree

Philosophiae Doctor

in the

Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

December 2014

Declaration of Authorship

I, MARC CLEMENT BOUWER, declare that this thesis, which I hereby submit for the degree Doctoral in Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed: _____

Date: _____

"If we knew what it was we were doing, it would not be called research, would it?"

Albert Einstein

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Abstract

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by Marc Clement Bouwer

The chemical ecology for key insect pests in South Africa's commercial *Pinus* and *Eucalyptus* plantations are explored in this thesis. The main aim was to discover and characterize semiochemical compounds that explain certain behaviours of the insects involved, and that could potentially be exploited in future for the manipulation of the insects' behaviour. Such formulations are sought after because they can be applied in environmentally friendly pest management techniques. Semiochemical interactions were studied in four different biological systems. These included the sex pheromone communication system for the Cossid moth, *Coryphodema tristis*; allelochemical interactions between the Eucalyptus weevil *Gonipterus* spp. and their *Eucalyptus* host; allelochemical interactions between *Sirex noctilio*'s, symbiotic fungus, *Amylostereum areolatum* and its biological control agent *Ibalia leucospoides*; and allelochemical interactions between the egg clutches of *Thaumastocoris peregrinus* and its biological control agent *Cleurocoides noackae*. Semiochemical compounds were found and identified through analysis techniques that included gas chromatography coupled to electroantennography (GC-EAD) and gas chromatography coupled to mass spectrometry (GC-MS). Some of these semiochemical interactions were complex involving combinations of different chemical cues, such as the host recognition cues identified for *Gonipterus* species and potential cues between *T. peregrinus* females and their egg clutches. Other specific interactions were found to be simpler and relied on a few specific chemicals. These included the sex pheromone communication between *C. tristis* adults and the interaction occurring between female *I. leucospoides* wasps and the mutualistic fungus of their prey, namely *A. areolatum*. Biological activity was investigated only for compounds with confirmed identity and included the sex pheromones of *C. tristis* and some of the chemicals identified from the egg clusters of *T. peregrinus*. These tests were conducted in laboratory and field conditions. Biological activity was proven for the identified sex pheromone of *C. tristis* during field trials conducted in 2011 and 2013. One of the chapters was published and another submitted for publication in a peer reviewed journal. Two provisional patents were also registered from this work.

Acknowledgements

I would like to thank the following people that have been involved in this project. Without them it would definitely not have been possible.

Firstly to Yvette Naudé and Niel Malan. You were always there to answer questions and help with matters that I myself could not do.

Secondly to Nico van Vuuren, Robin Muir and Johan Stegmann. Nico has helped tremendously with manufacturing of the insect flight tunnel while Robin and Johan helped with manufacturing glass apparatus.

Thirdly to my supervisors Prof Egmont R. Rohwer, Prof Bernard Slippers and Prof Mike J. Wingfield. Your continual motivation and advice was essential especially in interpreting difficult results and writing correctly. Also, for your motivation when I sometimes felt let down by unexpected results.

Lastly to the funding bodies: The Tree Protection Co-operative Programme (TPCP) and the Forestry Agricultural Biotechnology Institute (FABI). The National Research Foundation (NRF).

A special thanks goes to all the FABI people because their positive attitude sets a stage that creates success. I would also like to thank all Fabians that were involved in field trips and the collection of samples.

Contents

Declaration of Authorship i

Abstract iv

Acknowledgements v

List of Figures x

List of Tables xii

Abbreviations xiii

Symbols xiv

1 The application of insect semiochemicals for pest management in forestry 1

1.1 Abstract . . . 2
1.2 Introduction . . . 2
1.3 The chemical world as perceived by an insect . . . 5
1.4 Applications of semiochemicals in forestry . . . 6
1.4.1 Concepts and examples . . . 6
1.4.1.1 Monitoring . . . 6
1.4.1.2 Mass trapping . . . 7
1.4.1.3 Mating disruption . . . 8
1.4.1.4 Push-pull strategies . . . 10
1.5 Examples of semiochemical application in forestry . . . 10
1.5.1 Moths . . . 10
1.5.1.1 Gypsy moth, *Lymantria dispar* . . . 10
1.5.1.2 Codling moth, *Cydia pomonella*(L.) . . . 11
1.5.1.3 Oriental Fruit Moth or Peach Moth, *Grapholitha molesta* 12
1.5.2 Bark beetles . . . 12
1.5.2.1 *Ips* spp. . . . 12
1.5.2.2 *Dendroctonus* spp. . . . 13
1.6 Semiochemical use in South African forestry . . . 14
1.6.1 *Sirex noctilio* . . . 15
1.6.2 Pine emperor moth, *Nudaurelia cyfherea* Fabr *cyfherea* . . . 16

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

1.7 Other significant Semiochemical research in South Africa . . . 17
1.8 Challenges that influence the use of semiochemicals in forestry . . . 17
1.8.1 Challenges in pheromone research . . . 17
1.8.1.1 Analytical challenges in identifying the pheromone messages . . . 18
1.8.1.2 Odour blends and synergism . . . 19
1.8.1.3 Release rates and ratios of pheromone components . . . 20
1.8.2 Challenges in field application . . . 21
1.8.2.1 Trap placement and properties . . . 21
1.8.2.2 Difficulties in correlating trap capture with insect numbers and tree mortality in the field . . . 22
1.9 Conclusion . . . 23

2 Chemical signatures affecting host choice in the *Eucalyptus* herbivore, *Gonipterus* sp. (Coleoptera: Curculionidae) 24
2.1 Abstract . . . 25
2.2 Introduction . . . 25
2.3 Materials and Methods . . . 28
2.3.1 Insect samples . . . 28
2.3.2 *Eucalyptus* samples . . . 28
2.3.3 Volatile collection . . . 28
2.3.4 Electroantennography . . . 29
2.3.5 Gas chromatography coupled to electroantennography . . . 30
2.3.6 Gas chromatography coupled to mass spectrometry . . . 31
2.4 Results . . . 31
2.4.1 Electroantennography . . . 31
2.4.2 Gas chromatography coupled to electroantennography . . . 32
2.4.3 Gas chromatography coupled to mass spectrometry . . . 32
2.5 Discussion . . . 33
2.6 Acknowledgements . . . 36

3 Identification of the sex pheromone of the tree infesting cossid moth *Coryphodema tristis* (Lepidoptera: Cossidae) 46
3.1 Abstract . . . 47
3.2 Introduction . . . 47
3.3 Materials and Methods . . . 49
3.3.1 Sampling . . . 49
3.3.1.1 Insects . . . 49
3.3.1.2 Gland extraction . . . 49
3.3.1.3 Headspace samples . . . 49
3.3.2 Electroantennal responses . . . 50
3.3.2.1 Gas chromatography electroantennographic detection . . . 50
3.3.2.2 Dose response . . . 51
3.3.3 Characterization of chemicals . . . 51
3.3.3.1 GC-MS . . . 51
3.3.3.2 GC X GC-MS . . . 52
3.3.3.3 Double bond determination . . . 52

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

3.3.4 Field trial . . . 53
3.4 Results . . . 53
3.4.1 Electroantennal responses . . . 53
3.4.1.1 Gas chromatography electroantennographic detection . . . 53
3.4.1.2 Dose response . . . 54
3.4.2 Characterization of chemicals . . . 54
3.4.2.1 GC-MS . . . 54
3.4.2.2 GC X GC-MS . . . 55
3.4.3 Field trial . . . 55
3.5 Discussion . . . 56
3.6 Acknowledgements . . . 59

4 A potential semiochemical signal from *Amylostereum* species for *Ibalia leucospoides* 72
4.1 Abstract . . . 73
4.2 Introduction . . . 73
4.3 Methods and Materials . . . 74
4.3.1 Insects . . . 74
4.3.2 Amylostereum cultures . . . 75
4.3.3 Sampling . . . 75
4.3.4 GC-EAD . . . 75
4.3.5 GC-MS . . . 76
4.3.6 Chemical synthesis . . . 76
4.4 Results . . . 76
4.5 Discussion . . . 78
4.6 Acknowledgments . . . 80

5 Potential semiochemicals from the egg clutches of *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae) 85
5.1 Abstract . . . 86
5.2 Introduction . . . 86
5.3 Materials and Methods . . . 88
5.3.1 Collection of egg clutches . . . 88
5.3.2 Sampling of volatiles . . . 88
5.3.3 Gas chromatography coupled to mass spectrometry . . . 88
5.3.4 Quantification of egg-specific chromatographic peaks . . . 89
5.3.5 Bioassay . . . 89
5.4 Results . . . 90
5.5 Discussion . . . 91
5.6 Acknowledgements . . . 94

6 Summary 101

A Optimization and synchronization of GC-EAD and GC-MS 106
A.1 Theory and experimental results . . . 106

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

B Construction of wind tunnel for behavioural experiments 112
B.1 Wind tunnel construction . . . 112
B.1.1 Wind tunnel development . . . 113
B.2 Wind tunnel standardization . . . 114
B.2.1 Wind tunnel light . . . 114
B.2.2 Wind tunnel air speed . . . 117
B.3 Results . . . 118

C Pheromone release rate study of natural red rubber septa and methyl silicone rubber permeation devices as artificial pheromone dispensers for *Coryphodema tristis* 121
C.1 Introduction . . . 121
C.2 Materials and methods . . . 122
C.2.1 Natural red rubber septa . . . 122
C.2.2 Methyl silicone permeation devices . . . 122
C.3 Results . . . 123
C.3.1 Natural red rubber septa . . . 123
C.3.2 Methyl silicone permeation devices . . . 124
C.4 Discussion . . . 124

D Electron microscopy of *Coryphodema tristis* antennae 132
D.1 Introduction . . . 132
D.2 Methodology . . . 132
D.3 Results . . . 132

E Headspace time trial for *Coryphodema tristis* 136
E.1 Introduction . . . 136
E.2 Materials and methods . . . 137
E.2.1 Female moths . . . 137
E.2.2 Sampling . . . 137
E.2.3 GC-EAD analysis . . . 137
E.3 Results . . . 138

F Field trial tests of pheromones for *Coryphodema tristis* 148
F.1 Introduction . . . 148
F.2 Methods . . . 148
F.2.1 Field trial 2011 . . . 148
F.2.2 Field trial 2013 . . . 149

G Posters 150

Bibliography 154

List of Figures

2.1 Electrophysiological response of *G. scutellatus* to *E. globulus* volatiles . . 37
2.2 Electrophysiological response of *G. scutellatus* to *E. viminalis* volatiles . . 38
2.3 Electrophysiological response of *G. scutellatus* to *C. citriodora* volatiles . 39
2.4 *G. scutellatus* response to standard compounds 40
2.5 Bray-Curtis dissimilarity plot 41

3.1 Electrophysiological response of *Coryphodema tristis* males to pheromone gland extracts 60
3.2 Electrophysiological response of *Coryphodema tristis* males to female headspace samples 61
3.3 Electrophysiological response of *Coryphodema tristis* to standard compounds 63
3.4 Electrophysiological response of *Coryphodema tristis* to standard compounds 64
3.5 Electrophysiological response of *Coryphodema tristis* to standard compounds 65
3.6 Pheromone compound elucidation with GC-MS 66

4.1 GC-FID profile comparison of volatiles collected from *Amylostereum areolatum* 81
4.2 GC-EAD response of *Ibalia leucospoides* female to volatiles from *Amylostereum areolatum* 82
4.3 GC-EAD responses of *Sirex* males and females and *Ibalia* males to volatiles from *Amylostereum areolatum* 83
4.4 Mass spectrum of the unknown compound compared to synthesized compounds 84

5.1 Chromatogram of the headspace samples obtained from *Thaumastocoris peregrinus* eggs 97
5.2 Amounts of egg-specific volatiles measured from *Thaumastocoris peregrinus* eggs 98
5.3 Amounts of egg-specific volatiles measured from *Thaumastocoris peregrinus* eggs 99
5.4 Bio-assay results for *Thaumastocoris peregrinus* and *Cleurocoides noackae* 100

A.1 Van Deemter curve for the GC-EAD 108
A.2 Van Deemter curve for the GC-MS 109
A.3 Combined Van Deemter curves for the GC-EAD and GC-MS 111

B.1 Flight tunnel diagram by Miller and Roelofs (1978) 112

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

B.2 Flight tunnel design 1 by Erling Jirle 114
B.3 Flight tunnel design 2 by Erling Jirle 115
B.4 Flight tunnel design 3 by Erling Jirle 115
B.5 Custom made wind tunnel 116
B.6 Flight tunnel schematic 116
B.7 Flight tunnel light intensity calibration 119
B.8 Flight tunnel flow rate calibration 120

C.1 Estimated pheromone mass in red rubber septa 1 127
C.2 Estimated pheromone mass in red rubber septa 2 128
C.3 Estimated pheromone mass in silicone permeation devices 1 129
C.4 Estimated pheromone mass in silicone permeation devices 2 130

D.1 Scanning electron microscopy images of *C.tristis* female antenna 134
D.2 Scanning electron microscopy images of *C.tristis* male antenna 135

E.1 Glass wool time trial blank 139
E.2 Glass wool time trial night 1 140
E.3 Glass wool time trial night 2 141
E.4 Glass wool time trial night 3 142
E.5 Glass wool time trial night 4 143
E.6 Glass wool time trial night 5 144
E.7 Glass wool time trial all nights 145

G.1 IUFRO 2011 151
G.2 ESSA 2013 152
G.3 ESSA 2013 153

List of Tables

2.1 *Gonipterus* EAG experimental results . . . 42
2.2 Standard retention indexes . . . 43
2.3 Peak identities . . . 44
2.4 Peak abundance . . . 45

3.1 Kovats retention indexes of pheromone standards . . . 62
3.2 *Coryphodema tristis* headspace samples . . . 62
3.3 Field trial 2013 for *Coryphodema tristis* . . . 67
3.4 GC-MS runs of pheromone gland extract samples . . . 68
3.5 GC*GC-MS runs of pheromone gland extract samples . . . 69
3.6 Calibration curve parameters for FID data for *C. tristis* . . . 70
3.7 Dose response curve parameters for EAD data for *C. tristis* . . . 71

5.1 Retention times and Kovats retention indices of peaks found in egg headspace samples of *T. peregrinus* . . . 95
5.2 Regression equations used to quantify the confirmed compounds from *T. peregrinus* egg clutches . . . 95
5.3 Averaged quantities released from eggs in 60 minutes for all measurements 96

C.1 Pheromone dispenser release rate estimation . . . 131

E.1 Mass of glass wool used in time trial . . . 146
E.2 Male *Coryphodema tristis* response size to glass wool extracts . . . 147

F.1 Field trial treatments 2011 . . . 149

Abbreviations

DC	**D**irect **C**urrent
EAG	**E**lectro**A**ntenno**G**raphy
GC-FID	**G**ass **C**romatography **F**lame **I**onization **D**etector
GC-EAD	Coupled **G**ass **C**romatography-**E**lectro**A**ntennography **D**etection
GC-MS	**G**ass **C**romatography **M**ass **S**pectrometry
GLV	**G**reen **L**eaf **V**olatiles
VCC	**V**olatile **C**ollection **C**hamber
VOC	**V**olatile **O**rganic **C**ompounds

Symbols

H	plate height
m/z	mass to charge ratio
N	number of theoretical plates
σ	standard deviation
σ^2	variance
W	width of peak at base
$W_{1/2}$	width of peak at half height
τ	standard deviation in time units
t_r	retention time at peak apex
L	column length
μ	average linear rate of migration of the mobile phase in a column
υ	average linear rate of migration of a compound in a column
[ ]	concentration

Dedicated to my mother and father for their endless support and encouragement during my PhD. studies.

Chapter 1

The application of insect semiochemicals for pest management in forestry

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1.1 Abstract

Plantation forest industries based on non-native trees are under continual and growing threats from insect pests of various origins. Controlling these pests with environmentally friendly semiochemicals is an attractive alternative to using conventional pesticides. Semiochemicals can be used for monitoring insects, mass trapping them, disrupting their mating processes and pushing them out off valuable crops and pulling them into less valuable non-crops. Proven examples in the forestry arena are often for those species that rely on simple chemical signals with strong effects such as sex attractant pheromone blends. Other semiochemical signals such as kairomones and allomones have not been used as extensively in forestry mainly because their effects are very difficult to prove under field conditions. There are many unique challenges in developing semiochemical-based methods. These include difficulties in identifying and artificially mimicking chemical signals that are often chemically and spatially complex. Clearly, a sound understanding of chemical phenomena and the nature of pest biology are essential for successful identification and implementation. This requires a substantial developmental and research effort especially if success is desired in South African forestry environments.

1.2 Introduction

Eucalyptus and *Pinus* species have been introduced into many countries where they are grown in the absence of many of their native pests and pathogens. This has allowed for these fast growing trees to become the major crops for the production of solid wood and pulp products in many parts of the world. The pest free status of these crops, inevitably, does not last because insects are continually introduced into these plantations through human transportation activities (Lodge *et al.*, 2006; Keller *et al.*, 2011; Paine *et al.*, 2011). These introduced insects are not only a threat to the non-native plantation forests, but also to indigenous forest ecosystems (Waring and OHara, 2005).

Additional threats to exotic plantation forests are native insects that encounter these potential hosts (Paine *et al.*, 2011). This can have devastating effects. For example, various indigenous angiosperm-feeding moth species now feed on introduced conifer trees in Europe (Fraser and Lawton, 1994). In South Africa the Cossid moth, *Coryphodema tristis*, which is thought to be native to South Africa, is now causing damage to *Eucalyptus nitens* plantations in the country (Gebeyehu *et al.*, 2005). It is thought that *C. tristis* has undergone this shift due to the absence of natural enemies in the new host (Boreham, 2006). The pine brown tail moth, *Euproctis terminalis*, is another example

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of an indigenous insect that now feeds on *Pinus patula* trees (Geertsema and Van den Berg, 1973).

The damage caused by the native insects on their new foreign hosts can be variable but appears to be aggravated when the introduced tree has related species in the area of introduction (Roques *et al.*, 2006). This may be because related plants are more similar genetically to the introduced plant than unrelated plants. Such plants may carry insects that are easily capable of host range expansion onto the new hosts. Host range expansion may also occur for polyphagous insects that are able to utilize foreign plant material. Clearly introductions of foreign insects and host range expansion of local insects pose a significant and increasing threat to the sustainability of exotic forestry industries (Wingfield *et al.*, 2008).

Dealing with introduced and native pest threats to plantation forests is of paramount importance to the large industries that rely on them. Eradication is always preferred above management. However, eradication is usually only feasible in the early stages of pest establishment and if the affected area is small (Brockerhoff *et al.*, 2010). Integrated pest management (IPM) programmes are usually adopted to monitor and maintain the pests at tolerable levels.

Both eradication and management programmes require sensitive detection capabilities in order to function effectively (Brockerhoff *et al.*, 2006). In this regard, targeting the pest with a behaviour manipulating semiochemical can greatly enhance detecting capabilities of insect traps. This can be achieved by adding an irresistibly attractive chemical, such as a sex attractant, to the trap to enhance its luring effect. Successful monitoring in this way provides information that allows for optimal targeting and timing of pesticide spraying practices. Semiochemicals can also be used in other ways, for example, by mass trapping of insects if their population levels are high and suppressing population growth rate through mating disruption (Carde and Minks, 1995; El-Sayed, 2010). Effective semiochemicals are, therefore, highly valued in such practices.

Pheromone research in entomology had its birth when Henri Fabre in 1870 observed how male emperor moths were lured to a female moth on his balcony. He was able to show that the luring message must have been chemical rather than visual because males were lured to the spot after the female had left (Patlak *et al.*, 2003). Since then the term pheromone has been defined as a substance that is secreted by an animal to the outside and causes a specific reaction in a receiving individual of the same species (Karlson and Butenandt, 1959).

Pheromones have now been divided into different sub-types based on the type of reaction that they provoke in the receiving individual. Among these are the aggregation and sex

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pheromones that are effective for monitoring, mass trapping and mating disruption techniques. Pheromones are among the most useful types of semiochemicals because they are environmentally friendly, species specific and can be used relatively cheaply (Brockerhoff *et al.*, 2006; Witzgall *et al.*, 2010). However, there still remain many challenges in their implementation, even in orchards that are much smaller than large plantation forests (Suckling, 2000).

Other semiochemicals such as host kairomones are now being explored and can significantly enhance the effectiveness of pheromones. Various semiochemical based products are available for numerous forestry related insect pest species (El-Sayed *et al.*, 2006). Pheromones have been identified for nearly 530 Lepidopteran species (Ando *et al.*, 2004) and more than 1,600 different insect species in total (Patlak *et al.*, 2003). Despite the large number of semiochemicals known they are not necessarily used routinely in forestry.

A pertinent question that can be asked is: why are semiochemicals not used routinely in the forestry industry? The best available explanation is that the chemical signals and mechanisms involved in these messages are still not sufficiently well elucidated or understood for most pests and, therefore, they are difficult or even impossible to implement. The greatest challenge in developing semiochemical-based control strategies is the fact that chemical messages may consist of multiple chemicals that are imbedded in very complex mixtures, complicating their detailed characterization and thus their successful exploitation. For example, the light brown apple moth has nine different pheromone components in the pheromone gland, four of which were shown to influence the behaviour of males (El-Sayed *et al.*, 2011). Luckily some insect species can be lured with relatively simple chemical mixtures or even with a single compound. Take the case of the bark beetle *Dendroctonus valens* that can be lured by using only (+)-3-carene (Erbilgin *et al.*, 2007). This variability in the number of compounds needed to elicit a behavioural response in different insect species further complicates the matter.

This review specifically focuses on the use of semiochemicals for insect pest management in forestry. It briefly describes the general techniques used to monitor and control pests with semiochemicals. Examples of semiochemical control programmes in forestry are explored. The challenges that exist when using semiochemical methods for silvicultural pest management are considered and the potential use of such chemicals by the South African forestry industry is discussed.

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1.3 The chemical world as perceived by an insect

Much of our current understanding of the insect olfactory system comes from detailed studies of model insects such as the fruit fly, silk moth and locust. These studies have revealed the complex nature of the insect olfactory system and much of the information discussed below comes from them.

The ability to detect information from the environment is of great benefit to the survival of any living organism. Multi-cellular organisms (such as insects) have evolved various detector organs for this purpose. Each organ is able to tune into a sensory modality (for example: vision, olfaction and mechano- reception). Each type of organ converts information from each modality into a form that is processed in the central nervous system.

In the insect world chemical information, of a volatile nature at ambient temperature, is detected by the antennae or maxillary palp structures. These organs are able to selectively detect specific chemical molecules that may constitute a chemical signal. Information from the olfactory system can, together with the information from other sensory modalities, influence the behaviour of a specific insect in a specific environment (Dethier, 1982; Miller and Strickler, 1984).

The sensillum structure that can be found on, for example a moth antenna, is thought of as the most basic functional unit of the insect olfactory system (Boeckh, 1984). This structure consists of a outer cuticle with pores that lead to an internal liquid environment. Nerve cells are situated in this liquid environment and they are responsible for the conversion of a chemical signal into an electrical one. This is achieved through a mechanism that is not yet fully understood. It has been shown that these nerve cells inside the sensillum are capable of detecting certain molecules in the environment by expressing certain receptor proteins on their surfaces (Vogt, 2003). Each type of receptor protein is thought to have a variable extra-cellular domain with a specific conformation that leads to its specific selectivity.

Volatile organic molecules diffuse through the air and enter the sensillum through pores on its surface. A diverse array of odourant binding proteins (OBPs) in the aqueous layer are responsible for transporting non-polar odourant molecules to the receptor proteins on the surface of the neurons inside the sensillum (Vogt, 2003). These OBPs also protect the odourant from various olfactory degrading enzymes (ODEs) present in the aqueous layer (Leal, 2003). Negative charges present on the membrane surface area of the neuron cell are thought to induce a conformational change in the OBP and the odourant is expelled from the OBP and binds to the receptor on the surface (Leal, 2003). These OBPs, therefore, contribute to the selectivity of the olfactory system (Leal, 2003).

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The combined outputs of sensory neurons of each type (expressing a certain receptor or set of receptor proteins) aggregate in specific glomerulus in the olfactory lobe where the first level of processing begins (Carlsson and Hansson, 2003). The partially processed information is then conducted through projection neurons (PN) to the higher centers of the insect brain, such as the mushroom bodies and proto-cerebrum. Information is thought to be presented through various coding mechanisms in these centers (Carlsson and Hansson, 2003). Information may be processed or interpreted in these centers and could lead to various behavioural effects that can be observed after a given stimulus. Some chemical signals can have an attracting effect on the receiving insect under certain circumstances, while others may repel the insect. Chemical signals that influence behaviour in this way are especially useful once they are known because they can be used in different ways to manipulate and even control insect pests.

1.4 Applications of semiochemicals in forestry

1.4.1 Concepts and examples

Semiochemical-based pest management concepts are discussed below with relevant examples of their application in forestry. Fields other than forestry are included where no appropriate examples could be found for forest pests. For the purpose of clarity, these techniques are discussed separately, however, it should be noted that many of the examples mentioned here are of pests that are not controlled by one specific semiochemical-based technique alone. Techniques are typically used in unison for maximal effect and are sometimes also combined with pesticide spraying regimes.

1.4.1.1 Monitoring

It is often difficult to detect the presence of an insect pest directly in a plantation, due to the large surface area and large number of trees. Specific damage symptoms that are indicative of certain pest species are then used to make an indirect diagnosis. At the point where the pest or its damage is noticeable, pest population levels may already be large enough to cause loss in quantity and quality of wood production. A more direct measurement tactic, such as one using a sensitive monitoring trap, would be very valuable if it could detect pest presence before significant damage symptoms appear.

Waters (1986) described the four main points that embody the purpose of monitoring. Firstly, the monitoring process should be able to detect evidence of unusual insect activity; secondly, it should measure insect population abundance and distribution; thirdly, it

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should provide information to describe trends in crop damage that correlates with pest population levels; and finally, this information should guide other control methods and measure their effects. Traps baited with pheromones of insect pests are ideally suited for such monitoring purposes, because they can lure insects even at very low population densities (Waters, 1986).

Pheromones can be useful in both detecting insect presence and monitoring insect numbers. An example of the level of sensitivity of pheromones traps can be seen for the hermit beetle (*Osmoderma eremita*) and its predator the click beetle (*Elater ferrugineus*). These two endangered species are very hard to find in their natural oak forest habitat; however, pheromone traps that were developed for these beetles were shown to be highly efficient in detecting and even monitoring these rare insects (Larsson *et al.*, 2003; Svensson *et al.*, 2004). Pheromones traps have also been used successfully for a number of lepidopteran forest defoliators in the USA and Canada (Grant, 1991). These traps are valued specifically because they are cost effective and easy to use (Grant, 1991). Bark beetle population distributions are also successfully determined with pheromone based trapping methods (Hayes *et al.*, 2008). It is possible to correlate the number of individuals trapped with damage levels seen in the field. For example, trap numbers of the pine sawfly could be correlated with damage seen in the field, but only when the population density was high (Lyytikäinen-Saarenmaa *et al.*, 2006). There are many other similar monitoring examples.

There are a few disadvantages in using pheromone traps to monitor insect pest presence and population levels. This is because pheromones can be so effective in luring insects that they may even attract individuals from affected compartments into virgin areas (Lanier *et al.*, 1976). To alleviate this problem, traps should ideally be deployed at higher densities inside affected areas for monitoring purposes and at lower densities in the boundary area between affected and unaffected areas to capture stray individuals and to limit spreading of the pest (Tobin *et al.*, 2007). Correlating trap capture numbers with damage seen in the field can be difficult in some cases (Hayes *et al.*, 2008). Traps and pheromone lures can also be expensive and labour intensive to deploy.

1.4.1.2 Mass trapping

Mass trapping by definition refers to the attempt to trap as many individuals as possible within the affected area. The purpose here is to directly lower the population levels of the pest species through trapping. In this case, traps should have a large enough trapping capacity. Both sex- and aggregation pheromones are used for mass trapping, but aggregation pheromones that lure both sexes are advantageous (Witzgall *et al.*,

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

2010). Sex pheromones that attract only one of the sexes can be used in conjunction with other semiochemicals that may trap the opposite sex, see for example field trials with coddling moth pheromone (Knight *et al.*, 2005). Host kairomones have much potential here because they may act synergistically to increase the effect of a pheromone and are sometimes attractive to both sexes (Reddy and Guerrero, 2004).

Some of the best examples of mass trapping in forestry are programmes implemented for bark beetle control (Borden, 1989; Schlyter *et al.*, 2001). Pioneer beetles that land on a suitable tree typically produce aggregation pheromones that lure other beetles. These beetles are then able to overwhelm host tree defenses via mass attack, and sometimes pathogenic fungi that are carried and spread by the beetles (Paine *et al.*, 1997). Traps using other kairomones in conjunction with the bark beetle aggregation pheromones have been developed but these seem to loose their species specificity (Miller *et al.*, 2011). Such traps can be used to trap a larger diversity of insects.

There are a number of factors that contribute to the fact that mass trapping is not commonly used in plantation forestry. The major problem is the high density of traps that are needed to effectively trap sufficient individuals. This can be financially inviable given the large areas to be covered in a plantation forest environment. It is also often difficult to directly attribute the subsequent population decline to the mass trapping process itself, because numerous environmental factors can contribute to a natural population decline of the target species. Furthermore, predators of the target insect may also be lured and captured, which negatively affects natural biological control. It is thus important to ensure that molecules are incorporated into the traps that maximize the number of targeted insects caught relative to the predator insects (Aukema *et al.*, 2000).

1.4.1.3 Mating disruption

Mating disruption occurs when the original chemical message from the calling individual cannot be perceived or followed by the receiving individual (Witzgall *et al.*, 2010). Typically, the mate finding process is either delayed or halted in the treated area. Delaying mating may cause loss of reproductive potential in certain insect species (Stelinski *et al.*, 2009). Generally this is achieved by creating many pheromone point sources that release either the pheromone or pheromone analogues in the field. Point sources of a pheromone can be established by systematically dispersing pheromone dispensers in the affected area. Alternatively, it is possible to impregnate pheromones into some form of polymer matrix that is easily sprayed by aircraft. These matrices can be in the form of small polymer flakes (Thorpe *et al.*, 2000) or liquid polymer that sticks to the leaves of the trees being protected. Examples include matrix technologies such as

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

HERCON DISRUPT and SPLAT (Tcheslavskaia *et al.*, 2008) that are used for Gypsy moth (*Lymantria dispar*) control in forests of the USA and Canada.

Although the mechanisms underpinning mating disruption are still not fully understood in most cases, a number of modes of action have been proposed (Carde and Minks, 1995; Witzgall *et al.*, 2008). Generally these modes fall into two groups. The first concerns theories relating to malfunctioning sensory organs due to the over abundance of the pheromone. This may occur due to either adaptation or habituation of the sensory organ or even a sensory imbalance created by high levels of one or more components in the pheromone blend (Flint and Merkle, 1983; Bau *et al.*, 1999). The second group concerns theories that assume the message is being transmitted correctly, but behavioural manipulation occurs due to the presence of many point sources of the pheromone. Normally male moths fly into a pheromone plume with defined structure that allows them to orientate and navigate toward the source (Mafra-Neto and Cardé, 1994; Vickers, 2000). The male should not be able to orient correctly and would very seldom find a female if this plume structure is destroyed by the presence of large quantities of pheromone point sources in the field. The males consequently spend large amounts of energy and time following false trails. Male perception of true pheromone signals may be negatively influenced through a sensory imbalance that can be created by artificial pheromone present in the background. This background pheromone may also camouflage calling females.

The best examples of successful mating disruption are those of the pink bollworm (*Pectinophora gossypiella*) (Flint and Merkle, 1983; Boguslawski and Basedow, 2001), oriental fruit moth (*Grapholita molesta*) (Stelinski *et al.*, 2009) and the tomato pinworm (*Keiferia lycopersicella*) (Schuster *et al.*, 2000). The European pine shoot moth (*Rhyacionia* sp.) (Daterman *et al.*, 1975; Niwa *et al.*, 1988) and the Gypsy moth (*Lymantria dispar*) (Thorpe *et al.*, 2000) being the best examples in forestry. One of the main reasons why the Gypsy moth is so successfully controlled through mating disruption is because the females cannot fly, thus spread is only achieved by larval movement. Larvae of this pest are able to move in wind currents facilitated by strands of silk that the insect produces. Extensive lists of Lepidopteran pests controlled with mating disruption have been presented by Carde and Minks (1995) and Ando *et al.* (2004).

The major disadvantage of mating disruption is the fact that only one of the sexes is affected (Witzgall, 2001). Although mating disruption effectively lowers the probability that the two sexes would find each other, it never reduces these chances to zero. Therefore, the pest population would never disappear from the treated area.

1.4.1.4 Push-pull strategies

Push-pull strategies refer to the approach where attractive and repellent semiochemicals sources are used in unison (Cook *et al.*, 2007). Attractive semiochemical sources are placed outside of the protected crop whereas repellent sources are placed inside the protected area. Pheromones, kairomones and allomones can be used in combination or individually to produce the desired effect. These strategies are more efficient on a smaller scale and have been used successfully by small subsistence farmers in Africa (Hassanali *et al.*, 2008). For example, host and non-host kairomones are effectively used to remove lepidopteran maize stem borers through intercropping (Khan and Pickett, 2004).

Push-pull strategies have been used to control bark beetles on conifer trees in forestry (Cook *et al.*, 2007). The strategy has been used for *Ips paraconfusus* and *Dendroctonus ponderosae* (Lindgren and Borden, 1993; Shea *et al.*, 1995). Beetles can be trapped in designated trap trees that are inoculated with the aggregation pheromone while being pushed from infected trees by applying anti-aggregation pheromone (Borden, 1989).

1.5 Examples of semiochemical application in forestry

1.5.1 Moths

1.5.1.1 Gypsy moth, *Lymantria dispar*

The Gypsy moth was accidentally introduced into the USA from Europe between 1868 and 1869 by Léopold Trouvelot (Tobin *et al.*, 2007). The larvae of *L. dispar* are responsible for the defoliation of a wide diversity of deciduous trees including oak, aspen, poplar, willow, apple and others. The females are sedentary and produce a pheromone that lures male moths for mating.

The main compound from pheromone gland extracts in *L. dispar* was identified as *cis*-(7,8)-epoxy-2-methyloctadecane or disparlure (Bierl *et al.*, 1970). This compound was used in an extensive slow the spread campaign that relies on trap capture data to predict pest movement (Tobin *et al.*, 2007). In this campaign, a 170 kilometer zone between affected and unaffected areas was monitored with pheromone traps (Tobin, 2008). This zone was subdivided into a 100 kilometer action zone and a 70 kilometer evaluation zone closer to the boundary area. Traps were deployed at a high density with approximately 2 kilometers space between them in the action zone while traps were spaced between 3 and 8 kilometers in the evaluation zone (Tobin *et al.*, 2007). Stray populations that formed in the action zone could be detected and eradicated through various different

treatment techniques including, growth regulators, *Bacillus thuringiensis* kurstaki and nucleopolyhedrosis viruses (Reardon *et al.*, 1994, 1996). Mating disruption was also deployed in the action zone by spraying polymer flakes coated with a sticking agent. The mating disruption process appeared to be more effective when using a sticking agent that keeps the dispensers from falling to ground level, but this added to the costs and introduced difficulties in the application process (Thorpe *et al.*, 2000).

Other phenolic compounds have also been investigated as possible agonists or antagonists of the pheromone of *L.dispar* (Plettner and Gries, 2010). These compounds are useful because they can respectively increase or decrease the efficacy of the pheromone. Agonists may be used to enhance the luring effect of the pheromone trap while antagonists can be used to enhance the mating disruption effect.

1.5.1.2 Codling moth, *Cydia pomonella*(L.)

The codling moth *Cydia pomonella* (L.) is a pest of stone fruits (Witzgall *et al.*, 2008). Stone fruit orchards resemble the forest environment in many ways. Orchards resemble non-native plantation forests because they are usually non-native trees that are planted as a long term investment. The trees are planted in a similar design and are also often related to each other as is the case in the forestry environment.

The sex pheromone of the calling female codling moth was identified as (*E*8,*E*10)-dodecadien-1-ol later named codlemone (Roelofs *et al.*, 1971). The correct conformations around the double bonds were found by comparing electrophysiological responses of the male antenna to all the different possible isomers (Roelofs *et al.*, 1971). It has been shown that the female moth releases the pheromone at a rate of between 5 and 7 ng per hour during the calling phase (Bäckman *et al.*, 1997).

Codlemone is used to monitor male moths and appears to act synergistically with the pear ester (*E*,*Z*)-2,4-decadienoate (Knight *et al.*, 2005). This pear ester is also attractive to female moths, but not in combination with codlemone (Knight *et al.*, 2005). Codlemone is also used in an integrated pest management scheme in apple orchards in the USA (Brunner *et al.*, 2002). The use of mating disruption in areas with low population densities shows potential advantages over long term use of pesticides (Witzgall *et al.*, 2008). This includes a more effective use of bio-control agents for secondary pests (Brunner *et al.*, 2002).

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

1.5.1.3 Oriental Fruit Moth or Peach Moth, *Grapholitha molesta*

The only records of successful mating disruption in South Africa are those reported for the Oriental Fruit Moth, *Grapholitha molesta* (Kirsch and Lingren, 1993; Barnes and Blomefield, 1997). This species was introduced into South Africa in the late 1980s and causes damage to stone fruit trees grown in the Tulbagh valley in the Cape region (Kirsch and Lingren, 1993). The major sex pheromone component of this species was identified as (*Z*)-8-dodecenyl acetate (Roelofs and Comeau, 1969) and was later shown to be a blend of two acetates and two alcohol components (Cardé *et al.*, 1979). Mating disruption in this area was successful after only one season of application and the pheromone dosage could be halved in subsequent seasons after the initial success (Kirsch and Lingren, 1993).

1.5.2 Bark beetles

Some bark beetle species are known to be able to kill healthy trees through mass attack and some vector fungi that may cause a decline in wood quality. Bark beetles such as *Dendroctonus frontalis* and *Ips typographus* vector *Ophiostoma* and *Ceratocystis* species that can cause blue stain in the wood of certain conifer species (Paine *et al.*, 1997). Other bark beetles such as *Scolytus* spp. vector *Ophiostoma* spp. that cause Dutch Elm Disease; one of the most significant environmental disasters in Europe (Tomlinson and Potter, 2010). This has stimulated much research on these beetles, including work on their chemical communication and perception.

1.5.2.1 *Ips* spp.

Only *Ips confusus*, *I. paraconfusus* and *I. typographus* are considered here because these species are well-known serious pests of pine trees in the northern hemisphere and may vector blue stain fungi. *Ips confusus* and *I. paraconfusus* occur in the western pine forests of North America and are only distinguishable through molecular methods (Cognato *et al.*, 1995). *Ips confusus* mostly infests *Pinus edulis* and *P. monophylla*, while *I. paraconfusus* inhabits a larger number of pine species including the economically important *P. ponderosa* (EPPO/CABBI, 2006). These beetles typically breed in dead or dying pine trees and spread to healthy trees when epidemic situations prevail. They generally vector fungal species that cause blue stain in the wood, which degrades timber quality. Pine pitch canker, caused by *Fusarium subglutinans* (*Fusarium circinatum*), is a serious disease of Pine and is suspected to be vectored by *I. paraconfusus* in California (Storer *et al.*, 1994; Gordon *et al.*, 2001).

Male *Ips* beetles search for suitable trees to colonize. They tunnel into the wood and produce an aggregation pheromone that lures other males and females to the tree. Three different compounds were isolated from the frass produced by male *I. confusus* beetles. The male sex pheromone of *I. praconfusus* (at the time thought to be *I. confusus*) was isolated and identified in 1966 from 4.5 kg of frass (Silverstein *et al.*, 1966; Young *et al.*, 1973). Three different compounds were reported to be responsible for the aggregation behaviour, namely (-)-2-methyl-6-methylene-7-octen-4-ol (ipsenol), (+)-*cis*-verbenol and (+)-2-methyl-6-methylene-2,7-octadien-4-ol (ipsdienol). These three compounds did not produce the attraction behaviour when tested singly, but as soon as a mixture between ipsenol and either of the other compounds was made, the attraction behaviour was restored (Silverstein *et al.*, 1966).

Ips typographus, a bark beetle pest of spruce in northern Europe, can reach devastating population levels, especially after long periods of drought (Bakke, 1989) and severe storms where trees are uprooted or physically damaged in large numbers (Wermelinger, 2004). These natural phenomena are thought to impact negatively on host resistance and subsequently cause beetle populations to escalate. The aggregation pheromone 2-methyl-3-buten-2-ol in conjunction with *cis*-verbenol and ipsdienol was used to trap masses of *I. typographus* individuals in central Europe (Wermelinger, 2004), but the effectiveness of this method to reduce population numbers has been disputed. It was estimated that traps capture only around three to ten percent of the population within the treated area. This is increased by a factor of roughly 30 when the pheromone is applied to trap trees (Wermelinger, 2004) which implies a possible synergistic effect between the pheromone components and the host volatiles. Trap trees have been used as lures for bark beetles in Europe for more than 200 years.

1.5.2.2 *Dendroctonus* spp.

Aggregation pheromones of beetles in the genus *Dendroctonus* are produced by either the male or female insects, depending on the species. Such pheromones are used to lure high numbers of individuals to a host tree in order to overcome the host defense responses (Raffa and Klepzig, 1989).

Many of the pheromone molecules are common between related species. Specificity among species may be gained through differences in ratios of compounds that may be common between species For example, female *D. frontalis* produce an aggregation pheromone called frontalin (1,5-dimethyl-6,8-dioxabicyclo(3.2.1)octane) and trans verbenol. Frontalin is a chiral compound with two centres of chirality and it is produced in

different chiral ratios to gain specificity among the different species (Francke and Dettner, 2005). *D. frontalis* also produces an anti-aggregation pheromone called verbenone that prevents over colonization in the host tree. Another pheromone component, brevicomin, was found in a related species, *D. brevicomis*, and was also shown to be attractive to *D. frontalis*, suggesting that there may be inter-species communication occurring between sympatric bark beetles, which was not expected (Pureswaran *et al.*, 2008). These authors also showed that *D. frontalis* males produce exo-brevicomin even in the sympatric zone (Pureswaran *et al.*, 2008). It is now known that beetles using brevicomin as pheromone component utilize all degrees of information that is crated by the two chiral centers present in the molecule to express their species-specific pheromone messages (Francke and Dettner, 2005). Specificity is gained by changing ratios between enantiomers, diasteriomers and absolute amounts emitted (Francke and Dettner, 2005).

Some host volatiles have also been shown to function as kairomones and could potentially be used to trap these beetles. For example, (+)-3-carene has been shown to be a highly attractive terpene for *D. valens* (Erbilgin *et al.*, 2007). This was shown both in its endemic area, Nova Scotia through to California and the introduced area in China (Erbilgin *et al.*, 2007).

1.6 Semiochemical use in South African forestry

Eucalyptus and *Pinus* trees are the major species grown in commercial plantations in South Africa, accounting for more than 90% of the plantation forestry area (DWAF, 2009). These forests are relatively long term crops which, depending on the product, can take between six and twenty-five years to grow to a harvestable size. Plantations such as these are especially susceptible to insect pest outbreaks because they are often grown as monocultures (Wingfield *et al.*, 2008).

The use of insecticides such as organophosphates and carbamates, which are often persistent in the environment, can have many negative effects as they are often harmful to both pests and beneficial insects and can build up in natural water systems. Bird and fish populations may also be negatively affected by insecticides. Strong selective pressures exerted by insecticide can give rise to resistance in insect pest populations as with the codling moth (*Cydia pomonella*) in Europe (Witzgall *et al.*, 2008). Conventional insecticides should, therefore, be reserved as a last resort to control insect pest outbreaks. The use of environmentally friendly semiochemical- based methods to detect, predict and manage such outbreaks would be a valuable tool in such cases. However, apart from a few monitoring examples discussed below, no semiochemicals are currently being used by the South African forestry industry.

A number of reasons are thought to explain the slow acceptance and use of these techniques in South Africa (Nadel *et al.*, 2012). Funding these vital techniques is required for the identification and development of semiochemical based methods, specifically for forest insect pests in South Africa. Furthermore, semiochemical- based strategies developed in other parts of the world may not necessarily work under South African conditions. This may contribute to skepticism regarding the usefulness of these techniques. Estimating the financial effect of a semiochemical- based intervention is also very difficult. Moreover, the physical labour associated with deploying traps in the field and counting trapped insects may be inordinately expensive and is consequently avoided. This dilemma is unfortunate and should be rectified.

1.6.1 *Sirex noctilio*

Sirex noctilio is considered to be a major threat to pine plantations in South Africa. *Sirex noctilio* originates from the northern hemisphere where pine trees occur naturally. These wasps are thought to have entered South Africa in the Western Cape in 1994 (Tribe *et al.*, 1995). Females carry and infect pine trees with a symbiotic fungus (*Amylostereum areolatum*) and phytotoxic mucus (Ryan and Hurley, 2012). The fungus is essential for the survival of the larvae inside infested trees because it aids in the digestion of wood cellulose on which the larvae feed. The combination of both the fungus and the phytotoxic mucus results in tree death (Coutts, 1969; Ryan and Hurley, 2012).

Knowledge of how *Sirex* wasps behave in a plantation environment is essential if successful semiochemical- based methodologies are to be developed. It is well recognized that female wasps are attracted to pine trees that experience some form of stress (Madden, 1988; Villacide and Corley, 2012). This habit was subsequently exploited by foresters to trap females. Selected trees are purposefully stressed by either ring barking or injecting a phytotoxin (3,6-dichloro-2-methoxybenzoic acid) into the wood (Madden, 1988). Females are then preferentially lured to oviposit in such a tree. This method allows for selective inoculation with the biological control nematode, *Deladenus siricidicola*. Panel traps were subsequently developed in an attempt to mimic stressed pine trees (Bashford and Madden, 2012). These traps are baited with both α- and β-pinene. They are relatively efficient in detecting and monitoring the movement patterns *S. noctilio*, but they trap only females and must compete with stressed pine trees in the environment. Females of the biological control wasp, *Ibalia leucospoides*, are also attracted to these traps (Bashford and Madden, 2012).

A study of the antennal morphology in *S. noctilio* has shown that numerous contact chemo receptors are present on the antenna with a lower number of genuine volatile

chemo receptors (Crook *et al.*, 2008). Females were found to have more of the volatile chemo receptors when compared to males (Crook *et al.*, 2008). The high abundance of contact chemo receptors relative to other receptors has led to the hypothesis that a contact sex pheromone is used by these wasps. Subsequently, (*Z*)-7-heptacosene, (*Z*)-7-nonacosene, and (*Z*)-9-nonacosene were identified as contact sex pheromones (Böröczky *et al.*, 2009). These molecules were identified from body wash extracts of females and were found to induce copulation behaviour in males (Böröczky *et al.*, 2009).

The aggregation behaviour of male *S. noctilio* above the tree canopy is thought to be as a result of a positive phototaxis (Morgan *et al.*, 1966). A male aggregation pheromone (*Z*)-3-decen-1-ol was subsequently found in the headspace of trapped males, but not females, and was shown to be electrophysiologically active on both male and female antennae (Cooperband *et al.*, 2012). A combination of two minor components together with the major compound was shown to be attractive to both males and females in a wind tunnel (Cooperband *et al.*, 2012). This behavioural effect has significant implications for monitoring and possibly controlling *S. noctilio* and has stimulated a collaborative effort to show its efficacy in South Africa in field trials. Preliminary data suggest that trap efficiency is not as enhanced in the field. To date no effective long range sex pheromone could be identified for *S. noctilio*.

1.6.2 Pine emperor moth, *Nudaurelia cyfherea* Fabr *cyfherea*

There are two sub species of pine emperor moth that occur in South Africa. These are *N. cyfherea cyfherea* (Fabr.) and *N. cyfherea clarki* (Geertsema) (Geertsema and Van den Berg, 1973). The first sub-species is found mostly on *Pinus radiata* in the Cape region, while the second sub-species is found on *P. patula* in the Kwazulu-Natal and Mpumalanga regions (Geertsema and Van den Berg, 1973). These pine species constitute a large part of non-native South African plantations and the pine emperor moth is seen as a significant pest to the industry. The level of defoliation caused by these larvae can be predicted by determining the density of dead moths in the field (Geertsema, 1980).

Pigs are able to locate the pupae in the ground and were originally used as a control measure for this pest (Webb, 1958). Nevertheless, pigs were unable to control the pest in areas where the pupae were inaccessible. Pigs were also expensive to maintain and above all free roaming pigs damaged nearby vineyards (Webb, 1958; Geertsema and Van den Berg, 1973). An assortment of chemical sprays was investigated as control agents for pine emperor moth (Webb, 1958). The larvae also seemed to be affected by a number of RNA viruses (Tripconey, 1970; Hendry *et al.*, 1985). These virus particles induce larval

flaccidity and internal liquefaction. Neither of these techniques are sufficiently specific to be effective.

The pheromone of *N. cyfherea cyfherea* (Fabr.) was identified in the 1970s as (*Z*)-5-decenyl isovalerate (Henderson *et al.*, 1972, 1973). This pheromone was shown to be effective in luring males in field conditions, but no data on this effect was published. Unfortunately, subsequent to this early work, there have been no studies describing the nature of the attraction in the field.

1.7 Other significant Semiochemical research in South Africa

Semiochemical research in South Africa is basically driven by the laboratory for ecological chemistry situated at the University of Stellenbosh. A smaller group is now being developed at the Forestry and Agricultural Biotechnology Institute of the University of Pretoria. The group in Stellenbosh largely focuses on semiochemicals of dung beetles. Dung beetle males of the telecoprid group produce a sex attractant pheromone that is embedded in very complex organic material. This added complexity makes the analysis and identification of these pheromone compounds a very difficult task. Recent electroantennographic studies have identified three active components for the *Kheper subaeneus* species and five for the *K. bonellii* species (Burger and Petersen, 2002; Burger *et al.*, 2002, 2008). Propanoic acid, butanoic acid and 3-methylindole commonly named skatole seems to be common between these two species. Results from behavioural assays in the field have to date not been able to show statistically significant attraction between dung alone and dung supplemented with the identified semiochemicals (Burger and Petersen, 2002; Burger *et al.*, 2002, 2008).

1.8 Challenges that influence the use of semiochemicals in forestry

1.8.1 Challenges in pheromone research

The first step in using semiochemicals in pest control is to confirm that the behaviour of an insect is influenced by volatile compounds from a natural source. The next step is to identify the various different molecules that constitute the chemical message. After the identification process, the different compounds involved in the message must be mixed and tested for the behavioural effect. During this process a number of problems are likely to be encountered. These include:

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1.8.1.1 Analytical challenges in identifying the pheromone messages

The greatest challenge in developing semiochemical-based control strategies is the fact that chemical messages are usually very complex and hence difficult to elucidate and exploit. The analytical challenge of detailed interpretation of semiochemical messages is still a major bottleneck. The reason for this is that the analytical chemist must not only elucidate the identity of the molecules involved in these messages, but also the novelty that exists within the message for a given species. This is especially so if value is placed on the species specificity of the final semiochemical application.

Novelty within pheromone messages is necessary, because different species of insects need to be able to transmit their messages to others of the same species without confusion. Chemical novelty can exist in a number of forms. A unique molecule for one species, amongst all the other molecules from other species at the same locality and time, would be an ideal message because no other species would be able to interfere with the message. There are a number of examples where only one type of molecule is needed for a reaction in the recipient insect or receiver of the message. In these cases the semiochemical message must be released at a time when other insects do not release the same compound otherwise the message loses its specificity. For example, the gypsy moth (*Lymantria dispar*) is attracted only by the + enantiomer of dispalure while the - enantiomer is a strong behavioural antagonist (Miller *et al.*, 1977; Plettner *et al.*, 2000). *Ips pini* bark beetle populations in the Great Lakes region of the USA respond to *R*(-)-ipsdienol and the opposite enantiomer *S*(+)-ipsenol has a negative effect on attraction (Raffa and Klepzig, 1989).

Specificity such as that noted above is not always the case and researchers are now finding that most pheromone messages are multi-component. For example, moth pheromones are typically long chain carbon molecules with variable positions and geometries at unsaturated carbon-carbon bonds along the chain (Roelofs and Bjostad, 1984; Ando *et al.*, 2004). Minor components are often present and can be very important, because they may act synergistically with the major components in the pheromone message and may be critical for attraction.

A second way to generate novelty in pheromones is by using a unique ratio of non-unique molecules. It follows that the pheromone message should be used at a unique time or locality specific for each species producing the same molecules in their pheromone blends. Two closely related moth species could likely produce similar molecules in their pheromone blends, but in different ratios. An example of closely related species, some of which live and call in the same time period, is that of the small ermine moths, *Yponomeuta* sp. (Löfstedt *et al.*, 1991). Nine different species are known in Europe and

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

almost all of them have pheromone molecules that are common to each other. They differ only in the pheromone ratios that are produced and the presence or absence of certain minor components (Löfstedt *et al.*, 1991). These species can interbreed and it is predominantly thought that it is the unique minor components among them that maintain their isolation from each other (Löfstedt *et al.*, 1991).

It would appear that the composition of a pheromone for a specific species at one specific locality changes very slowly in time, purely through random mutations coupled to geographic isolation. The turnip moth, *Agrotis segetum*, was shown to have seventeen different possible pheromone compounds in the pheromone gland. Different combinations of some of these compounds are released and in different ratios for the population found in France as compared to other European countries (Löfstedt *et al.*, 1986; Stevens, 1998). However, sometimes a sudden major shift in pheromone component ratio can occur. The Asian corn borer, *Ostrinia furnacalis*, uses a mixture of (*Z*)- and (*E*)-12-tetradecenyl acetate, while the European corn borer, *Ostrinia nubilalis*, uses a mixture of (*Z*)- and (*E*)-11-tetradecenyl acetate (Roelofs *et al.*, 2002). Here it was shown that activation of one of the desaturation enzymes, leads to a different location of the double bond in the pheromone molecule and, therefore, the pheromone composition. Certain rare male moths may be present in a given population that respond to the opposite pheromone mixture (Linn Jr *et al.*, 2003, 2007). These rare males may then sustain a form of chemical isolation based on the pheromone and subsequently differentiation occurs from the parent population.

1.8.1.2 Odour blends and synergism

Synergism can be defined at a number of different levels. In behavioural terms synergism occurs when two or more compounds confer a combined effect greater than the sum of their separate effects on the behavioural response of an insect. These effects can be seen with bark beetle pheromones brevicomin (exo and endo-) and frontalin that are more effective when used in conjunction with myrcene, a host terpene (Bedard *et al.*, 1980). A more recent example of pheromone/kairomone synergism has been demonstrated for the codling moth. Adding the pear-derived host kairomone ethyl (*E*, *Z*)-2,4-decadienoate to codlemone enhances the attraction of traps (Knight *et al.*, 2005). This indicates that some host volatiles may be involved in enhancing pheromone messages. It has also been shown that bark beetles may be able to detect non-host angiosperm volatiles and thus, avoid these hosts (Zhang and Schlyter, 2004).

Another level of synergism occurs at the neuronal level of the olfactory system. Complex chemical signals are converted into electrical signals by a population of olfactory receptor

neurons (ORN) present in the olfactory sensilla on the antenna. Numerous ORN axons of different sub types converge in different glomeruli in the antennal lobe (AL). It is thought that this convergence of neurons enhances the signal to noise (s/n) ratio (Laurent *et al.*, 2001). Information is then conveyed through projection neurons (PN) into the higher centers of the insect brain including the mushroom bodies and the protocerebrum. These projection neurons can be thought of as a neuronal cell population. Different sets of these projection neurons encode and convey information from odours in apparently different encoding mechanisms that may function in certain insect species. One of these is based on a spatial temporal coding mechanism found in the locust (Laurent *et al.*, 2001).

Local field potential oscillations occur in certain parts of the insect brain. These oscillations are measurable in the mushroom bodies and are the result of many neurons that spike simultaneously in the antennal lobe (Carlsson and Hansson, 2003). The oscillations are irregular if no stimulus is given. It can be imagined that information from each PN is like an instrument in an orchestra. Each one contributes to the overall melody of the music and yet single instruments may change the melody at certain times. Clearly synergism exists here because information from each instrument can be distinguished not by the loudness of its signal, but rather by the relation it has to the other instruments (Laurent *et al.*, 2001).

These synergistic effects often make it very difficult to predict the behaviour of an insect when it is confronted by a combination of chemical substances. This is because the behaviour towards the mixture is often not just the sum of the behaviour towards the individual chemicals within the mixture.

1.8.1.3 Release rates and ratios of pheromone components

Many insects rely on the unique ratio of pheromone components that are generated by the calling individual and, therefore, the production process needs to be precisely controlled. Ratios between different compounds involved in a chemical signal are usually calculated from isolated samples that do not necessarily portray the true chemical signal and fail when behavioural tests are implemented. The true compound ratios should ideally be mimicked in a pheromone trap for optimal effect. This is not an easy task, mainly because molecules of different sizes have different vapour pressures and thus different release rates from the same type of dispensing matrix used within traps (Gut *et al.*, 2004). Pheromone molecules can also degrade if they are sensitive to light and antioxidants may have to be added into the dispenser (Millar, 1995). One way to achieve the correct release rates is by using different polymers for each compound. If, for example, two components of largely different vapour pressures and/or fuctionalities are needed in the

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pheromone blend, then two different polymers should be incorporated in the dispensing capsule or the relative amounts added to a one polymer dispenser should be adjusted to represent the blend more accurately. This can be done by estimating the relative vapour pressures of the components (Heath *et al.*, 1986).

Gut *et al.* (2004) described that changes in the molecular weight and/or functional group of a pheromone molecule influences its ability to partition between the air and for example pheromone dispensers and/or wax layers on leaf surfaces in the field. Here the partition coefficient was defined as the ratio of the concentration of the pheromone in the wax layer on a leaf divided by the concentration in the air immediately above the wax layer. In general, the partition coefficient increases log linearly as the molecular weight increases. The functionality of a pheromone molecule can also influence its partitioning behaviour. Should the functional group have a high degree of polarity then the partition coefficient would be large if the partitioning surface properties are polar. This difference in the partitioning behaviour of different pheromone molecules has a profound effect on the way in which pheromone is dispersed in the field. Additional factors that influence pheromone dispersal in the field are wind velocity, foliage density and temperature (Gut *et al.*, 2004).

Pulsation of pheromone plume was shown to have an effect on how certain lured moths lock on to the pheromone source (Baker *et al.*, 1985; Mafra-Neto and Cardé, 1994, 1996). For example, it could be shown that once the pheromone plume is pulsed in a flight tunnel, that the males of the Oriental fruit moth locate the pheromone source with less casting behaviour (Willis and Baker, 1984). This was also shown for the Almond moth, *Cadra cautella* (Mafra-Neto and Cardé, 1994). Moths therefore require the correct plume structure in order to be successfully lured into traps. This plume structure is defined by pheromone filaments that are interspersed with clean air and the frequency at which these filaments are released from a pheromone dispenser may be critical for close range selectivity and attraction.

1.8.2 Challenges in field application

1.8.2.1 Trap placement and properties

The large surface area that needs to be effectively monitored in the plantation environment may imply the use of many traps that are placed in a dense pattern which is usually not economically viable. Concepts such as the effective attractive radius (EAR) of a trap can be very useful in determining the optimal density of traps that are needed to monitor a pest successfully (Byers *et al.*, 1989). Traps are usually placed at strategic locations and at low density to minimize costs. The optimal location is not always

obvious because it is determined by the habits of the pest that is targeted. A sound knowledge of pest behaviour is essential. Some insect pests may fly preferentially at different levels above the ground. For example, males of the *Sirex noctilio* woodwasp species are known to aggregate above the pine tree canopy where they wait for females before mating (Morgan *et al.*, 1966). If it is desirable to monitor these males, then a monitoring trap should be placed as close as possible to the tree canopy. Placing the traps at such a height and monitoring them routinely would be labour intensive and expensive, but it should be considered if accurate results are desired. The colour of a trap and the amount of bait that is used can also significantly affect the number of insects that are caught. For instance black traps are far superior to yellow traps for the red palm weevil, *Rhynchophorus ferrugineus* (Abuagla and Al-Deeb, 2012).

1.8.2.2 Difficulties in correlating trap capture with insect numbers and tree mortality in the field

Pheromone monitoring with traps is useful only if the number of insects caught in a trap correlate well with the number of insects occurring in the field. This is achieved only when the trap does not saturate with trapped insects. Trap saturation is easily overcome by using larger traps, but the real problem is that traps do not maintain the same attractive force throughout the expected trapping time. Trapped insects may change the attractive nature of a trap because they can contribute to the volatile profile that is released from a trap. An added complication here is that the pheromone amount inside a trap usually decays exponentially from the moment the trap is placed in the field (Heuskin *et al.*, 2011). The release rate of different compounds depend on the environmental conditions (Kuhr *et al.*, 1972; Gut *et al.*, 2004). This can cause a trap to function correctly only for a short period. A zero order release rate for all the pheromone components is desired and many different dispensers have been developed, most of them with first order release rates (Heuskin *et al.*, 2011). The correct ratio of pheromone components should then be placed inside the releasing device so that components are released in the correct ratio in the field throughout the desired trapping time. This is probably one of the most serious pitfalls when designing a trap (Millar, 1995).

Tree mortality due to a specific pest insect is especially hard to quantify when monitoring with a trap. This is because tree mortality usually depends on many factors including many environmental factors that can cause stress in trees. These types of problems make it difficult to directly correlate the number of trapped individuals with the number of tree deaths. A good example of this is the number of bark beetles caught in pheromone baited funnel traps that were poorly correlated with tree mortality caused by bark beetle infestation (Hayes *et al.*, 2008).

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1.9 Conclusion

It is clear that plantation forest industries suffer from ever increasing insect pest problems. These challenges have to be addressed to ensure the sustainability of these industries in future. Semiochemical application provides an environmentally friendly way to address some of these problems. Techniques that have been developed and used in the forestry arena include monitoring, mass trapping and especially mating disruption. In theory these techniques sound very effective, and they are, but only in some cases. For example: sex attractant pheromones in the Lepidopteran order has been shown to be useful for the management of certain pests. Other semiochemicals such as allomones and kairomones are used but to a lesser extent because their usefulness is often more difficult to prove.

There are numerous examples of semiochemical application in the northern hemisphere where most of the fundamental research is performed and pest control techniques are developed. Few such developments are found in the Southern hemisphere. Successful development and application relies heavily on thorough understanding of pest biology, behaviour and chemical communication processes. Extensive multidisciplinary research that requires collaboration between entomologists, biologists, ecologists and chemists is essential for successful development. Such collaboration seems to be lacking especially from the South African forestry perspective where semiochemicals have been used for only two species to date: the Pine Emperor moth and the Sirex wood wasp.

The simulation of semiochemical signals is a very difficult task that requires increased attention. The necessary skills, equipment and collaboration have to be put in place in order to sustain a research field that could potentially solve the most urgent pest problems in the South African forestry industry. Attention should be focused on developing methods to investigate pest behaviour under firstly, controlled conditions and secondly in the field. The necessary chemical methodologies for finding, sampling and identifying potential semiochemical compounds must be expanded in future. Such expansion is possible by focusing on pest insects that are currently of economic importance in the South African forestry industry.

Chapter 2

Chemical signatures affecting host choice in the *Eucalyptus* herbivore, *Gonipterus* sp. (Coleoptera: Curculionidae)

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A version of this chapter was published in Arthropod Plant Interactions. DOI 10.1007/s11829-014-9327-y

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2.1 Abstract

It is well-known that herbivorous insects respond to host plant volatiles. Yet details of how these insects perceive the complex profile of volatiles from different potential host plants has not been studied for most insects. *Gonipterus* spp. are important pests of *Eucalyptus* worldwide, but differ in their preference for different species of this host. In this study we consider whether host volatiles affect the host choice for a *Gonipterus* sp., and we characterise the response of the female insect to the volatile profiles from these hosts in an electroantennographic (EAG) experiment. We sampled volatiles from freshly damaged leaves of three *Eucalyptus* species, and analyzed the profiles by gas chromatography coupled to electroantennography (GC-EAD) and gas chromatography coupled to mass spectrometry (GC-MS). Female weevils gave a mixed range of electrophysiological responses to volatile puffs from leaves of different tree species. This suggests that differences in volatile profiles of different trees play a role in how these beetles discriminate between potential hosts. GC-EAD analysis showed that responses were as complex as the volatile chemical compositions of the leaves. A number of these chemicals were identified and responses were mostly due to general green leaf volatiles. This was also evident from the fact that the insects showed a markedly greater response to the total volatile profile from freshly damaged leaves for all species. *G. scutellatus* females can therefore detect damaged leaves, which may indicate host quality. Host specificity information is further expected to lie in the relative differences in emission ratios and synergism between different host chemical compounds, rather than specific individual compounds.

2.2 Introduction

The *Eucalyptus* snout beetle originates from southeastern Australia and Tasmania, but has been introduced to numerous countries around the world (Tooke *et al.*, 1953). The insect feeds on leaves of *Eucalyptus* trees during both larval and adult stages and consequently can cause significant damage to susceptible trees (Tooke *et al.*, 1953; Richardson and Meakins, 1986). In many of these countries the beetles have led to significant losses in plantation forests (Mally *et al.*, 1924; Clark, 1932; Williams *et al.*, 1951; Hanks *et al.*, 2000; Rivera and Carbone, 2000; Lanfranco and Dungey, 2001; Loch and Floyd, 2001), including South Africa (Tooke *et al.*, 1953; Richardson and Meakins, 1986). The collective name *G. scutellatus* has often been used for the *Eucalyptus* snout beetle in the past, but it is known today that this name represents a species complex (Mapondera *et al.*, 2012). In South Africa, for example, the beetle has long been thought to represent the single species *Gonipterus scuttelatus*, but recent studies suggest that collections most

likely include *G. platensis* and an undescribed *Gonipterus* sp. 2 (Mapondera *et al.*, 2012).

The literature is unclear as to which *Eucalyptus* species is the preferred host for invasive *Gonipterus* spp. (Clarke *et al.*, 1998). For example, *E. globulus* has been reported as one of the most heavily damaged hosts for *Gonipterus* spp. in countries such as South Africa (Mally *et al.*, 1924; Tooke *et al.*, 1953; Richardson and Meakins, 1986), New Zealand (Clark, 1932), USA (Hanks *et al.*, 2000), Spain (Rivera and Carbone, 2000), Chile (Lanfranco and Dungey, 2001) and Australia (Loch and Floyd, 2001). It has, however, recently been shown that the beetles in South Africa survive better when feeding on *E. smithii* rather than *E. globulus*, which is in contrast to earlier reports (Newete *et al.*, 2011). Furthermore, in the native range of Tasmania, where a wider host range is available, *G. scutellatus* is reported to prefer *E. pulchella* above *E. globulus* trees (Clarke *et al.*, 1998). Host availability might thus be one of the factors influencing differences in reports about host preference of *Gonipterus* spp.

A number of reasons other than host availability might also influence differences in host preference reports. Techniques used to score damage by *Gonipterus* spp. are not standardized and are interpreted across long time scales and broad geographic ranges. The host preference of a range of cryptic, related species has also not yet been considered and studies prior to that of Mapondera *et al.* (2012) mostly refer to *G. scutellatus* in the broad sense. Environmental factors can also influence both the hosts and the beetles themselves (Clarke *et al.*, 1998). Temperature, for example, is known to influence the beetles activity levels (Tooke *et al.*, 1953) and the volatile emission rates of *Eucalyptus* trees (Guenther *et al.*, 1991; Nunes and Pio, 2001). Furthermore, many *Eucalyptus* spp. carry two distinct types of foliage, which have different physical (Brooker and Kleinig, 1996) and chemical (Guenther *et al.*, 1991; Nunes and Pio, 2001; Pio *et al.*, 2001) characteristics. These differences may influence the host choice of *Gonipterus* spp. (Richardson and Meakins, 1986; Cordero Rivera *et al.*, 1999).

Newete *et al.* (2011) showed that larvae of a *Gonipterus* sp in South Africa could survive on a number of *Eucalyptus* species including some species (e.g. *Corymbia citriodora* and others) that are not selected for oviposition. Larval survival and adult oviposition for *Gonipterus* in South Africa is, therefore, not necessarily correlated. The data provided by these authors, however, show that adult females preferentially lay eggs on *E. smithii*, *E. grandis*, *E. scoparia* and *E. viminalis* in the field (Newete *et al.*, 2011). The mechanism by which female *Gonipterus* spp. select oviposition material is largely unknown. What is known is that herbivorous insects are able to detect volatile organic compounds from plants (Visser, 1986; Metcalf and Metcalf, 1992; Dicke, 2000). Compounds that

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are commonly found around green plants include green leaf volatiles, monoterpenes, sequiterpenes and polyterpenes. It is thought that phytophagous insects may be able to select certain host plants based on these volatile chemicals (Bruce *et al.*, 2005). This could also be the case for *Gonipterus* spp. in South Africa.

Odours from damaged plant tissue have been found to play a role in the behaviour of a number of weevil species. For example, the vine weevil, *Otiorhynchus sulcatus*, is known to prefer plant material that has been damaged by other vine weevils. Furthermore, these beetles appear not to be able to distinguish between mechanically damaged and weevil damaged plant material (Van Tol and Visser, 2002). Research on the pepper weevil, *Anthonomus eugenii* has shown that these beetles are attracted to damaged plants and in particular to plants freshly damaged by their conspecifics (Addesso *et al.*, 2011). The sugarcane root-stalk borer weevil, *Diaprepes abbreviatus*, is also attracted to mechanically damaged plant tissue (Harari and Landolt, 1997).

Tooke *et al.* (1953), who studied *Gonipterus* (referred to as *G. scutellatus* in his studies) on *Eucalyptus* in South Africa, argued strongly that host selection behaviour of this insect was linked to some olfactory mechanism. He attempted to link the host preference of the insect to the essential oil composition of different *Eucalyptus* species by correlating the host susceptibility in the field to the major components in the essential oils made from these trees. This experiment, however, met with little success and Tooke (1953) could conclude only that the majority of preferred hosts had eucalyptol (cineol) in their essential oils.

If there is a host preference, as reported in the literature for *G. scutellatus* (which includes at least two different species), then it is likely that chemical cues might be involved in female host choice. These chemical cues could either be distinct or similar for each of the reported hosts. The aim of this study was to investigate the electrophysiological responses of females identified as *Gonipterus* sp. 2 (following Mapondera *et al.* (2012)) beetles to the total volatile bouquet originating from foliage of eleven different *Eucalyptus* spp. A further aim was to identify individual host volatiles that are electrophysiologically active for *Gonipterus* sp. 2 females. For the latter part of the study, volatiles were sampled from the damaged leaves of three *Eucalyptus* spp., two of reportedly susceptible hosts (*E. globulus* and *E. viminalis*) and one of a non-host, *Corymbia* (*Eucalyptus*) *citriodora* (Tooke *et al.*, 1953; Richardson and Meakins, 1986) by an adsorption process. GC-EAD active peaks were tentatively identified from the *E. globulus* volatile profile by GC-MS and confirmed with standards.

2.3 Materials and Methods

2.3.1 Insect samples

Gonipterus sp. 2 samples were obtained from a *Eucalyptus* plantation in Pretoria, South Africa near Tom Jenkins Drive (S25°44' 07,97 E28°14' 18.08). Only *Gonipterus* sp. 2 is known from this area and its identity has been confirmed using COI sequence data (Dr. J. Garnas unpublished, University of Pretoria, personal communication). Insects were fed on *E. smithii* and *E. globulus* foliage while being kept in wooden cages in a temperature controlled $20 - 25\,^{\circ}C$ room. Female insects were used in EAG recordings because they make the choice to find suitable oviposition material on which larvae will eventually develop. Females were identified based on the differences in the penultimate sternites as reported by Carbone and Rivera (1998).

2.3.2 *Eucalyptus* samples

Eleven *Eucalyptus* spp. were sampled from two sites in Pretoria. All species other than *E. saligna* have been reported as susceptible to infestation by *Gonipterus* spp. in South Africa by Tooke *et al.* (1953), Richardson and Meakins (1986) or Newete *et al.* (2011). *Eucalyptus grandis* is widely planted in South Africa and is also known to be a host (Rivera and Carbone, 2000) and it was, therefore, included in the analyses. *Corymbia citriodora* (previously also classified in *Eucalyptus*) was chosen to represent a non-host (Tooke *et al.*, 1953). Six of the sampled *Eucalyptus* spp. were found at the same site as the insects. The remaining five *Eucalyptus* species were obtained from the Forestry and Agricultural Biotechnology Institute (FABI, `http://www.fabinet.up.ac.za`) nursery at the University of Pretoria. Cross contamination between individual samples was avoided by separating them, upon sampling, in separately sealed poly-acetate cooking bags. These bags were stored in a fridge at $5\,^{\circ}C$ before the analyses were undertaken.

2.3.3 Volatile collection

Volatiles from the crushed juvenile foliage of three different *Eucalyptus* species were sampled through dynamic headspace sampling and adsorption onto standardized Tenax TA (200 mg) traps (MKIUNITY, Markes, Chemetrix, Midrand, South Africa). The sampling material was obtained from three trees at the same two sites in Pretoria. The leaves of each *Eucalyptus* sp. were cut into pieces of approximately 5 cm^2. The leaves were sampled for 30 minutes at a flow rate of 512 ml/min in duplicate for each *Eucalyptus*

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sp. and a sample blank was taken. The dry weight of the sampled leaves was measured as 6.1 g for *E. globulus*, 9.5 g for *E. viminalis* and 4.8 g for *Corymbia citriodora*.

2.3.4 Electroantennography

All electrophysiological recordings were made with an EAG detector system (Syntech, Hilversum, The Netherlands). Live female beetles were used in these recordings because a decline in antennal sensitivity was observed when antennae were removed (data not shown). Individual beetles were secured with cotton wool inside a micropipette tip with only the head and antenna protruding from the end of the pipette tip. The pipette was secured to a mounting device and a dissection microscope and micro-manipulator were used to position and connect glass capillary microelectrodes to the insect antenna and head. The recording electrode was connected to the tip of the club shaped antenna with the reference electrode connected to the eye on the opposite side of the insects head. Ag/AgCl electrodes were made from silver wire that were immersed in a 0.1 M KCl electrolyte solution with 2 % PVP (polyvinyl pyrolidone) added to prevent desiccation. The entire preparation was moved to within one centimeter from a glass stimulus delivery tube. Filtered and humidified air was blown onto the insect preparation through the stimulus delivery tube at a flow rate of 150 ml/min and sample volatiles were introduced into this air flow 170 mm upstream from the antennal preparation as 0.4 second puffs, at 30 ml/min at puff maximum.

Clean surgical blades were used to cut a 1 cm^2 piece of leaf from each of the eleven different *Eucalyptus* spp. samples. Each leaf piece was inserted into a different Pasteur pipette and an empty pipette was used as a sample blank. A blank recording was made before and after sets of five sample recordings for each of the *Eucalyptus* samples. Each of the samples was freshly damaged after the first five recordings by mechanically scraping the cuticle of the leaf with a clean piece of glass. Five additional recordings were subsequently made of the freshly damaged plant material. A recovery period of one minute was allowed between each individual recording. The entire experiment was repeated three times with three different female insects. The order in which these recordings were made was kept constant for all three insects.

The absolute response intensity (mV) of each recording was measured. Four outliers (1.5 X Inter Quartile Range) were identified and discarded from the analysis. All blanks and respective recordings for each *Eucalyptus* species were pooled and a global ANOVA analysis was done based on deflection intensity. Dunnetts test was used for joint ranking

(control group = blank) with an α level equal to 5% in order to determine which *Eucalyptus* species had larger responses than the blank recordings. Tukey Honestly Significant Difference test was used to assign letters of significance.

2.3.5 Gas chromatography coupled to electroantennography

All GC-EAD recordings were made with the same EAG detector system as reported above (Syntech) coupled to an Agilent 6890N gas chromatography system (Chemetrix, Midrand, South Africa). EAD signals were recorded at a sampling rate of a 100 samples per second. A 10 times external amplification was used and the low cut-off filter was set to 0.05 Hz on the software. High frequency noise was digitally removed, after the recording was made, by adjusting the low pass filter after the run to allow only a window of 0.05 Hz to 3 Hz to pass. These settings were used for all thermally desorbed samples. Samples were injected onto a 60 m DB 624 column (J & W scientific, ID: 0.25 μm, film: 1,4 μm) with a thermal desorption system (MKIUNITY, Markes, Chemetrix, Midrand, South Africa) at a 17:1 split ratio. The transfer line between the thermal desorption system and GC was kept at 190 °C. Nitrogen was used as carrier gas and constant column head pressure of 20.1 psi was used during separation. The GC oven was kept at 40 °C for 7 minutes and increased at 5 °C per minute to a maximum of 260 °C.

Antennae were removed at their bases from live female *Gonipterus* sp. 2 beetles using a surgical blade. A dissection microscope and micromanipulator were used to position and connect glass capillary microelectrodes to the insect antennae. The recording electrode was connected to the tip of the club shaped antenna with the reference electrode connected to the base of the removed antenna. Ag/AgCl electrodes were prepared as reported above. The GC effluent was introduced into the air steam 90 mm upstream from the preparation. The transfer line between the GC and EAD detector was kept at a maximum temperature of 260 °C. Six GC-EAD recordings were performed for each *Eucalyptus* sp. in order to identify repeatable responses in the EAD data.

GC-EAD responses to the standard compounds as tentatively identified with the GC-MS analysis (described below) were also confirmed on the GC-EAD system by liquid injection of a mixture (1000 ppm) made in dichloromethane (n = 9). The liquid injector was operated in split mode (20:1) at a temperature equal to 200 °C. In order to avoid the automatic baseline correction, direct current (DC) recordings were performed during these liquid injection runs. Baseline correction was performed on the resulting EAD data (Time constant r = 0.85) (Slone and Sullivan, 2007) and retention indices were used to match peaks between the two sample injection methods.

2.3.6 Gas chromatography coupled to mass spectrometry

GC-MS analysis was done in order to tentatively identify some of the EAD active peaks. A Thermo Quest trace GC 2000 series coupled to a Finnigan Polaris ITD and a Perkin Elmer thermal desorption system with an identical 60 m DB624 analytical column as was used during GC-EAD. A split ratio equal to 43.7:1 was used during desorption of samples on the GC-MS system. Helium was used as carrier gas and the average linear velocity was matched with the GC-EAD system isothermally at 130 °C and required a column head pressure of 16.0 psi. The oven of the GC-MS system was set at 40 °C for 7 minutes and increased at 5 °C/minute to a maximum of 260 °C. The transfer line between the thermal desorption system and GC was kept at 190 °C and the transfer line between the GC and MS was kept at 260 °C. The Finnigan Polaris ITD was operated with an ion source temperature equal to 200 °C and 70 eV ionization energy. The mass scan range was 50-285 m/z. Tentative identities were assigned based on a mass spectral comparison to library spectra and known retention indexes (Nist 2.0c, 2004). Sixteen standard reference compounds were purchased from reputable suppliers for confirmation of compound identity. Peak area was calculated by integration of the total ion chromatogram if the peaks were pure. Mass fragments were used when compounds could not be resolved from their total ion chromatograms.

Statistical analysis was conducted in R version 3.0.2 on relative percentage peak areas of the peaks confirmed with reference standards only (metaMDS, Vegan package, Oksanen *et al.* (2013)). This gives a relative representation of identified compound distribution for each sample based upon these standards. Bray-Curtis distances (Bray and Curtis, 1957) were calculated and used to locate the relative positions of species within a multidimensional space. Non-metric multidimensional scaling (NMDS) was used to find a low dimensional representation with a maximum distance between points represented on the first dimension. This type of comparison was used successfully in other similar studies (Proffit and Johnson, 2009; Kotze *et al.*, 2010).

2.4 Results

2.4.1 Electroantennography

Results of the EAG experiment with leaves before mechanical scraping, showed that female beetles had a significantly greater responses to *E. viminalis*, *E. smithii* and *E. tereticornis* when compared to blank recordings. *E. tereticornis*, *E. smithii*, *E. globulus*, *E. robusta* and *E. camaldulensis* did not give statistically significantly different responses

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from each other. *E. globulus*, *E. robusta*, *E. camaldulensis*, *E. grandis*, *E. saligna*, *E. scoparia*, *E. punctata* and *C. citriodora* could not be statistically separated from blank recordings (Table 1).

The EAG response of the beetles to the total volatile profile of freshly damaged leaves showed that all the *Eucalyptus* spp. tested elicited a response that was significantly greater than blank recordings. Among these, *E. globulus*, *E. tereticornis*, *E. viminalis*, *E. robusta*, *E. smithii*, *E. camaldulensis* and *E. scoparia* elicited larger EAG responses compared to the non-host *Corymbia citriodora*, which showed the smallest responses (Table 1).

2.4.2 Gas chromatography coupled to electroantennography

Electroantennogram responses observed for the chromatograms of the different *Eucalyptus* spp. revealed that there are many different peaks that elicited responses from the female *Gonipterus* sp. 2 antennae. Many of these peaks were common for the three different *Eucalyptus* species that were sampled, but they occurred in different ratios for each species (Figure 1, 2, 3).

Responses to the standard compounds revealed that (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, eucalyptol, γ-terpinene, α-pinene, 2-phenylethanol, benzyl acetate and ethyl phenylacetate were correctly identified as being antenna-active compounds. These standard compounds were confirmed to give measurable electrophysiological responses from the female antenna. The largest responses, amongst these, were observed for the green leaf volatiles (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate and phenolic compounds 2-phenylethanol, benzyl acetate and ethyl phenylacetate (Figure 4). Some of the compounds in the standard mixture were confirmed as antenna-active, but could not be detected in the chromatographic profiles of any of the *Eucalyptus* samples tested in this experiment. These included camphene, β-pinene, 3-carene and m-cymene. These compounds were tentatively identified as being present in these profiles. However, retention index differences between the sample peaks and standard compounds showed that the initial tentative identification, which was based on library mass spectra, was incorrect.

2.4.3 Gas chromatography coupled to mass spectrometry

Standard compounds were confirmed to be correctly identified through retention index matches on both the GC-EAD and GC-MS systems (Table 2, 3 and 4). Mass spectral comparisons between standards and unknowns were also used to confirm tentative

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identities (see table 4 for relative ion distributions of major ions). Inconsistency in retention indices (large difference in KI between the two systems) was observed for the alcohols 2-phenylethanol and (*Z*)-3-hexen-1-ol. These inconsistencies could be explained by column surface activity on the GC-MS instrument, which caused band broadening of alcohols through hydrogen bonding. Four of the identified compounds co-eluted under these chromatographic parameters. These include (*E*)-2-hexenal that co-eluted with (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate that co-eluted with 3-carene.

Non-metric multidimensional scaling (NDMS) was used to plot samples in two dimensions in such a way that the distance between the points portrayed the relative differences between samples (Supplementary Figure 1). A larger distance is associated with a larger degree of dissimilarity. Caution was applied when interpreting these results since they are based on the presence and relative abundance of the 16 investigated compounds for only 2 samples of each species (stress ≈ 0). This plot separates the three sampled species by grouping them together based only on the presence and relative abundance of the identified compounds. The analysis separated *E. globulus* from *C. citriodora* and *E. viminalis*, largely based on the presence of 2-phenyl ethanol, benzyl acetate, ethyl phenylacetate and terpenyl acetate. These compounds were not detected in *E. viminalis* and *C. citriodora*. Limonene, eucalyptol, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate played a role in separating *E. viminalis* from the other two species. *Corymbia citriodora* was mainly separated from *E. globulus* and *E. viminalis* due to the influence of γ-terpinene, which was present in relatively larger proportions.

2.5 Discussion

Results of this study showed that host volatiles could play a significant role in host choice for the *Eucalyptus* pest, *Gonipterus* sp. 2 in South Africa. This was evident from measurable electroantennogram responses from the beetles to virtually all the *Eucalyptus* spp. tested, especially when the leaves were freshly damaged. A number of specific volatiles to which the beetle responded were also identified.

Significantly larger antennal responses were recorded from freshly damaged leaves of *E. globulus*, *E. tereticornis*, *E. viminalis*, *E. smithii*, *E. camaldulensis* and *E. scoparia*, when compared to freshly damaged *C. citriodora* leaves on which the beetle is known not to feed (Newete *et al.*, 2011). Consistent with these results, the same *Eucalyptus* spp. have also been reported as being preferred hosts for *G. scutellatus* s.l. in South Africa (Mally *et al.*, 1924; Tooke *et al.*, 1953; Richardson and Meakins, 1986; Newete *et al.*, 2011). *Eucalyptus dorrigoensis*, *E. nitens*, *E. scoparia*, *E. viminalis*, *E. grandis* and *E. smithii* were also found to bear more *G. scutellatus* eggs when compared to the

other species surveyed from the field (Newete *et al.*, 2011). Most of these species are known to occur near the suspected region of origin of the insect in eastern Australia (Newete *et al.*, 2011). Female beetles, therefore, appear to be able to detect hosts that resemble some of the species found in their original habitat.

There are a number of factors that could result in the increased response magnitude observed to freshly damaged leaves. For example, different volatiles and mixtures of volatiles can be released after damaging the leaves (Kalberer *et al.*, 2001). The differences in EAG responses observed between the different *Eucalyptus* species could arise due to different volatiles that are either unique to each species of tree or common between them. It is also possible that *Gonipterus* females detect volatiles that originate specifically from the damaged foliage. Green leaf volatiles are known to originate from enzymatic reactions that occur when plant material is damaged (Galliard and Matthew, 1977; Matsui *et al.*, 2000). These types of volatiles are known to stimulate the antennae of various phytophagous insects (Visser, 1986; Metcalf and Metcalf, 1992) and they are almost ubiquitous among all green plants. For example, certain phytophagous spider mites (*Tetranychus urticae*) are known to be attracted to foliage damaged by conspecific mites (Pallini *et al.*, 1997). This is also known for weevils such as the vine weevil, *Otiorhynchus sulcatus*, which is strongly attracted to foliage that has been damaged by its conspecifics (Van Tol *et al.*, 2002). It is, therefore, possible that weevils such as *Gonipterus* spp. detect these compounds, because they convey information regarding the stress levels and general health of a potential host plant (D'Alessandro and Turlings, 2006).

Gas chromatographic investigation of the damaged *Eucalyptus* leaves revealed that many of the volatiles that originate from leaves stimulate the antenna of the *Gonipterus* sp. 2 females, as could be seen in the complex EAD traces matching the isolated volatiles. Compounds that were identified and confirmed as being antennally active for *Gonipterus* sp. 2 females included (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, α-pinene, camphene, β-pinene, (*Z*)-3-hexenyl acetate, 3-carene, limonene, eucalyptol, γ-terpinene, 2-phenylethanol, benzyl acetate and ethyl phenylacetate. These compounds are almost ubiquitous among all green plants (Metcalf and Metcalf, 1992; Bruce *et al.*, 2005).

The fact that *Gonipterus* sp. 2 detects a range of common compounds from *Eucalyptus* leaves may be explained by the high number of different *Eucalyptus* species that have been reported as hosts for this insect see Clarke *et al.* (1998), which would require a general mechanism to identify the hosts more broadly. It is possible that *Gonipterus* sp. 2 distinguishes different *Eucalyptus* host species based on the relative emission rate and ratio differences of common compounds emitted from potential host trees. Unique combinations and ratios of some host volatiles could indicate more and less preferred

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hosts for *Gonipterus* sp. 2. If this is true, then the antenna would need a high degree of selectivity and sensitivity toward such volatiles. This phenomenon is known for other insect species. For example, females of the moth Manduca sexta are able to distinguish host species and quality based on host plant odour profiles (Späthe *et al.*, 2013). The necessary selectivity appears to be present in the antenna or sensory periphery for that species (Späthe *et al.*, 2013).

Results of this study showed that *Gonipterus* sp. 2 female antennae give relatively larger responses for the green leaf volatiles ((*Z*)-3-hexen-1-ol, (*E*)-2-hexenal and (*Z*)-3-hexenyl acetate) when compared to terpenes, including α-pinene, β-pinene, 1,8-cineol and γ-terpinene. This shows that the antennae are more sensitive to these compounds than towards the identified terpenes. This finding is consistent with an EAG study conducted on the vine weevil, *Otiorhynchus sulcatus*, which was shown to give larger EAG responses to (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal, 2-phenylethanol and (*Z*)-3-hexenyl acetate, but showed weak responses towards terpenes (Van Tol *et al.*, 2002). Measurement of single sensillum responses (SSR) in other weevil species have shown that these insects have specific neurons that are specialized for certain sets of volatiles. A SSR study on the white clover seed weevil (*Apion fulvipes*) has shown temporal differences in the response patterns of different receptor neuron classes towards different compounds (Andersson *et al.*, 2012) including many compounds identified in the present study. These response differences were speculated to aid in discrimination of different odour filaments that the insect encounters as it flies. *Apion fulvipes* was also shown to possess a class of olfactory receptor neurons that specifically responds to damaged leaf odours (Andersson *et al.*, 2012). In another weevil study, Blight *et al.* (1995) was able to show single sensillum responses to many of the same compounds as those identified in the present study, for the cabbage seed weevil (*Ceutorhynchus assimilis*). It is thus possible that *Gonipterus* sp. 2 uses a similar mechanism and similar receptor sets to discriminate different host odours.

A number of the identified compounds that elicited EAD responses in the *Gonipterus* sp. 2 females are also known to be antennally active for other insect species. These include 2-phenyl ethanol and (*Z*)-3-Hexen-1-ol that were EAG active for the Colorado potato beetle, *Leptinotarsa decemlineata* (Weissbecker *et al.*, 1999) and (*Z*)-3-hexen-1-ol, α-pinene, β-pinene, cymene, 1,8-cineole, and limonene being EAD active for the *Eucalyptus* woodborer, *Phoracantha semipunctata* (Barata *et al.*, 2000). Five of the antenna- active compounds identified in the present study (2-phenylethanol, 1,8-cineol, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal) were found to be EAG active for the cabbage seed weevil (*Ceutorhynchus assimilis*) by Blight *et al.* (1995). 2-Phenylethanol was also identified as being antenna- active for the pollen beetle, *Astylus atromaculatus* and was shown to be behaviourally attractive to that species (Van den Berg *et al.*, 2008). Two

of the identified compounds, (*Z*)-3-hexenyl acetate and 2-phenyl ethanol, was found to be behaviourally attractive to adult tea weevil, *Myllocerinus aurolineatus*, females (Sun *et al.*, 2012). These compounds could, therefore, be some of the volatiles that distinguish potential hosts for *Gonipterus* sp. 2 females. It is interesting that these two compounds were also in part responsible for separating the three species based on their presence and relative abundance within the damaged leaf profiles.

Although the behavioural function of the volatiles on *Gonipterus* sp. 2 remains unknown, our results have shown that there is a chemical interaction between *Gonipterus* sp. 2 female antennae and volatiles isolated from different *Eucalyptus* species. The olfactory interaction between *Gonipterus* sp. 2 females was further shown here to be very complex, but is mainly based on common green leaf volatiles that are released once the leaves are damaged. A number of electrophysiologically active volatile compounds were identified, and it is expected that some of these compounds may be involved in the insect's behaviour, in particular female host choice. There is a possibility that some of the identified chiral terpenes (for example α-pinene, β-pinene, 3-carene, camphene and limonene) add an extra layer of complexity to the host selection behaviour for *Gonipterus* sp. 2. Enantiomeric ratios could also differ between crushed and non-crushed leaves of a single *Eucalyptus* species. Chiral separation to determine enantiomeric ratios could shed light on these complexities in future.

2.6 Acknowledgements

We are grateful to the members of the Tree Protection Co-operative Programme (TPCP), the THRIP initiative of the Department of Science and Technology (DST), South Africa and the National Research Foundation (NRF) for providing financial support for this study. We also thank Dr. Jeff Garnas for assistance with some of the statistical tests and for providing unpublished data pertaining to the identification of the *Gonipterus* sp. used in this study.

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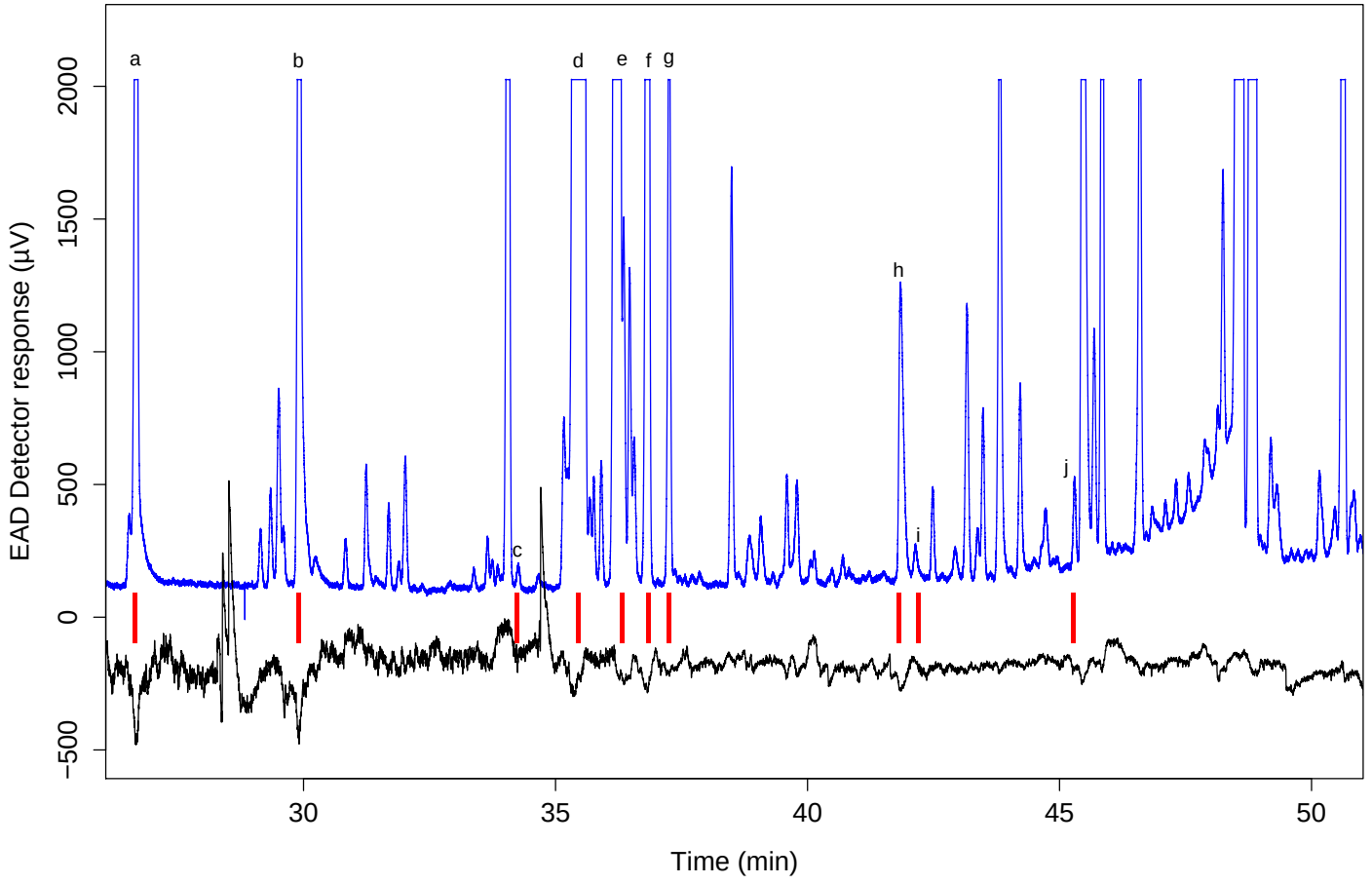

FIGURE 2.1: FID chromatographic peaks (top trace) for *E. globulus* leaf volatiles and corresponding EAD responses (bottom trace) of *Gonipterus* sp. 2 antenna. Vertical lines correspond to elution times of peaks that were investigated in order to identify electrophysiologically active compounds. Peak letters correspond to those listed in Tables 3 and 4.

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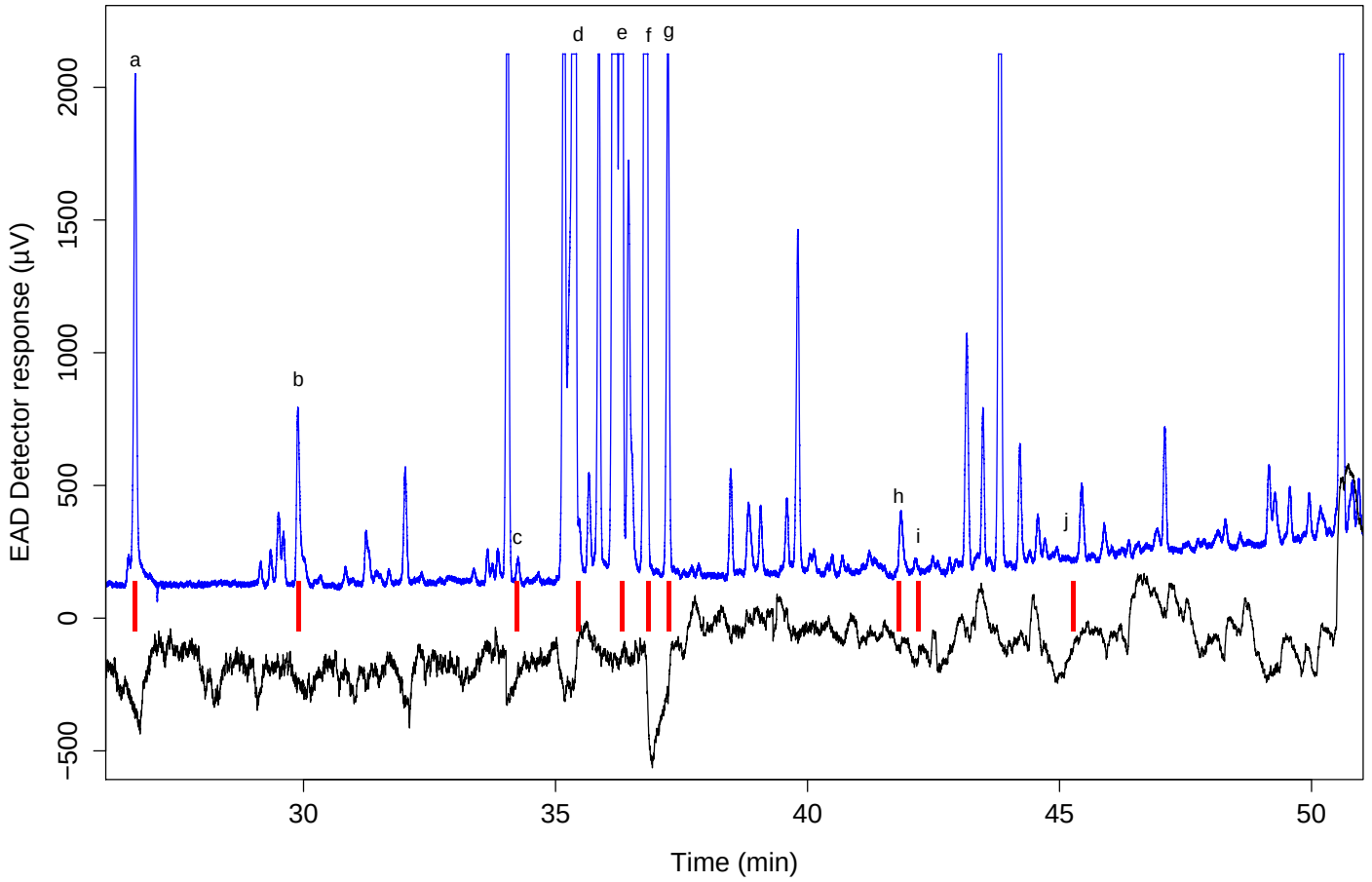

FIGURE 2.2: FID chromatographic peaks (top trace) for *E. viminalis* leaf volatiles and corresponding EAD responses (bottom trace) of *Gonipterus* sp. 2 antenna. Vertical lines correspond to elution times of peaks that were investigated in order to identify electrophysiologically active compounds. Peak letters correspond to those listed in Tables 3 and 4.

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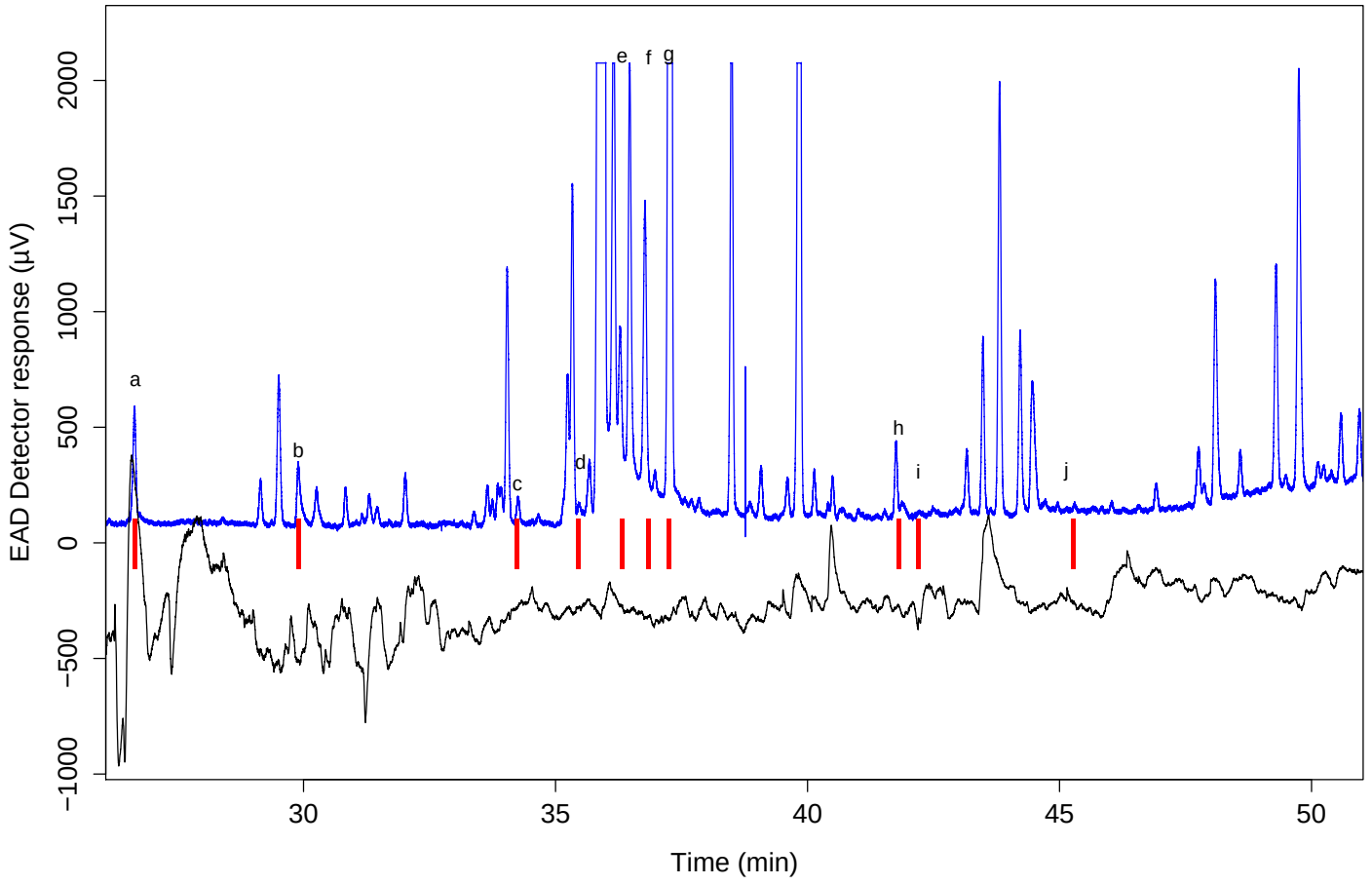

FIGURE 2.3: FID chromatographic peaks (top trace) for *C. citriodora* leaf volatiles and corresponding EAD responses (bottom trace) of *Gonipterus* sp. 2 antenna. Vertical lines correspond to elution times of peaks that were investigated in order to identify electrophysiologically active compounds. Peak letters correspond to those listed in Tables 3 and 4.

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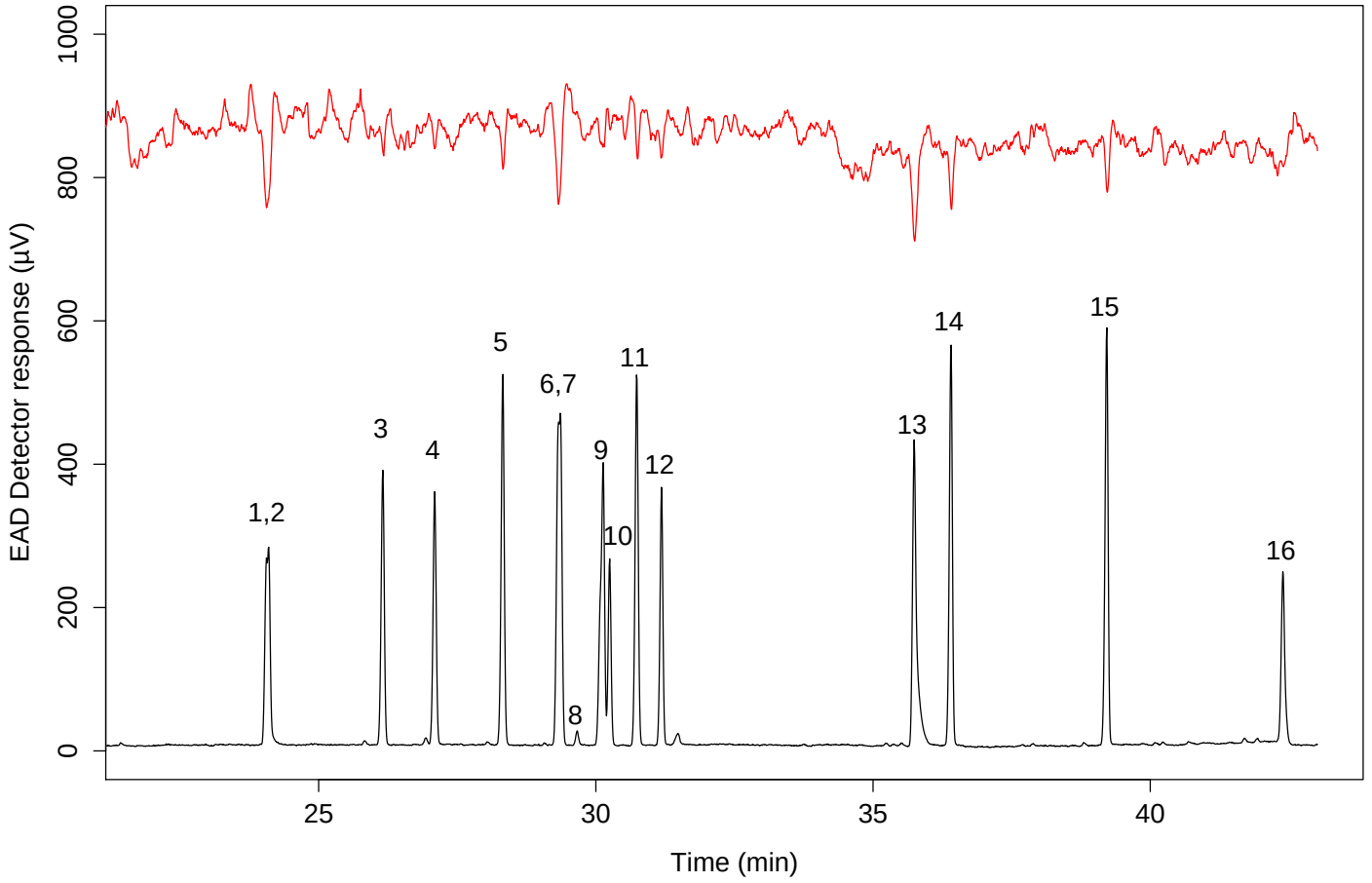

FIGURE 2.4: GC-EAD responses toward identified standard compounds after liquid injection at 20.0psi (25 ng at EAD). Peak numbers refer to standard numbers in Tables 2, 3 and 4. The top trace is the EAD response and the bottom trace is the FID response.

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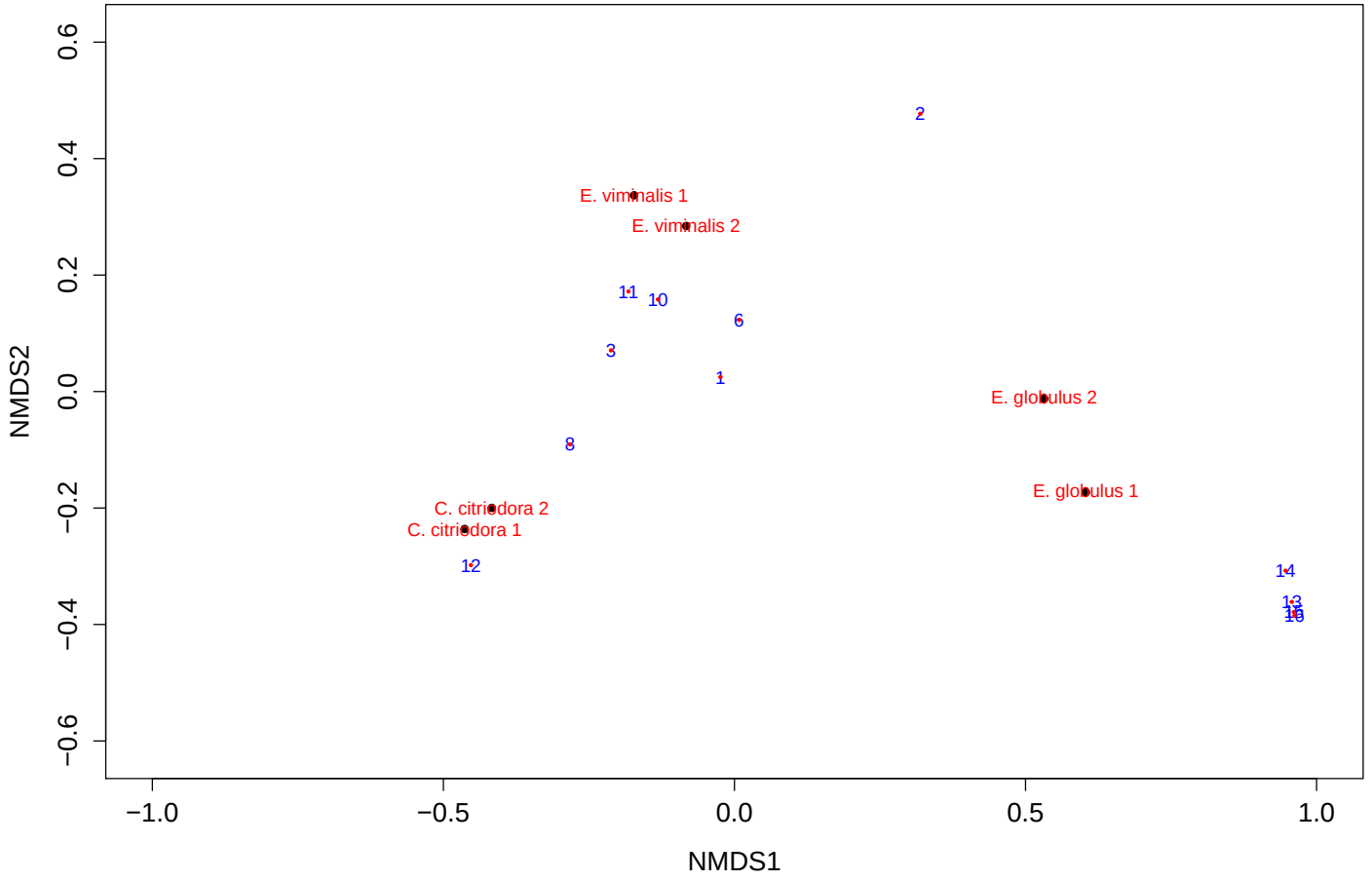

FIGURE 2.5: Non metric multidimensional scaling (NMDS) plot based on the mass spectral integration data for the 16 compounds confirmed with reference standards for each species. The plot shows the Bray-Curtis distance rotated so that the variance is maximized on the first dimension. Numbers indicate compound identity as in Tables 2, 3 and 4. Stress ≈ 0.

TABLE 2.1: Differences between EAG response magnitude to the different leaf treatments. Freshly damaged refers to leaves that were mechanically scraped before recordings were made

Level	n	Mean (mV)	Std Dev (mV)	Letters of significance*
freshly damaged *E. globulus*	16	0.768	0.201	A
freshly damaged *E. tereticornis*	15	0.760	0.186	A
freshly damaged *E. viminalis*	13	0.745	0.232	AB
freshly damaged *E. robusta*	15	0.715	0.110	AB
freshly damaged *E. smithii*	15	0.715	0.165	AB
freshly damaged *E. camaldulensis*	15	0.666	0.148	ABC
freshly damaged *E. scoparia*	14	0.655	0.247	ABC
freshly damaged *E. punctata*	15	0.634	0.208	ABCD
freshly damaged *E. saligna*	15	0.593	0.134	ABCD
freshly damaged *E. grandis*	15	0.558	0.137	BCD
E. viminalis	15	0.514	0.165	CDE
E. tereticornis	15	0.483	0.126	CDEF
E. smithii	15	0.457	0.133	DEF
freshly damaged *C. citriodora*	15	0.453	0.143	DEF
E. globulus	14	0.360	0.088	EFG
E. robusta	15	0.331	0.079	EFG
E. camaldulensis	15	0.327	0.076	FG
blank	72	0.297	0.132	G
E. grandis	15	0.256	0.064	G
E. saligna	15	0.230	0.042	G
E. scoparia	15	0.199	0.088	G
E. punctata	15	0.178	0.035	G
C. citriodora	15	0.174	0.062	G

*Levels with the same letter are not significantly different, Tukey HOD $p < 0.05$

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TABLE 2.2: The standard compounds, purities and Kovats retention indexes as calculated for the different instruments and injection methods that were used.

Standard No	Name	Cass no	% Purity	GC-EAD* KI liquid	GC-EAD* KI thermal	GC-MS* KI thermal
1	(*E*)-2-hexenal	6728-26-3	98	910	910	912
2	(*Z*)-3-hexen-1-ol	928-96-1	99	910	910	912
3	α-pinene	80-56-8	98	959	958	959
4	camphene	79-92-5	99.3	982	980	981
5	β-pinene	127-91-3	99.5	1012	1011	1011
6	(*Z*)-3-hexenyl acetate	1708-82-3	99	1038	1038	1038
7	3-carene	13466-78-9	96.5	1038	1038	1038
8	α-terpinene	99-86-5	94	1046	1046	1047
9	m-cymene	535-77-3	99	1058	1058	1058
10	limonene	5989-27-5	99.3	1058	1058	1058
11	eucalyptol	470-82-6	-	1074	1073	1074
12	γ-terpinene	99-85-4	97	1085	1085	1085
13	2-phenylethanol	60-12-8	-	1209	1209	1226
14	benzyl acetate	140-11-4	99.5	1229	1228	1231
15	ethyl phenylacetate	101-97-3	99	1311	1311	1314
16	terpenyl acetate	80-26-2	-	1411	1411	1413

*Gas Chromatography (GC), Electroantennography detector (EAD), Mass spectrometry (MS)

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TABLE 2.3: Identities of the compounds associated with EAD active peaks and the retention indices for these peaks for comparison between the three tree species analysed on the GC-EAD instrument.

			E. globulus		*E. viminalis*		*C. citriodora*	
Peak[a]	Standard No	Compounds	Rt (min)	KI	Rt (min)	KI	Rt (min)	KI
a		C6 alcohol	26.66	842.3	26.67	842.3	26.62	841.3
b	1	(*E*)-2-hexenal	30.01	912.7	29.87	909.4	29.87	909.3
	2	(*Z*)-3-hexen-1-ol	present	-	-	-	-	-
	3	α-pinene	32	958.2	31.99	957.8	31.99	957.9
	4	camphene	-	-	-	-	-	-
c	5	β-pinene	34.24	1010	-	-	-	-
d	6	(*Z*)-3-hexenyl acetate	35.55	1043	35.44	1040	35.31	1037
	7	3-carene	-	-	-	-	-	-
	8	*alpha*-terpinene	present	-	35.7	1047	35.65	1045
e	10	limonene	36.32	1062	36.22	1060	36.13	1057
		cymene	present	-	36.35	1063	36.25	1061
f	11	eucalyptol	36.85	1076	36.81	1075	36.75	1073
g	12	γ-terpinene	37.25	1086	37.24	1085	37.25	1086
h	13	2-phenylethanol	41.81	1209	41.83	1209	-	-
i	14	benzyl acetate	42.45	1227	-	-	-	-
j	15	ethyl phenylacetate	45.27	1310	-	-	-	-
	16	terpenyl acetate	48.6	1415	-	-	-	-

[a]Peak letters displayed in table refer to the investigated peaks that were selected from the initial antennal responses as in Figure 2.1 to 2.3

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TABLE 2.4: Identities and relative abundances of the compounds associated with EAD active peaks and the Kovats retention indices for these peaks for comparison of the three tree species analysed on the GC-MS instrument.

			E. globulus (n=2)			*E. viminalis* (n=2)			*C. citriodora*(n=2)		
Peak[a]	Standard No	Compounds[b]	Rt (min)	KI	Area % ± std	Rt (min)	KI	Area % ± std	Rt (min)	KI	Area % ± std
a		C6 alcohol	27.63	844.3		27.63	844.2		27.62	844	
b	1	(*E*)-2-hexenal m/z 100*, 55(100), 56 (25), 57 (20) 67(21), 69 (57), 70 (25), 80 (25), 83 (67)	30.82	912.5	3.02 ± 3.37	30.83	912.7	1.13 ± 0.23	30.82	912.4	0.67 ± 0.37
	2	(*Z*)-3-hexen-1-ol m/z 100*, 55(100), 56 (25), 57 (20) **67** (21), 69 (57), 70 (25), 80 (25), 83 (67)	present	-	1.73 ± 0.74	-	-	1.26 ± 0.67	-	-	0 ± 0
	3	α-pinene m/z 136*, 77 (38), 79 (28), 91 (100), 92 (50), 93 (64), 105 (13)	32.83	959.3	1.85 ± 1.37	32.83	959.1	3.50 ± 0.07	32.82	958.9	1.87 ± 0.24
	4	camphene m/z 136*, 67 (28), 77 (31), 79 (52), 91 (70), 93 (100), 107 (23)	-	-	0 ± 0	-	-	0 ± 0	-	-	0 ± 0
c	5	*β*-pinene m/z 136*, 77 (51), 79 (44), 80 (24), 91 (100), 93 (84), 107 (14), 107 (14), 121 (19)	-	-	0 ± 0	-	-	0 ± 0	-	-	0 ± 0
d	6	(*Z*)-3-hexenyl acetate m/z 142*, 65 (10), **67** (100), 82 (11)	36.15	1040	21.78 ± 7.80	36.12	1039	19.77 ± 6.05	36.11	1039	4.55 ± 2.13
	7	3-carene m/z 136*, 65 (20), 67 (96), 77 (48), 79(47), 91 (100), 92 (40), **93** (72), 105 (18), 121 (23)	-	-	0 ± 0	-	-	0 ± 0	-	-	0 ± 0
	8	α-terpinene m/z 136* (70), 77 (43), 79 (39), 91 (100), 93 (92), 105 (32), 107 (12), 121 (71)	36.44	1047	1.34 ± 0.06	36.43	1047	2.67 ± 0.83	36.41	1047	2.97 ± 0.18
e	10	limonene m/z 136*, **67** (100), 79 (60), 91 (73), 92 (36), 93 (67), 94 (58), 107 (28), 119 (29), 121 (20)	36.93	1060	7.15 ± 0.53	36.89	1059	13.81 ± 0.25	36.87	1058	4.18 ± 1.86
		cymene m/z 134* (32) , 67 (31), 79 (25), 91 (64), 115 (15), 117 (34), **119** (100)	-	-	0 ± 0	-	-	0 ± 0	-	-	0 ± 0
f	11	eucalyptol m/z 154*, 67 (35), 69 (31), 79 (24), 81 (67), 93 (100), 107 (27), 108 (35), 111 (30), 139 (70)	37.53	1075	14.2 ± 3.47	37.51	1075	38.32 ± 8.09	37.5	1074	11.89 ± 2.29
g	12	γ-terpinene m/z 136* (27), 77 (39), 79 (27), 80 (12), 91 (100), 92 (32), 93 (65), 105 (15), 121 (25)	37.95	1086	6.98 ± 3.23	37.94	1086	19.53 ± 0.62	37.94	1086	73.86 ± 7.07
h	13	2-phenylethanol m/z **122*** (11), 65 (23), 91 (100), 92 (67)	43.05	1228	0.11 ± 0.01	-	-	0 ± 0	-	-	0 ± 0
i	14	benzyl acetate m/z **150*** (14), 77 (12), 79 (40), 89 (21), 90 (14), 91 (23), 108 (100)	43.17	1231	0.04 ± 0.02	-	-	0 ± 0	-	-	0 ± 0
j	15	ethyl phenylacetate m/z 164* (28), 65 (16), 91 (100), 92 (15), 105 (10), 136 (14)	45.92	1314	1.33 ± 0.08	-	-	0 ± 0	-	-	0 ± 0
	16	terpenyl acetatem/z 196*, 67 (18), 79 (28), 91 (46), 92 (29), 93 (99), 105 (17), 107 (24), 108 (22), 121 (100), 136 (40)	49.04	1415	40.48 ± 4.91	-	-	0 ± 0	-	-	0 ± 0

[a]Peak letters displayed in table refer to the investigated peaks that were selected from the initial antennal responses as in Figure 2.1 to 2.3. [b]Mass fragments are indicated with relative intensities for unknown peaks and standards if not detected in the samples. *The molecular ion is given first followed by fragments in ascending mass order. Ions in bold were used to extract peaks and for integration purposes if peaks were not resolved in the total ion chromatogram.

Chapter 3

Identification of the sex pheromone of the tree infesting cossid moth *Coryphodema tristis* (Lepidoptera: Cossidae)

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A version of this chapter was submitted to PLOS ONE and two patents were registered from work done in this chapter.

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3.1 Abstract

The cossid moth (*Coryphodema tristis*) has a broad range of native tree hosts in South Africa. The moth recently moved into non-native *Eucalyptus* plantations in South Africa, on which it now causes significant damage. Here we investigate the chemicals involved in pheromone communication between the sexes of this moth in order to better understand its ecology, and with a view to potentially develop management tools for it. In particular, we characterise female gland extracts and headspace samples through coupled gas chromatography electroantennographic detection (GC-EAD) and two dimensional gas chromatography mass spectrometry (GCxGC-MS). Tentative identities of the potential pheromone compounds were confirmed by comparing both retention time and mass spectra with authentic standards. Two electrophysiologically active pheromone compounds, tetradecyl acetate (14:OAc) and *Z*9-tetradecenyl acetate (*Z*9-14:OAc) were identified from pheromone gland extracts, and an additional compound (*Z*9-14:OH) from headspace samples. We further determined dose response curves for the identified compounds and six other structurally similar compounds that are common to the order Cossidae. Male antennae showed superior sensitivity toward *Z*9-14:OAc, *Z*7-tetradecenyl acetate (*Z*7-14:OAc), *E*9-tetradecenyl acetate (*E*9-14:OAc), *Z*9-tetradecenol (*Z*9-14:OH) and *Z*9-tetradecenal (*Z*9-14:Ald) when compared to female antennae. While we could show electrophysiological responses to single pheromone compounds, behavioural attraction of males was dependent on the synergistic effect of at least two of these compounds. Signal specificity is shown to be gained through pheromone blends. A field trial showed that a significant number of males were caught only in traps baited with a combination of *Z*9-14:OAc (around 95% of the ratio) and *Z*9-14:OH. Addition of 14:OAc to this mixture also improved the number of males caught, although not significantly. This study represents a major step towards developing a useful attractant to be used in management tools for *C. tristis* and contributes to the understanding of chemical communication and biology of Cossidae.

3.2 Introduction

The quince borer *Coryphodema tristis*, Drury, 1782 (Lepidoptera: Cossidae) is native to South Africa. The larvae of this moth has long been known to be a pest of grape vine, apple, quince and sugar pear trees, especially in the Cape Town region (Petty, 1917; Meyer, 1965). Recently, *C. tristis* was reported from *Eucalyptus nitens* plantations near the Lothair/Carolina area in the Mpumalanga province (Gebeyehu *et al.*, 2005). Here the larvae cause considerable damage to the trees through extensive tunnelling in the

wood. *Coryphodema tristis* is, therefore, regarded as a serious emerging threat to the forestry industry in South Africa for which a control programme needs to be developed.

The distribution and population levels of *C. tristis* are difficult to monitor. The greater part of the life cycle, up to eighteen months, is spent as larvae inside the trunks of infested trees (Gebeyehu *et al.*, 2005). Population levels and the extent of the damage can be confirmed only by felling infested trees, which is obviously not ideal for large-scale assessments. Adult moths are active for a short period in early spring. This narrow period of emergence provides an opportunity to monitor the extent of the infestation indirectly, if a trapping tool were available for the adults.

Sex pheromones are widely used for trapping insects (Hegazi *et al.*, 2009). Lures impregnated with pheromones are typically much more efficient and specific than traps using physical attractants (e.g. light) or with lures imitating chemical signals from the host (kairomones). Pheromones can also be used to disrupt mating by flooding the environment with pheromone to confuse the lured sex (Carde and Minks, 1995). No published studies are available on the pheromone communication of *C. tristis*.

Pheromone attraction between males and females in Lepidoptera is common and well known (Jurenka, 2003). Female moths store pheromone molecules or precursors within pheromone glands, that may be dorsally located in the abdomen (for example, the Tiger moth, *Holomelina lamae*, Freeman, 1941), with pores opening in glandular cells between the eighth and ninth abdominal segments (Ma and Ramaswamy, 2003). Glandular tubercle structures have been found in other cossid species and possibly function to produce close range pheromones or defense chemicals (Davis, 2006). Moth pheromones in general are released at specific times to attract a mate. This is occasionally associated with calling behaviour such as wing fanning while the ovipositor is exposed to the atmosphere.

Pheromones have been identified for cossid species including the Carpenter moth, *Cossus insularis* (Chen *et al.*, 2006), the European goat moth, *Cossus cossus* (Capizzi *et al.*, 1983) and the Sandthorn carpenter worm, *Holcocerus hippophaecolus* (Fang *et al.*, 2005). The identified pheromone components are typically C12-C18 chain length acetates, aldehydes or alcohols with one or two unsaturated positions along the chain. Single compounds either do not attract males (Fang *et al.*, 2005) or rarely attract as many males in the field as mixtures (Capizzi *et al.*, 1983). This suggests that moths classified in this order generally rely on specific pheromone blends for attraction.

Coryphodema tristis males are smaller than females and have plumose antennae, while the females have more simple antennae (see Appendix D). This dimorphism in antenna structure suggests that females attract males using pheromones as is typical in the

Lepidoptera, including Cossidae. The aim of this study was thus to identify possible pheromone compounds used in sexual communication in *C. tristis*. To achieve this goal, we analyzed female gland extracts and headspace samples through both GC-EAD, GC-MS and GC x GC-MS methods. Electrophysiologically active peaks were identified by both retention times and mass spectra. Relative ratios of the active compounds were calculated and dose-response curves set up through GC-EAD for both male and female insects. A field trial was then used to test the biological activity of these compounds.

3.3 Materials and Methods

3.3.1 Sampling

3.3.1.1 Insects

Infested *Eucalyptus nitens* logs were collected from a plantation near Lothair, South Africa (GPS S 26 18.633, E 30 37.389). The logs were transported to the University of Pretoria and maintained in cages (115 X 65 X 58 cm) in an insectary. This facility had a controlled temperature (20-25 °C) and photoperiod (12 hours photo-/scotophase), which was maintained while awaiting emergence of the moths. Male and female moths were separated after emergence.

3.3.1.2 Gland extraction

A total of 62 glands were removed from virgin female moths when calling behaviour was observed. Typically, female moths exposed their ovipositors in a series of short intervals while fanning their wings. The glands were removed with clean forceps while gently squeezing the abdomens of the female moths. Twelve glands were extracted in n-hexane and acetone respectively and 38 glands were extracted in dichloromethane. Solvent volumes of 100 μl were used in each case and each gland was extracted for approximately 1 minute to avoid additional contamination that can occur.

3.3.1.3 Headspace samples

Headspace samples were collected from individual females overnight. Each female (n = 6) was placed on approximately 0.5 g of silane treated glass wool (Supelco, South Africa) inside a custom made glass sampling chamber that was wrapped with foil to minimize any light disturbances at night. Air was filtered through activated carbon before entering

the chamber (13.5 by 5.5 by 5.5 cm, 150 ml internal volume) and passed through the sampling chamber at a flow rate of 47 ± 2.4 ml/min (std). A sampling pump (SKC, South Africa) was used to draw air through the sampling apparatus and through a glass Gerstel thermal desorption sampling trap (17.8 cm x 6 mm x 4 mm ID) containing 60 mm bed length Tenax TA (35 m^2/g). Teflon tubing was used for all connections. Samples were taken for a period of 826 ± 38 min (Std) each night. The glass wool inside the sampling chamber was extracted with analytical grade dichloromethane the following morning (see Appendix E).

3.3.2 Electroantennal responses

3.3.2.1 Gas chromatography electroantennographic detection

GC-EAD recordings were made with an EAD system (Syntech, Hilversum, The Netherlands) coupled to an Agilent 6890N gas chromatography system (Chemetrix, Midrand, South Africa). The antennae from virgin male moths (between 2 and 3 days old) were removed with a surgical blade and the antennal tips were removed in the same manner. This procedure allowed for a superior connection to the tissues inside the antennae. The antennae were coupled to two Ag/AgCl capillary glass electrodes filled with an electrolyte solution made by dissolving 3 ml of Spectra®electrolyte gel into 50 ml of distilled water.

The antennal preparation was placed as near as possible (≈ 2 mm) to the outlet of the stimulus delivery tube of the EAD system. Purified and humidified air was allowed to pass through the stimulus delivery tube at a flow rate of 180 ml/min and 2 μl of the gland extract was injected at a split ratio of 5:1 (300 °C) onto a 30 m HP 5 analytical column (J & W scientific, 0.32 mm ID, 0.25 μm). Half of the sample was directed to the antennal preparation through a Y-quartz (Agilent, PN:5181-3398) splitter at the end of the analytical column and the other half to the FID (300 °C). Direct current recordings were made with a ten times external amplification in all cases and baseline drift was removed by plotting the derivative of the EAD data as described in Slone and Sullivan (2007).

The GC was operated in constant pressure mode at 16 psi (He) (see Appendix A). Two GC oven methods were used. The first oven method (40 °C for one minute and ramped to 300 °C at 10 °C per minute) was used to scan through samples to determine the retention time of electrophysiologically active peaks. The main electrophysiologically active peak eluted at 17.08 minutes when using this method. After the initial EAD screening, the run time was shortened to allow the peak of interest to elute at 9.11 min.

The shortened oven method was as follows: 120 °C for 1 min and ramped to 220 °C at 10 °C per minute.

3.3.2.2 Dose response

GC-EAD dose response curves were determined by using the GC (120 °C, 1 min to 240 °C at 20 °C/min) to deliver the individual pheromone components at increments of 0.181, 1.81, 18.1, 181 ppm. Considering the split ratio of 10:1 in the inlet and the 50% split at the end of the column then 9.09, 90.9, 909, 9090 pg were delivered to the EAD preparation. Live males and females (n = 5) were first wrapped in cotton wool and then in dental wax (Utility wax strips white, Wright Millners) with the head and antennae protruding at the one end. Each insect was coupled to the EAD detector by connecting the reference electrode at the base of the antenna and the recording electrode at the tip of the right antenna. This preparation minimized movement of the moths and the decline in antennal sensitivity observed for removed antennae. GC runs were conducted on a 30 m, 0.32 mm ID, 0.25 μm film thickness, HP 5 (J & W Scientific, Agilent) capillary column at 16 psi. The EAD software (GcEad32 V4.3, Syntech, Hilversum, The Netherlands) was used to measure both the FID and EAD response size in terms of the peak height (μV) for the recorded direct current data. The GC was used as a delivery device during these dose response experiments, given the advantages when compared to the normal EAG technique
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R version 3.1.0 software package was used to plot dose response curves and to calculate the associated statistical parameters by means of ANCOVA. Dose response parameters were treated as continuous variables and it was assumed that the EAD data should have a log linear relationship with concentration levels (Struble and Arn, 1984). Flame ionization detector data was log(μV+1) transformed to preserve homogeneity among variances of residuals.

3.3.3 Characterization of chemicals

3.3.3.1 GC-MS

Twenty-seven of the gland extracts that were made in dichloromethane were analyzed on a GC-MS instrument and the remaining samples were analyzed on a GC X GC-MS instrument. The gland extracts were analyzed on a 30 m, 0.32 mm ID, 0.25 μm film thickness, HP 5 (J & W Scientific, Agilent) capillary column with a GC-MS system (Agilent 6890A-5975C MSD). The inlet (250 °C) was operated in splitless mode (7 psi,

He, constant pressure) and 1 μl of the sample was injected (see Appendix A). The oven was operated as follows: 40 °C for three minutes to 300 °C for three minutes at a rate of 20 °C/min. Kovats retention indexes were calculated and matched between the GC-EAD instrument and the GC-MS instrument.

3.3.3.2 GC X GC-MS

Samples were injected (250 °C) at a 5:1 split ratio onto a GC X GC-TOFMS (Pegasus, Leco) system with a 30 m, 0.25 μm ID, ZB5 analytical column for the first dimension and a RXI 17 (1 m, 0.1 μm film, 0.1 ID) column for the second dimension (primary oven 120 °C to 225 °C at 10 °C/min, secondary oven offset at 10 °C). Helium was used as a carrier gas at 16 psi (constant pressure) for analysis carried out on the ZB 5 capillary column. This analysis was repeated with a polar ionic liquid column (SLB-IL-100, 30 m, 0.25 mm, 0.2 μm) to facilitate the separation of the expected Z and E form of the unsaturated component in the gland extract mixture. Here, a 1.59 m ZB 5 column (0.1 μm ID, 0.1 μm Film) was used as the second column. A constant flow rate (1 ml/min) was used during experiments with the ionic liquid column.

An additional GC x GC-TOFMS (Pegasus, Leco) analysis was done at a split ratio of 10:1 (250 °C) with a SLB-IL-111 column (30 m, 0.250 mm, 0.2 μm) coupled to a ZB 5 (1.190 m, 0.100 mm, 0.1 μm) as second column. The instrument was operated in constant flow (1 ml/min, He) mode with the primary oven 50-230 °C at 10 °C/min and the secondary oven 25 °C (10 °C/min) hotter than primary oven. This analysis was done after the previous analysis for extra confirmation with reference standards. The relative ratios of the compounds in the extracts were calculated by using the two dimensional peak integration results. Standard compounds were obtained in pure form from Insect Science^{TM}(South Africa, Tzaneen) and the identities of the peaks were confirmed based on retention times on the ZB5, SLB-IL-100, SLB-IL-111 and HP5 columns.

3.3.3.3 Double bond determination

Dimethyl disulfide (DMDS) adducts of the pheromone extracts (one sample from each solvent used) and standards (1000 ppm in n-hexane) were made according to Buser *et al.* (1983), except that the reaction was carried out at 60 °C for 48 hours, not at 40 °C overnight. The double bond location of the unsaturated pheromone component in the gland extracts was confirmed by the characteristic ions and retention times when compared to the standard compounds analyzed on the GC X GC-MS system with the IL 100 and DB 5 column combination.

3.3.4 Field trial

The attractiveness of the identified pheromone compounds were assessed in a field trial (for 2011 results see Appendix F). The trial was conducted at two sites near the area where original moths had been collected. The trial was undertaken between 27 September and 16 November 2013. Nine treatments were arranged in two stratified random block designs. A pheromone permeation device was constructed with a glass capillary tube (10 mm by 1.5 outer diameter by 0.8 mm Id, ± 5 μl internal volume) with one 30 mm long methyl silicone rubber tube (2.16 outer diameter by 1.02 mm ID, functioned as a permeation membrane) attached to both ends creating a loop to form a seal. Pure pheromone compounds and mixtures were dispensed within each permeation device with three pheromone compounds (*Z*9-14:OAc : *Z*9-14:OH : 14:OAc) in the following volumetric ratios: treatment one 1 : 0 : 0; treatment two 0.94 : 0 : 0.06; treatment three 0.99 : 0 : 0.01; treatment four 0 : 1 : 0; treatment five 0.94 : 0.06 : 0; treatment six 0.06 : 0.94 : 0; treatment seven 0.95 : 0.025 : 0.025. Dispensers without pheromone compounds were blank treatments (treatment eight) and newly emerged female insects were used as a positive control (treatment nine). All treatments were replaced twice during the course of the trial (approximately once every 2 weeks).These permeation devices were hung from a wire inside the dispenser area of yellow bucket funnel traps (Insect Science^{TM}, South Africa, Tzaneen). Traps were hung at a height of 4 m from standing *E. nitens* trees and they were arranged in a grid pattern with approximately 10 m between traps within the two plantations. Differences between treatments were determined with Steel-Dwass method for non-parametric multiple comparisons.

3.4 Results

3.4.1 Electroantennal responses

3.4.1.1 Gas chromatography electroantennographic detection

GC-EAD investigation of the gland extracts revealed a large (1884.44 ± 434.68 μV, N = 9, ± SE) EAD response of the male antenna to the only peak above the FID detection limit. This provided an estimate of the retention time that could be associated with physiologically relevant peaks in the chromatogram. The response was in most cases well above the noise level of the EAD detector and occurred at 9.11 minutes after optimization of the chromatographic parameters (Figure 3.1). The retention index of this peak was calculated as 1797 and matched with the retention index of the standards for Z and *E*9-14:OAc on this system.

Two electroantennographic responses were observed for the glass wool extracts from the female headspace (Figure 3.2). The larger response of the two occurred at 5.61 minutes. The retention index of this peak was calculated as 1671.7 on this system. Literature comparison of this retention index suggested that the compound was either *E*11-14:OH or *Z*10-14:OH
citepmarques1 and it was later confirmed to be *Z*9-14:OH. The smaller response occurred at 6.38 minutes (KI = 1799.0) and coincided with the elution time of *Z*9-14:OAc on the GC-EAD system. No chromatographic peak could be observed at this response time on both the GC-EAD and GC-MS systems.

3.4.1.2 Dose response

Males and females were treated with similar stimuli for each compound to allow for a direct comparison of the differential sensitivity in the antennae of males and females (Figures 3.3, 3.4, 3.5 and Supplementary Table 3.6). There were five zero values at lower concentrations for *Z*9-14:Ald, *Z*9-14:OH and *Z*11-14:OH. These zero values caused the p value of the interaction term (dose:sex) to become significant only for *Z*11-14:OH ($F = 6.487$, $p = 0.015$). The EAD results confirmed that there was a difference between the sensitivity of male and female antennae (Table 3.6). In general males showed larger responses to *Z*9-14:OAc, *Z*7-14:OAc, *E*9-14:OAc, *Z*9-14:OH and *Z*9-14:Ald when compared to females. Females were more sensitive only for 14-Ac. Male and female antennae did not show a differential sensitivity for *Z*11-14:OH. Antennal responses were found to be log linear especially for the male antennae and for those compounds that showed larger responses (for example *Z*9-14:OAc and *Z*7-14:OAc).

3.4.2 Characterization of chemicals

3.4.2.1 GC-MS

A large peak was found at elution time of 11.802 minutes during the GC-MS investigation with a smaller peak eluting at 11.857 minutes (Supplementary Table 3.4). The calculated retention indexes compared well with the literature values for both *Z*9-14:OAc and/or *E*9-14:OAc and 14:OAc for the smaller peak (Table 3.1). The library search indicated that the first peak was either Z and/or *E*9-14:OAc and it was possible that they may have co-eluted on the DB 5 column (Marques *et al.*, 2000). The second peak was tentatively identified as 14:OAc and this compound and *Z*9-14:OAc was later confirmed with the reference standards. *Z*9-14:OAc was the dominant component in the gland extracts of

females and was present in a relative ratio of 94.4 ± 3.8 % : 5.6 ± 3.7 % (mean ± SD, N = 27) when compared to 14:OAc.

Mass spectral comparison of the peak found in the headspace with the NIST library indicated that the compound was either *Z*9-14:OH or *Z* or *E*11-14:OH. This peak had the same retention index of 1667 (Table 3.2) as was reported for *Z*9-14:OH (Marques *et al.*, 2000). Using a reference standard, this peak was later confirmed to be *Z*9-14:OH.

3.4.2.2 GC X GC-MS

Separation of the compounds present in the gland extracts was achieved with the polar column (SLB-IL-111) used in the GC x GC-MS instrument. The mass spectrum and retention times of the unknown peaks were compared with those obtained for the standard compounds and the library hit spectrum (Table 3.2). The characteristic ions (m/e = 348 (M^+), 231, 117) of the DMDS adducts revealed the double bond location between the ninth and tenth carbon atoms of the unsaturated C14 acetate (Figure 3.6). The geometry around the double bond was confirmed to be *Z* and not *E* as compared to retention time differences between derivatised individual standards and samples.

The ratio of the compounds in the gland extracts was found to be slightly different when samples were analyzed on the two-dimensional instrument. This column combination could separate *E*9-14:OAc and *Z*9-14:OAc and the results showed that *E*9-14:OAc could not be detected in any of these extracts (Supplementary Table 3.5). The fact that *E*9-14:OAc was not detected in these extracts, suggests that it is not part of the pheromone blend of *C. tristis*. These analyses revealed a ratio of 1.13 ± 1.31 : 98.87 ± 1.31 (14:OAc : *Z*9-14:OAc, mean ± SD, N = 33). Two of the dichloromethane extracts did not contain the compounds above the detection limit and were thus not included in the calculation of the ratio.

3.4.3 Field trial

Results of the field trial showed that the attractiveness of some of the artificially baited traps was enhanced, if compared to blank traps. A total of 122 male moths were caught in the field trial and 102 of these were caught using treatment 5 and 7, which contained *Z*9-14:OAc, *Z*9-14:OH (94: 6 %) and *Z*9-14:OAc, *Z*9-14:OH, 14:OAc (95: 2.5: 2.5 %) respectively. A greater number of males were caught when all three components; *Z*9-14:OAc, *Z*9-14:OH, 14:OAc, identified from the gland and headspace samples were used in the pheromone lures (treatment no 7), although this did not differ significantly (p = 0.6667, Steel-Dwass) from treatment 5 (Table 3.3). No moths were caught in blank

treatments and only two of 30 female *C. tristis* moths that were used as positive control lured a small number (8) of male moths.

3.5 Discussion

Analysis of pheromone gland extracts from the cossid moth, *C. tristis* in this study revealed that *Z*9-14:OAc and 14:OAc were possible pheromone candidates. Headspace samples contained the additional compound *Z*9-14:OH. All three of these compounds were detected by male antennae. Pheromone lures containing at least *Z*9-14:OAc and *Z*9-14:OH in a specific ratio significantly increased the number of males caught in the field trials, confirming the biological activity of these two compounds. The addition of 14:OAc to lures also increased the number of moths trapped, although this difference was not statistically significantly different from lures containing *Z*9-14:OAc and *Z*9-14:OH. These results represent a major step towards developing an environmentally friendly monitoring and management tool for *C. tristis* in South Africa.

Behavioural data were difficult to collect for *C. tristis* under laboratory conditions (see Appendix B). Adults of this species are available only for a very short period (approximately 1.5 months) each year. Females reared from field-collected infested logs were often observed to have a few males in close proximity (often less than 10 cm) inside cages, but despite intensive monitoring, mating was never observed. Delaying the emergence period through lowering the ambient temperature of logs was attempted, but this often resulted in fungal growth over the logs and only a few stunted moths that emerged. These observations suggest that the change in environmental conditions, such as lack of some environmental cues or other changes brought about when moths are reared in captivity, may have a direct influence on the mating behaviour of *C. tristis*.

The dose response experiments indicated that the double bond position, its geometry and the functional group play an important role in the recognition system of the male *C. tristis*. In general, the male antennal response size became larger when the double bond position was moved to the seventh and ninth carbon positions. Their antennae were also more sensitive to the acetate functionality and the Z geometry around the double bond (see for example the responses to *Z*9-14:OAc compared to *E*9-14:OAc and *Z*9-14:OH). These results confirmed that the pheromone receptors present on the male antennae are selectively sensitive to *Z*9-14:OAc, *Z*7-14:OAc, *E*9-14:OAc and *Z*9-14:OH when compared to females. Similar electrophysiological patterns are known for other moths residing in this family of the Lepidoptera. For example, European goat moth (*Cossus cossus*) males show greater antennal responses towards C12 acetates with the *Z*-geometry at the double bond at the fifth carbon as compared C12 alcohols (Capizzi

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et al., 1983); and males of the Sandthorn Carpenterworm (*Holcocerus hippophaecolus*) also show larger antennal responses to *Z*7-14:OAc when compared to the corresponding alcohol (Fang *et al.*, 2005). The compounds that elicit larger antennal responses were also the main pheromone components for these species.

We expected the antennal response to have a log linear relationship to stimulus concentration at levels below saturation (Mayer *et al.*, 1984; Struble and Arn, 1984). This was indeed the case for male responses to *Z*9-14:OAc and *Z*7-14:OAc and both male and female responses to *Z*11-14:OAc. This result suggests that the tested pheromone concentration was below the saturation level for at least these compounds.

Pheromone concentration plays an important role in the selectivity process of the antennae. For example, Mayer (Mayer, 1993) could show that three different pheromone molecules (for *Trichoplusia ni*) stimulate three different receptor neurons at physiologically relevant concentration levels, but all three neurons were sensitive to all three compounds at concentrations above physiological levels. Our results indicate that female antennae saturate rapidly when compared to males especially for *Z*9-14:OAc, *E*9-14:OAc and *Z*7-14:OAc. Males should, therefore, have greater numbers of receptors for these compounds on their antennae when compared to females, which possibly relates to the larger surface area of their antennae (see Appendix D).

Female moth antennae are in most cases expected to be less sensitive to their own pheromone molecules (for example the silk worm moth *Bombix mori*, (Schneider, 1957) and the turnip moth, *Agrotis segetum*, (Hansson *et al.*, 1989), but exceptions of pheromone auto-detection in females do occur (Schneider *et al.*, 1998). Our dose response experiments showed that males are more sensitive than females to most of the tested pheromone compounds other than 14:OAc. The results show that there is indeed an auto-detection that occurs for *C. tristis* females, but this was detected only at the higher concentrations tested. The 14:OAc exception (where females showed larger responses than males) can be attributed to the large antennal response that males had to *Z*9-14:OAc, in comparison to females that showed very small responses to this compound. It is known from EAG experiments that the antennae of some moths, for example *Trichoplusia ni*, (Payne *et al.*, 1970) need between 30 and 60 seconds to recover to the original response magnitude. We thus hypothesize that *C. tristis* male antennae were still recovering to baseline potential during the 14:OAc stimulation period because the *Z*9-14:OAc peak eluted 8.3 seconds before 14:OAc in our chromatograms. This short time interval between stimuli probably did not give the male antenna sufficient time to recover before the elution of 14:OAc. It is therefore regarded as an artifact of the methodology.

Pheromone molecules similar to those that were identified here have been found to be components of pheromones in other moth species residing in the sub-family Cossinae.

These include the European goat moth, *Cossus cossus* (Capizzi *et al.*, 1983) and the sandthorn carpenterworm, *Holcocerus hippophaecolus* (Fang *et al.*, 2003, 2005). The similarity of pheromone compounds utilized in Cossinae, including *C.tristis* indicate that these molecules are relatively common and that specificity lies in unique combinations. For example, Fang et al., (Fang *et al.*, 2005) found that the individual compounds *Z*7-14:OAc and *E*3-14:OAc failed to attract males in the field. However, mixing them in a 1:1 ratio restored their attraction. The results of the present study show that *C. tristis* males also rely on such a synergistic ratio for attraction to occur. Attraction is enhanced when both the acetate (*Z*9-14:OAc) and alcohol (*Z*9-14:OH) are present within the artificial lures. The function of 14:OAc in the pheromone blend is unknown at this stage, but traps containing a small fraction of this compound caught a greater number of male moths.

Pheromone compounds in moth species seem to be derived from a common biological pathway that utilizes long chain fatty acid precursors, which are chain-shortened, desaturated, reduced and acetylated by various combinations of enzymes. Variations in these combinations give the final pheromone blend that a specific species utilizes (Tillman *et al.*, 1999; Jurenka, 2003; Ishikawa *et al.*, 1999; Takanashi *et al.*, 2000). Small variations in these biological pathways seem to be present for closely related species. For example, species of *Ostrinia* produce pheromone blends that are very similar, especially if they are found in the same habitat (Ishikawa *et al.*, 1999; Takanashi *et al.*, 2000). Specificity is in this case is gained by the addition of certain unique minor components. Another example is the subtle differences in blends that occur between two strains of the European corn borer *O. nubilalis* that differ only in the ratio of *E* to *Z*11-14:OAc (Ishikawa *et al.*, 1999). The similarity of the compounds present in these biosynthetic pathways to those found in the present study makes it probable that *C. tristis* females use similar pathways to produce their species-specific pheromone blend.

This study revealed the identity of three electrophysiologically and behaviourally active compounds in the cossid moth *C. tristis*, two of which were found in the female gland extracts, *Z*9-14:OAc and 14:OAc, and another in the headspace, *Z*9-14:OH. It is possible that some components, *Z*9-14:OAc and 14:OAc, of the pheromone are stored within the gland and another, *Z*9-14:OH, is produced at the time of calling. Blends as opposed to single compounds were more effective for luring male moths into traps in the field. Future work should focus on enhancing trap efficiency by optimizing the lure release rates to match that of a calling female moth (see Appendix C). It is also possible that additional undiscovered minor components could be present in the pheromone plumes of calling females. These compounds were below detection capabilities of the present study, but they may yet be discovered.

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3.6 Acknowledgements

We thank Dr. Brett Hurley and Mr. Hardus Hatting for their help in collecting field samples and Yvette Naudé, Department of Chemistry, University of Pretoria for assistance in analyzing samples. Dr Chris Moore, Department of Agriculture, Fisheries and Forestry, Queensland, Australia for preliminary GC-MS analyses. Members of the Tree Protection Co-operative Programme (TPCP), the THRIP initiative of the Department of Trade and Industry and the DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB) are acknowledged for financial support. This work was registered in RSA provisional patents No. F2014/00749, F2014/00750 and F2014/03257.

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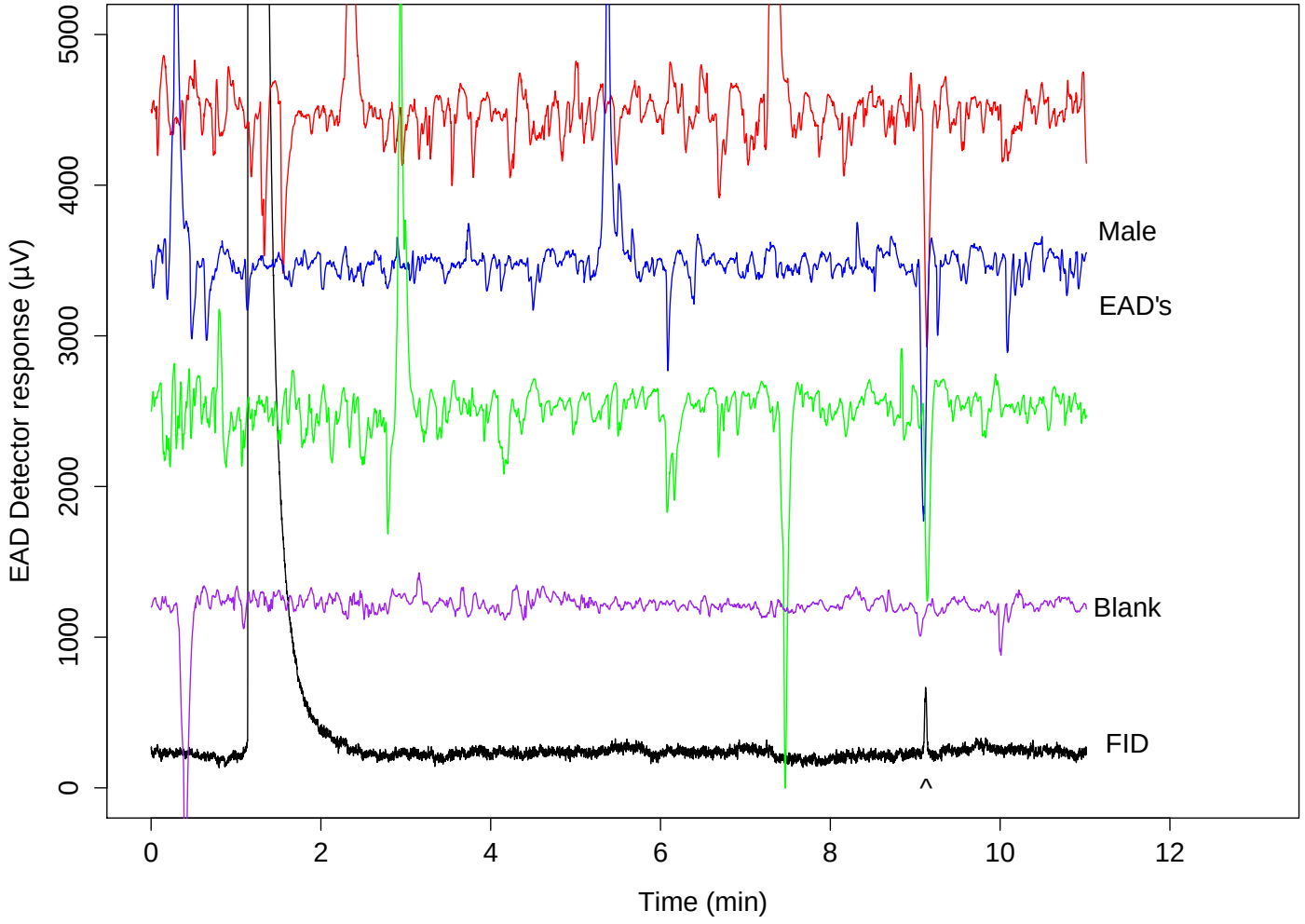

FIGURE 3.1: GC-EAD responses of *Coryphodema tristis* male antennae to gland extracts. The arrow indicates the peak of interest in the FID signal. Bottom is the response to the blank. (EAD response at 9.11 min: 1884 μV $\pm$ 435, mean $\pm$ SE, N = 9)

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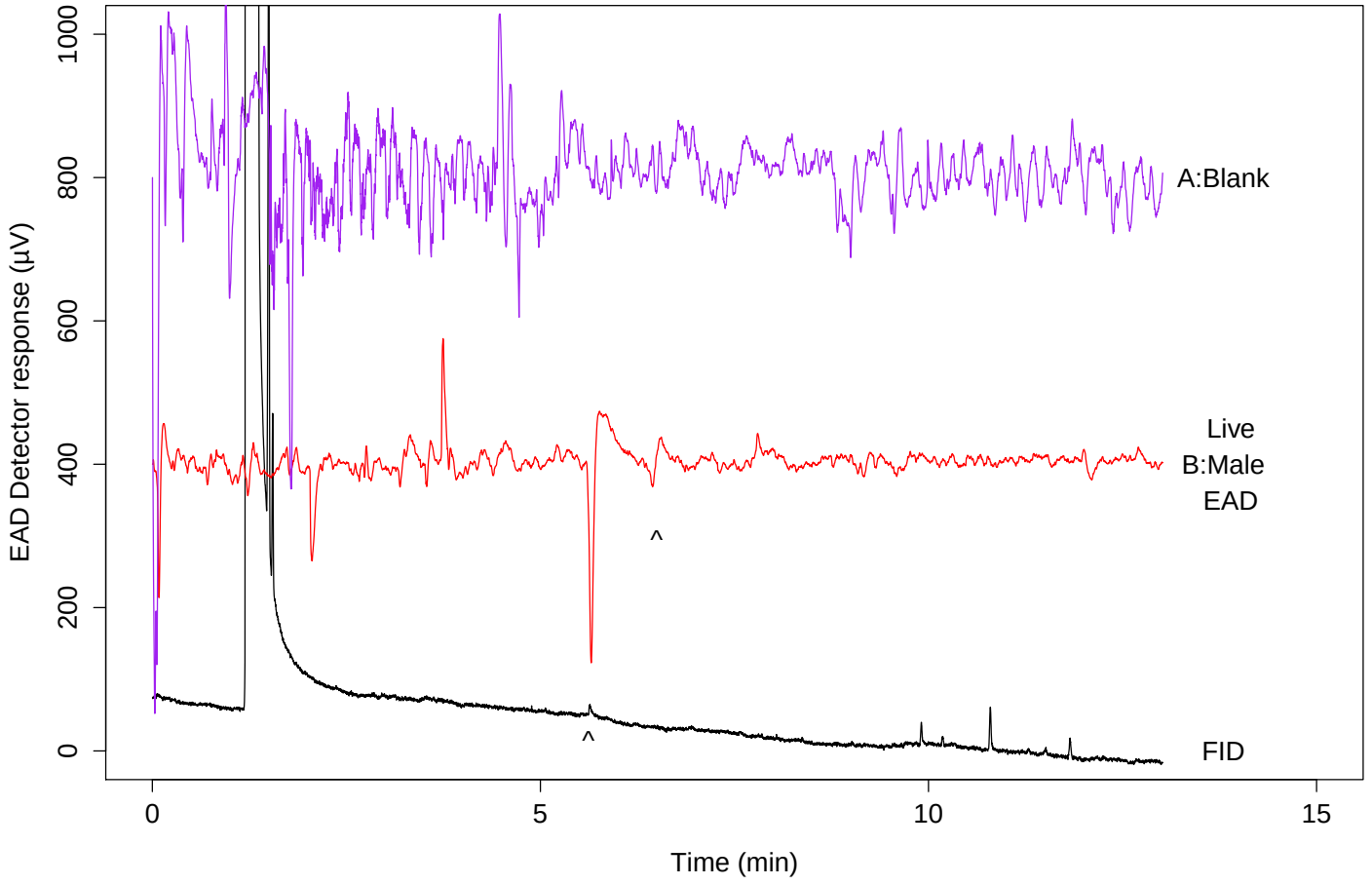

FIGURE 3.2: GC-EAD response of *Coryphodema tristis* male antennae to one of the glass wool extracts of the female headspace. The arrow indicates the peak of interest in the FID signal and the presence of a smaller second response. A: The response to the blank. B: the averaged response of four different sample recordings. (EAD response at 5.61 min: 590 μV $\pm$ 50.33, mean $\pm$ SE, N = 4)

TABLE 3.1: Standards and Kovats retention indexes as compared to literature values (HP5, 0.32 mm, 0.25 μm)

Compound	KI GC-MS	KI GC-EAD	KI Lit
*Z*9-14:Ald	1604.7	1605.6	1603
*Z*9-14:OH*	1666.1	1665.4	1667
*Z*11-14:OH*	1678.4	1676.1	1678
*Z*5-14:OAc	1789.9	1788.5	1790
*Z*7-14:OAc	1792.0	1790.5	1792
*Z*9-14:OAc	1799.8	1798.7	1801
*E*9-14:OAc	1800.4	1798.5	1801
14:OAc	1809.5	1808.7	1811
*Z*11-14:OAc	1811.7	1809.7	1812

* peak start time used to calculate KI

GC-MS, oven 40, 3 min 300 @ 20 °C, 3 min, 7 psi, He, 48.9 cm/sec, Butane 80 °C

GC-EAD, oven 120, 1 min 300 @ 20 °C, 3min, 16 psi, He, 47.1 cm/sec, Butane 80 °C

KI Lit (Marques *et al.*, 2000), oven 100, 1 min 275 @ 5C,

3 min, 18.6 psi, He, 29.4 cm/sec, DB5, 0.25 mm, 0.25m

TABLE 3.2: Retention time and Kovats retention index of active compound found in *C. tristis* headspace samples

	Headspace		Glass wool	
Sample	RT (min)	KI	Rt (min)	KI
1	12.652	1667.0	11.162	1667.2
2	12.630	1666.1	11.162	1667.2
3	12.603	1667.2	11.170	1667.3
4	12.614	1669.1	*	*

*Not detected

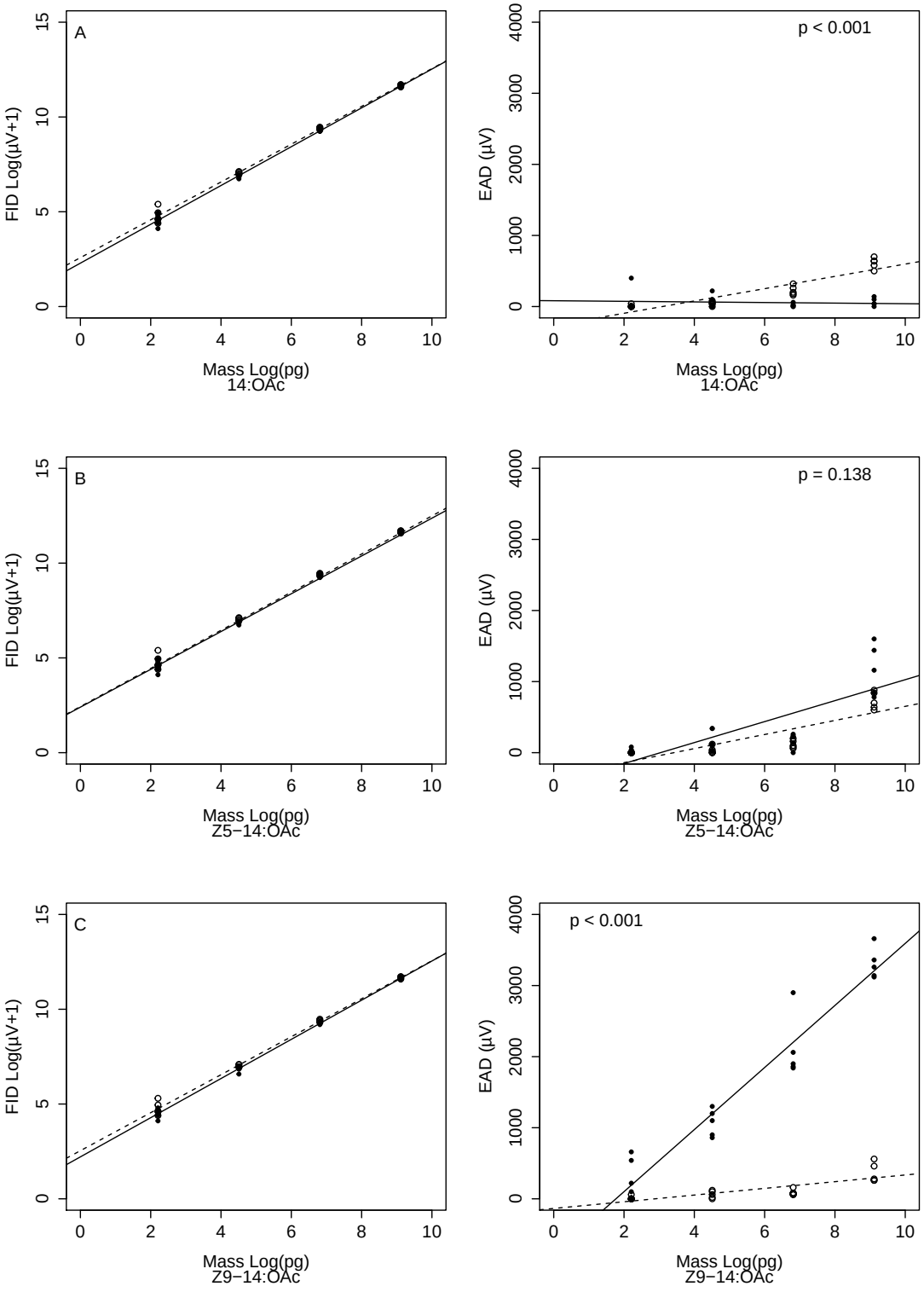

FIGURE 3.3: Fitted dose response curves of the FID compared to the EAG response of live *Coryphodema tristis* for A: tetradecyl acetate, B: *Z*5-tetradecenyl acetate and C: *Z*9-tetradecenyl acetate. (Dashed lines = Female, Solid lines = Male, N = 5)

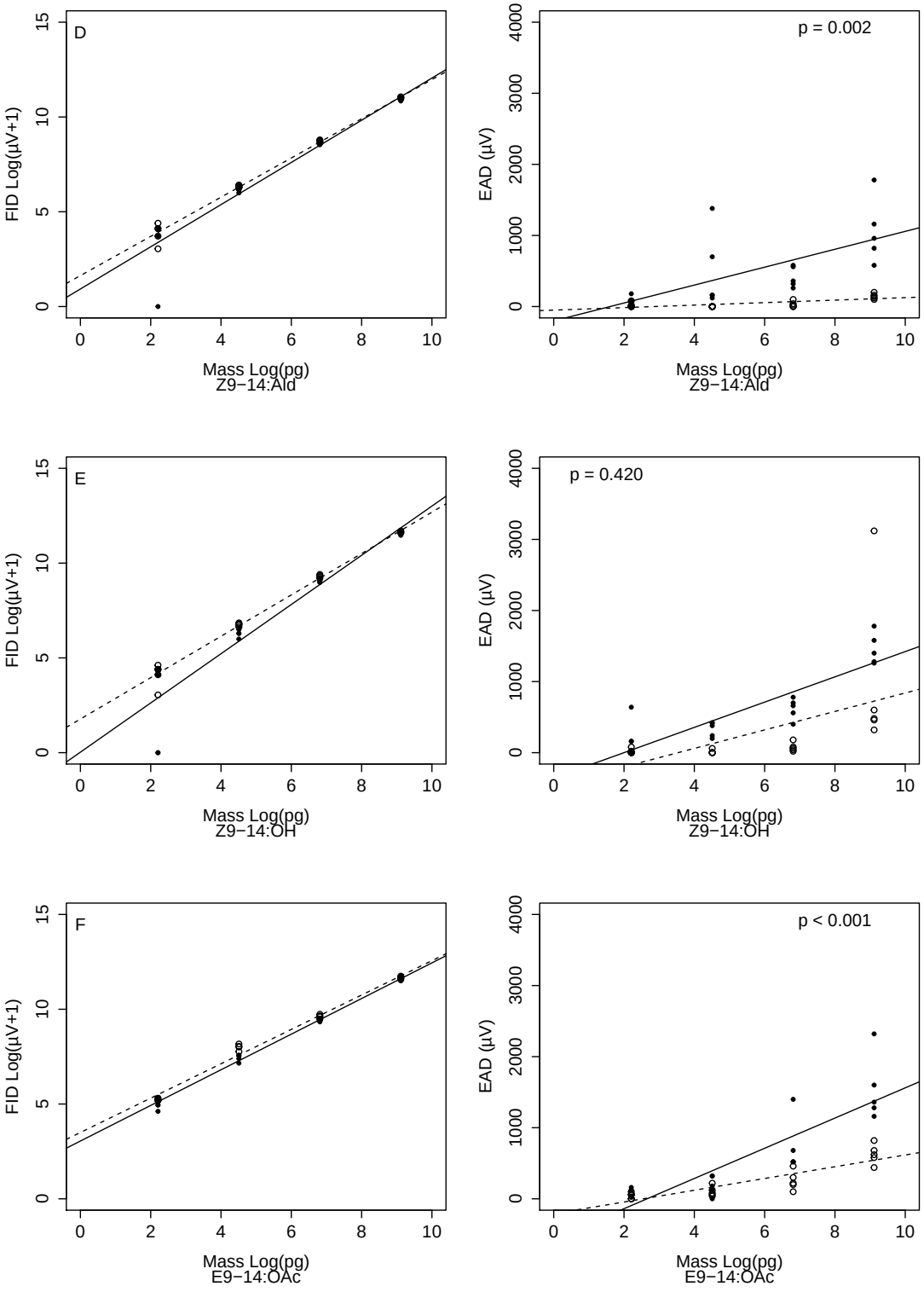

FIGURE 3.4: Fitted dose response curves of the FID compared to the EAG response of live *Coryphodema tristis* for D: *Z*9-tetradecenal, E: *Z*9-tetradecenol and F: *E*9-tetradecenyl acetate. (Dashed lines = Female, Solid lines = Male, N = 5)

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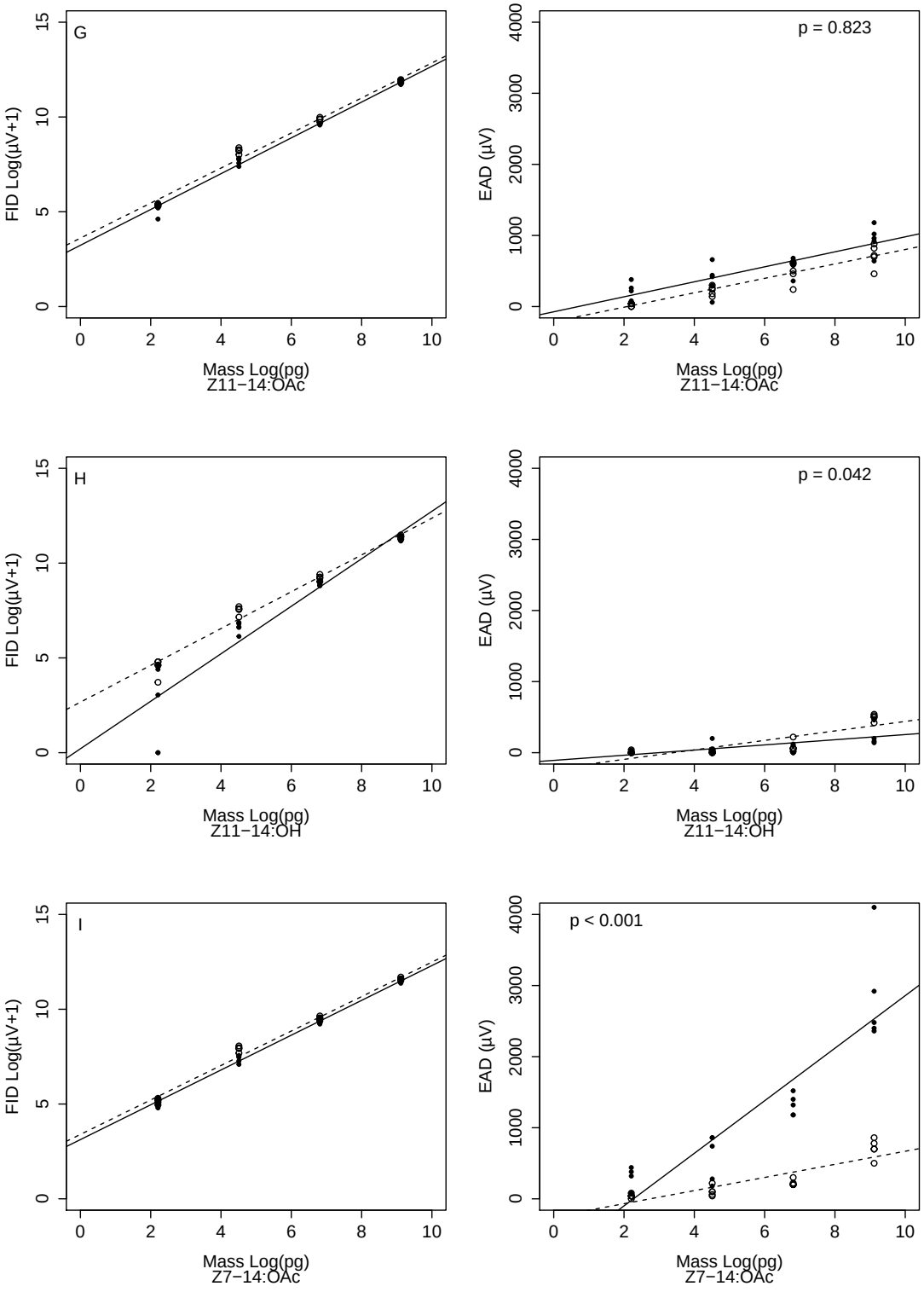

FIGURE 3.5: Fitted dose response curves of the FID compared to the EAG response of live *Coryphodema tristis* for G: *Z*11-tetradecenyl acetate, H: *Z*11-tetradecenol and I: *Z*7-tetradecenyl acetate. (Dashed lines = Female, Solid lines = Male, N = 5)

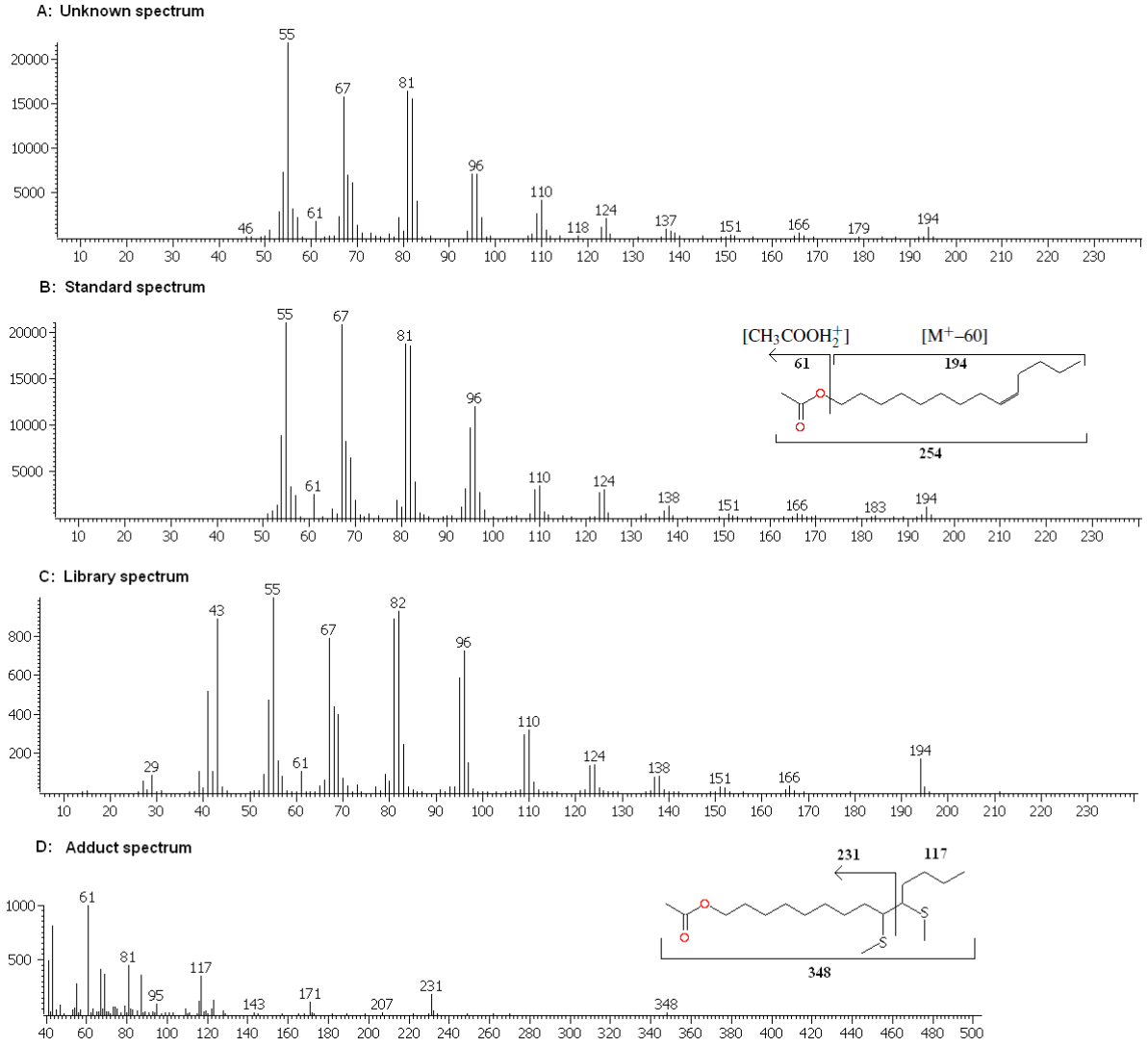

FIGURE 3.6: A: The mass spectrum found for the unknown component in one of the hexane gland extracts. B: The mass spectrum of the standard compound *Z*9-14:OAc. C: The library comparison to the gland sample. D: The mass spectrum of the DMDS adduct from a sample in n-hexane.

TABLE 3.3: Field trial treatment ratios, by volume, and number of males caught per treatment

n	Treatment	*Z*9-14Ac	*Z*9-14Ol	14Ac	# Males	Mean	StDev	Letters*
10	1	1	0	0	2	0.2	0.6	AB
10	2	0.94	0	0.06	6	0.6	0.7	AB
10	3	0.99	0	0.01	3	0.3	0.5	AB
10	4	0	1	0	0	0	0	A
10	5	0.94	0.06	0	39	3.9	4.6	BC
10	6	0.06	0.94	0	1	0.1	0.3	A
10	7	0.95	0.025	0.025	63	6.3	3.4	BC
10	8 (Blank)	0	0	0	0	0	0	A
10	9 (Female)	0	0	0	8	0.8	2.2	AB

*Rows with the same letters are not statistically significantly different Steel-Dwass, $p < 0.05$

TABLE 3.4: GC-MS runs HP5 column (0.32 mm, 0.25 μm)

	RT (min)		KI		Relative area %	
Gland extract #	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2
1 CH2Cl2	11.800	11.855	1800.7	1810.8	95.83	4.17
2 CH2Cl2	11.801	11.858	1800.9	1811.4	95.26	4.74
3 CH2Cl2	11.803	*	1801.3	*	100.00	0.00
4 CH2Cl2	11.801	11.857	1800.9	1811.2	95.87	4.17
5 CH2Cl2	11.801	11.857	1800.9	1811.2	94.99	5.01
6 CH2Cl2	11.801	11.859	1800.9	1811.6	94.89	5.11
7 CH2Cl2	11.800	11.858	1800.7	1811.4	93.24	6.76
8 CH2Cl2	11.801	11.856	1800.9	1811.0	95.82	4.17
9 CH2Cl2	11.801	11.854	1800.9	1810.7	95.85	4.15
10 CH2Cl2	11.800	11.856	1800.7	1811.0	91.69	8.31
11 CH2Cl2	11.803	11.858	1801.3	1811.4	88.17	11.83
12 CH2Cl2	11.801	*	1800.9	*	100.00	4.17
13 CH2Cl2	11.801	11.856	1800.9	1811.0	95.64	4.36
14 CH2Cl2	11.801	11.859	1800.9	1811.6	90.07	9.93
15 CH2Cl2	11.807	*	1802.4	*	86.33	13.67
16 CH2Cl2	11.802	*	1801.5	*	100.00	0.00
17 CH2Cl2	11.801	11.855	1801.3	1811.2	88.32	11.68
18 CH2Cl2	11.801	11.856	1801.3	1811.4	94.25	4.17
19 CH2Cl2	11.802	11.858	1801.5	1811.8	95.37	4.63
20 CH2Cl2	11.801	11.857	1801.3	1811.6	96.21	3.79
21 CH2Cl2	11.803	11.860	1801.7	1812.1	92.04	7.96
22 CH2Cl2	11.800	11.858	1801.1	1811.8	94.47	4.17
23 CH2Cl2	11.802	11.859	1801.5	1811.9	89.07	10.93
24 CH2Cl2	11.802	*	1801.5	*	100.00	0.00
25 CH2Cl2	11.801	11.858	1801.3	1811.8	91.46	8.54
26 CH2Cl2	11.805	*	1802.0	*	100.00	0.00
27 CH2Cl2	11.801	11.858	1801.3	1811.8	94.58	5.42
Mean	11.802	11.857	1801.2	1811.4	94.42	5.62
STD	0.002	0.002	0.4	0.4	3.84	3.71

*not detected

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TABLE 3.5: Chromatographic peak data of pheromone gland extract samples analyzed on the GC*GC-MS

	Peak properties								
	14-Ac		(E)-9-14Ac		(Z)-9-14Ac		Relative % abundance		
Sample	RT (min; min)	Area counts	RT (min; min)	Area counts	RT (min; min)	Area counts	14-Ac	(E)-9-14Ac	(Z)-9-14Ac
Standard	13.583; 0.041	171549396	14.000; 0.037	147041084	14.250; 0.035	88428705	~	~	~
Hexane 1	13.583; 0.041	350624	*; *	*	14.250; 0.035	28094959	1.23	0.00	98.77
Hexane 2	13.500; 0.042	882207	*; *	*	14.167; 0.036	38712355	2.23	0.00	97.77
Hexane 3	13.583; 0.041	1427817	*; *	*	14.250; 0.035	34970830	3.92	0.00	96.08
Hexane 4	13.500; 0.041	1167997	*; *	*	14.167; 0.036	60985357	1.88	0.00	98.12
Hexane 5	13.667; 0.040	328929	*; *	*	14.250; 0.035	47731186	0.68	0.00	99.32
Hexane 6	13.583; 0.041	849920	*; *	*	14.250; 0.035	47382408	1.76	0.00	98.24
Hexane 7	*; *	0	*; *	*	14.167; 0.035	7141605	0.00	0.00	100.00
Hexane 8	13.583; 0.041	443637	*; *	*	14.167; 0.036	15707471	2.75	0.00	97.25
Hexane 9	13.583; 0.041	3066217	*; *	*	14.250; 0.035	90953684	3.26	0.00	96.74
Hexane 10	13.583; 0.041	444551	*; *	*	14.250; 0.035	10859798	3.93	0.00	96.07
Hexane 11	13.500; 0.041	127346	*; *	*	14.167; 0.036	12881962	0.98	0.00	99.02
Hexane 12	13.500; 0.041	2336049	*; *	*	14.167; 0.036	62774371	3.59	0.00	96.41
CH2Cl2 1	*; *	0	*; *	*	14.250; 0.035	5307144	0.00	0.00	100.00
CH2Cl2 2	13.583; 0.041	326683	*; *	*	14.250; 0.035	16885920	1.90	0.00	98.10
CH2Cl2 3	*; *	0	*; *	*	14.250; 0.035	1009261	0.00	0.00	100.00
CH2Cl2 4	*; *	0	*; *	*	14.250; 0.035	1976077	0.00	0.00	100.00
CH2Cl2 5	*; *	0	*; *	*	14.250; 0.034	497466	0.00	0.00	100.00
CH2Cl2 6	13.667; 0.039	696656	*; *	*	14.250; 0.034	20533924	3.28	0.00	96.72
CH2Cl2 7	*; *	0	*; *	*	*; *	*	0.00	0.00	0.00
CH2Cl2 8	*; *	0	*; *	*	14.250; 0.034	1512664	0.00	0.00	100.00
CH2Cl2 9	13.667; 0.039	999618	*; *	*	14.250; 0.034	67194242	1.47	0.00	98.53
CH2Cl2 10	*; *	0	*; *	*	*; *	*	0.00	0.00	0.00
CH2Cl2 11	*; *	0	*; *	*	14.250; 0.034	462868	0.00	0.00	100.00
Acetone 1	13.667; 0.039	221875	*; *	*	14.250; 0.034	15773472	1.39	0.00	98.61
Acetone 2	*; *	0	*; *	*	14.250; 0.034	14819562	0.00	0.00	100.00
Acetone 3	13.667; 0.039	149329	*; *	*	14.250; 0.034	15827857	0.93	0.00	99.07
Acetone 4	13.667; 0.039	146493	*; *	*	14.250; 0.034	21128628	0.69	0.00	99.31
Acetone 5	*; *	0	*; *	*	14.250; 0.034	2446136	0.00	0.00	100.00
Acetone 6	*; *	0	*; *	*	14.250; 0.033	4691304	0.00	0.00	100.00
Acetone 7	*; *	0	*; *	*	14.250; 0.034	1038531	0.00	0.00	100.00
Acetone 8	*; *	0	*; *	*	14.250; 0.034	4195781	0.00	0.00	100.00
Acetone 9	*; *	0	*; *	*	14.250; 0.034	11413688	0.00	0.00	100.00
Acetone 10	13.583; 0.039	310316	*; *	*	14.250; 0.034	29085257	1.06	0.00	98.94
Acetone 11	*; *	0	*; *	*	14.250; 0.034	8952914	0.00	0.00	100.00
Acetone 12	13.583; 0.039	46352	*; *	*	14.250; 0.034	10527993	0.44	0.00	99.56
Average		434019				21620505	1.13	0.00	98.87
STD		706456				22885255	1.31		1.31

*not detected
Primary column: SLB-IL-111 column (30 m, 0.250 mm, 0.2 μm)
Secondary column: ZB 5 (1.190 m, 0.100 mm, 0.1 μm)

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TABLE 3.6: Calibration curve parameters for FID for *C. tristis* males and females

			Intercept (Log(μV+1)				Slope (Log(μV+1)/Log(pg))				Fit parameters	
Compound	Sex	R^2	Estimate	Std. Error	t value	P value	Estimate	Std. Error	t value	P value	F ratio	P value
14:OAc	F	0.9947	2.58	0.10	24.92	<0.001	0.99	0.017	59.92	<0.001	3591	<0.001
*E*9-14:OAc	F	0.9864	3.50	0.15	23.07	<0.001	0.91	0.024	37.17	<0.001	1382	<0.001
*Z*11-14:OAc	F	0.9849	3.61	0.16	22.09	<0.001	0.92	0.026	35.20	<0.001	1239	<0.001
*Z*11-14:OH	F	0.9771	2.66	0.21	12.59	<0.001	0.97	0.034	28.51	<0.001	812.9	<0.001
*Z*5-14:OAc	F	0.9994	2.43	0.03	72.03	<0.001	1.01	0.005	185.08	<0.001	34250	<0.001
*Z*7-14:OAc	F	0.9858	3.40	0.16	21.83	<0.001	0.91	0.025	36.33	<0.001	1320	<0.001
*Z*9-14:OAc	F	0.9954	2.54	0.10	26.27	<0.001	1.00	0.016	64.46	<0.001	4155	<0.001
*Z*9-14:Ald	F	0.9914	1.63	0.14	11.89	<0.001	1.03	0.022	46.84	<0.001	2194	<0.001
*Z*9-14:OH	F	0.9885	1.77	0.17	10.59	<0.001	1.09	0.027	40.51	<0.001	1641	<0.001
14:OAc	M	0.9961	2.29	0.09	25.13	<0.001	1.02	0.015	69.85	<0.001	4879	<0.001
*E*9-14:OAc	M	0.9938	3.05	0.11	28.67	<0.001	0.94	0.017	55.00	<0.001	3025	<0.001
*Z*11-14:OAc	M	0.9921	3.23	0.12	26.82	<0.001	0.94	0.019	48.73	<0.001	2375	<0.001
*Z*11-14:OH	M	0.8807	0.21	0.66	0.315	0.756	1.25	0.105	11.89	<0.001	141.3	<0.001
*Z*5-14:OAc	M	0.9928	2.40	0.12	19.89	<0.001	1.00	0.019	51.34	<0.001	2636	<0.001
*Z*7-14:OAc	M	0.9947	3.12	0.10	32.81	<0.001	0.92	0.015	59.97	<0.001	3596	<0.001
*Z*9-14:OAc	M	0.9969	2.21	0.08	26.79	<0.001	1.03	0.013	77.75	<0.001	6045	<0.001
*Z*9-14:Ald	M	0.9193	0.93	0.47	1.974	0.064	1.11	0.075	14.75	<0.001	217.6	<0.001
*Z*9-14:OH	M	0.898	0.03	0.62	0.045	0.965	1.30	0.100	12.98	<0.001	168.4	<0.001

*Fit calculations were based on five recordings for each compound at each concentration level

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TABLE 3.7: Linear fit to EAD dose response curves for *C. tristis* males and females

			Intercept (μV)				Slope (μV/log(pg))				Fit parameters	
Compound	Sex	R^2	Estimate	Std. Error	t value	P value	Estimate	Std. Error	t value	P value	F ratio	P value
14:OAc	F	0.8224	-267.75	57.04	-4.694	<0.001	86.51	9.17	9.432	<0.001	88.97	<0.001
*E*9-14:OAc	F	0.7354	-210.12	70.11	-2.997	0.008	82.69	11.27	7.335	<0.001	53.80	<0.001
*Z*11-14:OAc	F	0.8550	-212.33	59.35	-3.578	0.002	101.45	9.54	10.630	<0.001	113.00	<0.001
*Z*11-14:OH	F	0.6755	-227.64	65.14	-3.495	0.003	66.71	10.47	6.368	<0.001	40.56	<0.001
*Z*5-14:OAc	F	0.6836	-338.56	94.96	-3.565	0.0021	99.02	15.27	6.485	<0.001	42.05	<0.001
*Z*7-14:OAc	F	0.7521	-253.20	74.91	-3.380	0.003	92.24	12.04	7.658	<0.001	58.65	<0.001
*Z*9-14:OAc	F	0.5998	-135.51	53.93	-2.513	0.022	47.08	8.67	5.429	<0.001	29.47	<0.001
*Z*9-14:Ald	F	0.4803	-50.33	25.33	-1.987	0.06264	17.55	4.07	4.308	<0.001	18.56	<0.001
*Z*9-14:OH	F	0.2021	-459.60	335.20	-1.371	0.187	129.90	53.90	2.411	0.027	5.81	0.027
14:OAc	M	-0.0122	81.60	54.97	1.484	0.155	-4.17	8.84	-0.472	0.643	0.22	0.6428
*E*9-14:OAc	M	0.7187	-563.74	187.70	-3.003	0.008	212.46	30.18	7.039	<0.001	49.55	<0.001
*Z*11-14:OAc	M	0.7194	-73.95	93.01	-0.795	0.437	105.45	14.96	7.051	<0.001	49.71	<0.001
*Z*11-14:OH	M	0.3980	-108.54	61.31	-1.770	0.094	36.31	9.86	3.683	0.002	13.56	0.002
*Z*5-14:OAc	M	0.5844	-447.94	174.21	-2.571	0.019	147.49	28.01	5.265	<0.001	27.72	<0.001
*Z*7-14:OAc	M	0.7847	-838.80	274.30	-3.058	0.007	369.70	44.10	8.382	<0.001	70.25	<0.001
*Z*9-14:OAc	M	0.9335	-772.37	165.91	-4.655	<0.001	436.55	26.68	16.364	<0.001	267.80	<0.001
*Z*9-14:Ald	M	0.4193	-201.99	204.17	-0.989	0.336	125.95	32.83	3.836	0.001	14.72	0.001
*Z*9-14:OH	M	0.7570	-355.05	142.45	-2.492	0.023	177.71	22.91	7.758	<0.001	60.19	<0.001

*Fit calculations were based on five recordings for each compound at each concentration level

Chapter 4

A potential semiochemical signal from *Amylostereum* species for *Ibalia leucospoides*

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4.1 Abstract

The parasitic wasp, *Ibalia leucospoides*, is globally considered to be an important biological control agent for the invasive pine infesting woodwasp, *Sirex noctilio*. The parasitoid females need to locate *S. noctilio* oviposition sites in pine trees for their own oviposition. It is known that they accomplish this partly through detecting the presence of the symbiotic fungus, *Amylostereum areolatum*, which *S. noctilio* inoculates into trees during oviposition. It is also known that some Siricids, notably *Xeris* spp., detect *Amylostereum* volatiles. It is, however, not known whether *S. noctilio* responds to *Amylostereum* volatiles. In this study, we isolated volatiles from *A. areolatum* and *A. chailletii* cultures in order to investigate the electrophysiological response patterns of both *S. noctilio* and *I. leucospoides* in a gas chromatography coupled to electroantennography (GC-EAD) experiment. A prominent chromatographic peak, which was not detected in blank samples, elicited repeatable responses only from *I. leucospoides* females. GC-MS analyses suggested that the unknown compound is a sesquiterpene, 1,3,4,5,6,7-hexahydro-2,5,5-trimethyl-2H-2,4a-Ethanonaphthalene. The tentative identity could not be confirmed using a reported synthesis from a related sesquiterpene. This unknown sesquiterpene is thought to indicate the presence of the *Amylostereum* fungi for *I. leucospoides* females and may have a behavioural effect on the wasps. A second peak was also shown to elicit a response from *I. leucospoides*, but its concentration was below the detection limit. This research provides a foundation for the identification of the behaviourally active volatiles produced by *Amylostereum* spp. and that could be used in future for monitoring and management purposes.

4.2 Introduction

The *Sirex noctilio* woodwasp was accidentally introduced into South Africa and first reported in 1994 (Tribe *et al.*, 1995). This insect has become a significant pest in pine plantations because it can, together with its phytotoxic mucus and fungal symbiont, kill trees on a large scale (Slippers and Wingfield, 2011). The female woodwasp deposits a symbiotic fungus, *Amylostereum areolatum*, and mucus into the tree during oviposition. The development of the larvae is dependent on the predigestion of carbohydrates from the wood by the fungus, and is therefore obligately linked to it (Madden, 1988).

A large number of the pine plantations in South Africa consist of monocultures planted in dense stands for pulp production. Species that are commonly used include *Pinus patula*, *P. taeda*, *P. elliotii* and *P. radiata* (DAFF, 2009), most of which are considered susceptible to *S. noctilio* infestation (Hurley *et al.*, 2007). One of the biological control

agents that has been introduced into South Africa in an attempt to control the pest is the egg parasitoid wasp, *Ibalia leucospoides*. The parasitoid females locate oviposition channels made by the *Sirex* females and lay their eggs into and/or close to the eggs of *S. noctilio* (Tribe and Cillié, 2004). The *I. leucospoides* larvae then feed on and kill the eggs or early instar larvae of *S. noctilio*.

The mechanism that the *I. leucospoides* female wasps use to find the oviposition holes made by *S. noctilio* is not well understood. It has been shown that females preferentially tap with their antennae and probe with their ovipositor near *Amylostereum* fungal cultures on agar disks (Spradbery, 1974). In more recent Y-tube bioassay experiments, it was shown that *I. leucospoides* females are attracted to fungus alone and fungus grown in pine logs (Martinez *et al.*, 2006). It is also known that and unfed *I. leucospoides* females prefer fungus over a control when given a choice (Pietrantuono *et al.*, 2012). These studies suggest that there is a specific semiochemical signal that originates from fungal cultures that affects the oviposition behaviour of *I. leucospoides* females. A previous study by Bryant (2010) tentatively identified a number of sequiterpenes and aromatic volatiles produced by *A. areolatum*. These volatiles were classified as potential semiochemicals for both *S. noctilio* and *I leucospoides* but they failed to attract *S. noctilio* and *I leucospoides* in traps in field conditions (Cucura, 2013).

The aim of this study was to identify possible semiochemical signals that may play a role in the host finding behaviour of *I. leucospoides* and *S. noctilio* females (see section 1.6.1). This was achieved by comparing headspace profiles of samples obtained from Petri dishes with no fungal growth with those containing growth from *Amylostereum* sp. Electrophysiological responses of both males and females of *S. noctilio* and *I. leucospoides* to the fungal volatiles were investigated. An attempt was then also made to identify the peaks to which the wasps respond using a reported synthesis (Kitchens *et al.*, 1972).

4.3 Methods and Materials

4.3.1 Insects

Ibalia leucospoides and *Sirex noctilio* insects emerged from *P. patula* billets that were collected at Rooihoogte (GPS: S25.64232, E 30.34592). Insects were sexed before GC-EAD recordings. *Ibalia leucospoides* females have a sharper angle at the rear dorsal tip of the abdomen whereas males have a rounded abdomen. *Sirex noctilio* males are easily recognized by an orange-yellow band on the abdomen, which is absent in female wasps.

4.3.2 Amylostereum cultures

Amylostereum areolatum (CMW 41237, isolated 26-04-2012) and *Amylostereum chailletii* (CMW 41613, isolated 8-06-2011) fungi were cultured in 90 mm Petri dishes on three different media types. PDA (39 g/l), 39 g PDA plus 10 g dried *P patulla* sawdust and 20 g agar plus 10 g dried *P patulla* sawdust. Cultures were maintained for 14 days at 23 ± 1 °C before sampling of volatiles commenced.

4.3.3 Sampling

Two sampling methods were used to collect volatiles from the cultures. The first method was a passive sampling where a sterilized activated charcoal tube (ORBO^{TM}-32, 100/50 mg) was added to each Petri dish for a period of 24 hours. Passive sampling was conducted on all media types, using both *A. areolatum* and *A. chailletii*, as well as Petri dishes containing uninoculated agar as controls. A dynamic headspace sampling was also performed where filtered air was drawn through a custom-made glass apparatus that was designed to house a small 40 mm diameter Petri dish. Dynamic headspace sampling was used to collect volatiles from *A. areolatum* grown on PDA agar only, as well as from the control PDA Petri dishes. The charcoal inside the tubes was extracted twice with 1ml n-hexane (Analytical grade, Sigma Aldrich). Samples were concentrated under gentle stream of nitrogen gas until the meniscus of the solvent touched the bottom of the 2 ml amber, wide opening screw top, glass vial (Agilent Technologies). These samples were stored at −5 °C until GC-EAD or GC-MS analysis.

4.3.4 GC-EAD

Recordings were made with an EAD system produced by Syntech (Hilversum, The Netherlands) coupled to an Agilent 6890N gas chromatography system (Chemetrix, Midrand, South Africa). The antennae of males and females of *S. noctilio* and *I. leucospoides* were removed with a surgical blade. The antennae were coupled to two Ag/AgCl capillary glass electrodes filled with Beadle-Ephrussi-Ringer solution (129 NaCl: 4.7 KCl: 1.9 CaCl2 mmol/l de-ionized distilled water).

The antennal preparation was placed as near as possible to the outlet of the stimulus delivery tube of the EAD system. Purified and humidified air was allowed to pass through the stimulus delivery tube at a flow rate of 180 ml/min and 1 μl of the samples were injected splitless (300 °C) onto a 30 m HP 5 analytical column ((0.32 mm ID, 0.25 μm film, J & W scientific). Half of the sample was directed to the antennal preparation through a quartz splitter (Agilent, PN:5181-3398) at the end of the analytical column

and the other half to the FID (300 °C). Direct current recordings were made with a ten times external amplification in all cases and baseline drift was removed by plotting the derivative of the EAD data as described in Slone and Sullivan (2007). The GC was operated with constant pressure at 16 psi (He). The GC oven was kept at 60 °C for one minute and ramped (20 °C/min) to 300 °C (2min).

4.3.5 GC-MS

Samples were injected (He, 7 psi, constant pressure) split-less onto a 30 m HP 5 (0.32 mm ID, 0.25 μm film, J & W scientific) analytical column. The GC (Agilent 6890A) oven was kept at 60 °C for one minute and ramped (20 °C/min) to 300 °C (2min). The MS (5975 C Inert MSD) analyzer (250 °C, mass range 40-550 m/z) was operated in positive ion mode with an electron impact ionization source (70 eV). Tentative identities of peaks unique to the samples, after comparison to the system blank, were obtained by matching Kovats retention indices and mass spectra to the NIST 2008 MS database. Kovats retention indices were calculated through linear interpolation between n-alkanes (1 μl, 38 ppm).

4.3.6 Chemical synthesis

In order to confirm the identity of the unknown peak we attempted to synthesize the compound from a structurally related sesquiterpene ((-)-Thujopsene, cas no 470-40-6, 97% pure, Sigma Aldrich). Chemical synthesis was followed as described in Kitchens *et al.* (1972). The sulfuric acid catalysis procedure was followed as in entry six (Kitchens *et al.*, 1972). Briefly, 50 mg (-)-Thujopsene (cas no 470-40-6) was added drop wise to a mixture (40 °C) of acetic acid (20 mg) and sulfuric acid (50 mg). The mixture was agitated and kept at 40 °C for three hours before neutralizing with an equivalent of NaOH (1.7 Mol/l). This mixture was extracted twice with 400 μl diethyl ether. Half of the diethyl ether extract was concentrated under gentle steam of nitrogen. One μl of the up-concentrated product was dissolved in 1000 μl dichloromethane and diluted one hundred-fold with dichloromethane before 1 μl was analyzed on the GC-MS system.

4.4 Results

Gas chromatographic analysis revealed that all the samples had complex profiles. Most of the peaks were present in the system blank and these peaks were regarded as background. Background peaks present in the system blanks were ignored in further analyses. It is

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possible that other peaks of fungal origin may have been present in these samples, but these were either obscured by other co-eluting peaks or below detection capabilities of the methods and instruments used.

A prominent peak was observed (RT 6.79 min GC-EAD, RT 6.34 min GC-MS) that was not present in the system blank (Figure 4.1). This peak was present in samples of both *A. areolatum* and *A. chailletii*, although *A. chailletii* appeared to produce the compound in lower quantities when compared to *A. areolatum* (Figure 4.1). The retention index for this peak was calculated as 1394 on the GC-EAD and 1393 on the GC-MS system.

The prominent peak elicited a repeatable electroantennogram response from *Ibalia leucospoides* female (n = 5, individuals) antennae (PDA/Wood: 107 ± 67 V, n = 3, PDA: 80 ± 42 V, n = 2). Dynamic headspace sampling further confirmed the response of *I. leucospoides* females and showed an additional response to a peak at RT 6.74 min KI 1387 on the GC-EAD instrument (Figure 4.2). No chromatographic peak could be observed on the GC-MS for this second response,but the response coincided with the retention index of isolongifolene (cas no: 1135-66-6) a sesquiterpene with 3 ring structures and one double bond (Adams *et al.*, 2007).

Ibalia leucospoides males (n = 2, individuals) and *Sirex noctilio* males (n = 5, individuals) and females (n = 8, individuals) did not show a detectable response to the same peak to which *I. leucospoides* females responded (Figure 4.3).

The detectable peak that was associated with the response of *I. leucospoides* females was tentatively identified as a sesquiterpene, 1,3,4,5,6,7-hexahydro-2,5,5-trimethyl-2H-2,4a-Ethanonaphthalene. This identification was equivocal because the retention index did not match that reported in literature (KI 1334 (Raal *et al.*, 2011), KI 1310 (Aliboudhar *et al.*, 2013)).

The acid catalyzed isomerization reaction of (-)-thujopsene produced five major products. Two of these products had higher match factors to 1,3,4,5,6,7-hexahydro-2,5,5-trimethyl-2H-2,4a-Ethanonaphthalene (the library hit of the unknown NIST reg no 26182) than the unknown (Figure 4). The retention indices of these two product compounds (Product 1 = KI 1473, NIST match = 90.4 % and product 2 = KI 1494, NIST match = 91.4 %) were sufficiently different from the unknown retention index (KI 1393, NIST match = 78.1 % to tentative identity provided above) and from the reported retention indices previously published (Raal *et al.*, 2011; Aliboudhar *et al.*, 2013). These differences led to conclude that the compound is either not present in the library or that we could not synthesize the suspected compound as reported in Kitchens *et al.* (1972). This prompted an accurate mass analysis, which confirmed that the unknown compound

has the empiric formula C15H24 (mass 204.18719), which implies any combination between four double bonds and/or ring structures.

4.5 Discussion

The results of this study showed that the *S. noctilio* fungal symbiont *Amylostereum areolatum* produces a specific sesquiterpene that can be detected by *I. leucospoides* females. *I. leucospoides* males and both *Sirex noctilio* males and females were not equally sensitive to the same sesquiterpene. *Amylostereum chailletii*, a fungal associate of other Siricid wasps was also shown to produce this sesquiterpene, but in lower relative quantities when compared to *A. areolatum*. Both fungal species appear to be able to synthesize the compound on agar, without the need for pine wood as a substrate. These findings suggest that the unknown sesquiterpene may be part of the signal that indicates the presence of *Amylostereum* species to *I. leucospoides* parasitoid females.

This study is the first to demonstrate a repeatable electroantennogram response from *I. leucospoides* females to a specific but currently unknown sesquiterpene. The volatile profile of *A. areolatum* fungus was investigated in two previous studies (Bryant, 2010; Cucura, 2013). Bryant (2010) focused on compounds that elicited electroantennogram responses from *I. leucospoides* and *S. noctilio* antennae. His study tentatively identified other compounds such as *p*-anisaldehyde, *m*-ethylacetophenone and *p*-ethylacetophenone. The latter two compounds were then tested as possible attractants in field conditions, but without success. Cucura (2013) also tested these compounds plus linalool, anisaldehyde, geraniol, acetaldehyde and two host compounds, α-pinene and δ-3-carene, as potential attractants for *S. noctilio* and *I. leucospoides* in the field, but also without success. These additional compounds used by Cucura (2013) were previously tentatively identified by Bryant (2010). These compounds were also tested in conjunction with a commercial *Sirex* lure provided by Alphascents, Inc (Portland, OR) (Cucura, 2013). However, neither of these studies mention the presence of the unknown sesquiterpene found in the present study. It is possible that this compound was either misidentified or below the detection capabilities of the instruments used in the previous studies.

It has been known for some time that two- week-old *A. areolatum* and *A. chailletii* cultures that are grown on potato dextrose agar disks elicit antennal palpating and ovipositor probing behaviour from *Ibalia leucospoides* females (Spradbery, 1974). The attraction of the female wasps to the *A. areolatum* fungus was also shown in four separate experiments by Martinez *et al.* (2006). These authors showed that the wasps are able to detect the presence of *A. areolatum*, regardless of whether it is present within pine logs or simply grown on agar media (Martinez *et al.*, 2006). These observations

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are consistent with present findings because the unknown compound was detected in fungal samples grown on PDA media. The concentration of the fungal odour (Martinez *et al.*, 2006) and whether the *I. leucospoides* females were previously fed (Pietrantuono *et al.*, 2012) appeared to play a role in the attraction mechanism between *A. areolatum* and *I. leucospoides*. These findings are interesting because *A. areolatum* appears to manufacture more of the unknown compound than *A. chailletii*. The presence of this compound in both *Amylostereum* species confirms the belief (Spradbery, 1974; Martinez *et al.*, 2006) that *I. leucospoides* can detect both species.

It has recently been shown that Siricids in North America can exchange strains and even species of *Amylostereum*, and not infrequently (Hajek *et al.*, 2013; Wooding *et al.*, 2013; Olatinwo *et al.*, 2013). This suggests that Siricid wasps at least sometimes oviposit in close proximity to previous ovipositions. This could be accidental, but it is feasible to consider a fitness advantage associated with the ability to detect previous successful attacks and a ready food source for developing larvae. Other Siricids, specifically *Xeris*, have been shown to be attracted to previous inoculations of *Amylostereum* in wood (Fukuda and Hijii, 1997). Present results, however, did not show an antennal response that could be attributed to a detectable peak of fungal origin. It is possible that *S. noctilio* females rely on a synergistic combination of volatiles from both the host pine tree and their own symbiotic fungus when searching for potential oviposition sites. These volatiles of fungal origin may have been below the levels that were detectable. It is also possible that *S. noctilio* females are receptive only to signals of fungal origin after mating has occurred. It was unknown whether *S. noctilio* females had mated prior to these experiments.

Electroantennography with both *S. noctiolio* and *I leucospoides* antennae was difficult. It is suspected that complications arise during the antennal removal process possibly because the hard outer shell of the antennae cracks and damages the neurons during this process. Recordings with live insect preparations were also attempted, but failed due to lack of adequate connections to the antenna and the uncontrollable movement of the insects. On the other hand, recordings made with removed antennae limited the number of antennal recordings per antenna to between one and four, depending on the antennal lifetime. The large number of contaminating peaks from the substrate present in these samples further complicated the analyses. These contaminating peaks could elicit electroantennographic responses, but it was not possible to attribute the responses to volatiles of fungal origin. Therefore, only peaks not detected within the system blank samples could be confirmed to be of fungal origin. Contaminating peaks are attributed to sources such as the agar medium, the charcoal traps and the solvent impurities that were detected once samples were concentrated. These limitations should be considered in future studies.

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The identity of the sesquiterpene found in this study is currently unknown and further structural elucidation of the compound is needed before any behavioural tests can be conducted. Such a structural elucidation would require isolation of the compound in larger quantities for NMR or X-ray crystallography analysis. A more detailed analysis of the volatiles produced by both these *Amylostereum* species would possibly reveal other compounds that may be detected by both *S. noctilio* and *I. leucospoides*. These unknown volatile compounds represent the ideal candidate semiochemicals that could potentially be used to enhance monitoring trap efficiencies in future.

4.6 Acknowledgments

-We thank Dr Brett Hurley and Mr. Hardus Hatting for their help in collecting field samples and Dr Yvette Naudé, Department of Chemistry, University of Pretoria for assistance in analyzing samples. Members of the Tree Protection Co-operative Programme (TPCP), the THRIP initiative of the Department of Trade and Industry and the DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB) that provided financial support for this study.

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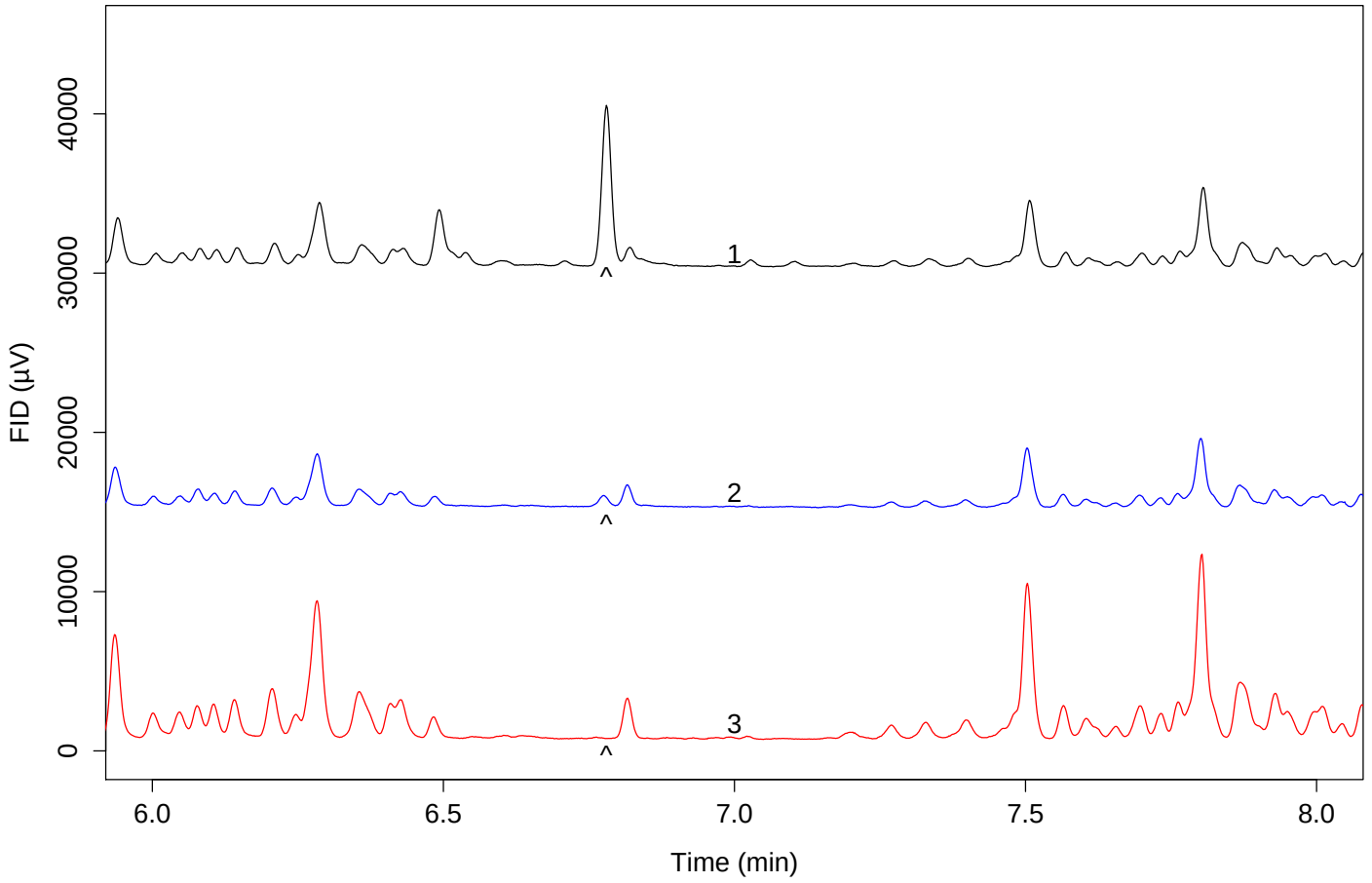

FIGURE 4.1: GC-FID profile comparison of volatiles collected from *Amylostereum* species. Chromatograms show the region of interest for comparison purposes between samples obtained from *Amylostereum areolatum* fungus (1), *Amylostereum chailletii* fungus (2) and a system blank (3). Notice the difference in the relative amount of the peak indicated with the ∧ between *Amylostereum areolatum* and *Amylostereum chailletii* and the absence of this peak in the system blank.

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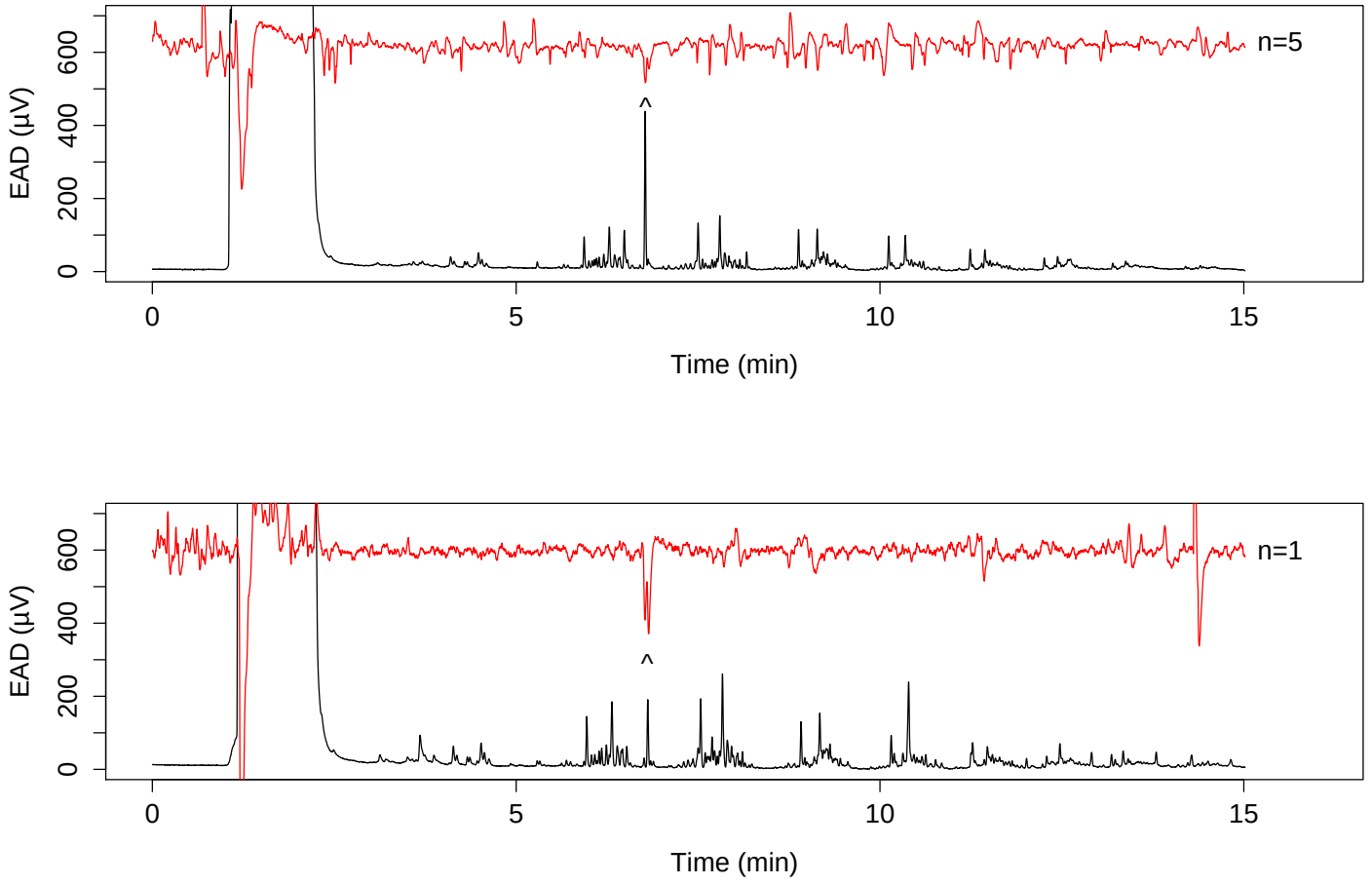

FIGURE 4.2: Top: Averaged GC-EAD response of *Ibalia leucospoides* females to *Amylostereum areolatum* fungus volatiles that were sampled passively. Bottom: Antennal response of *Ibalia leucospoides* female to volatiles collected from headspace of *Amylostereum areolatum* through dynamic headspace sampling. Note the responses around 6.79 minutes.

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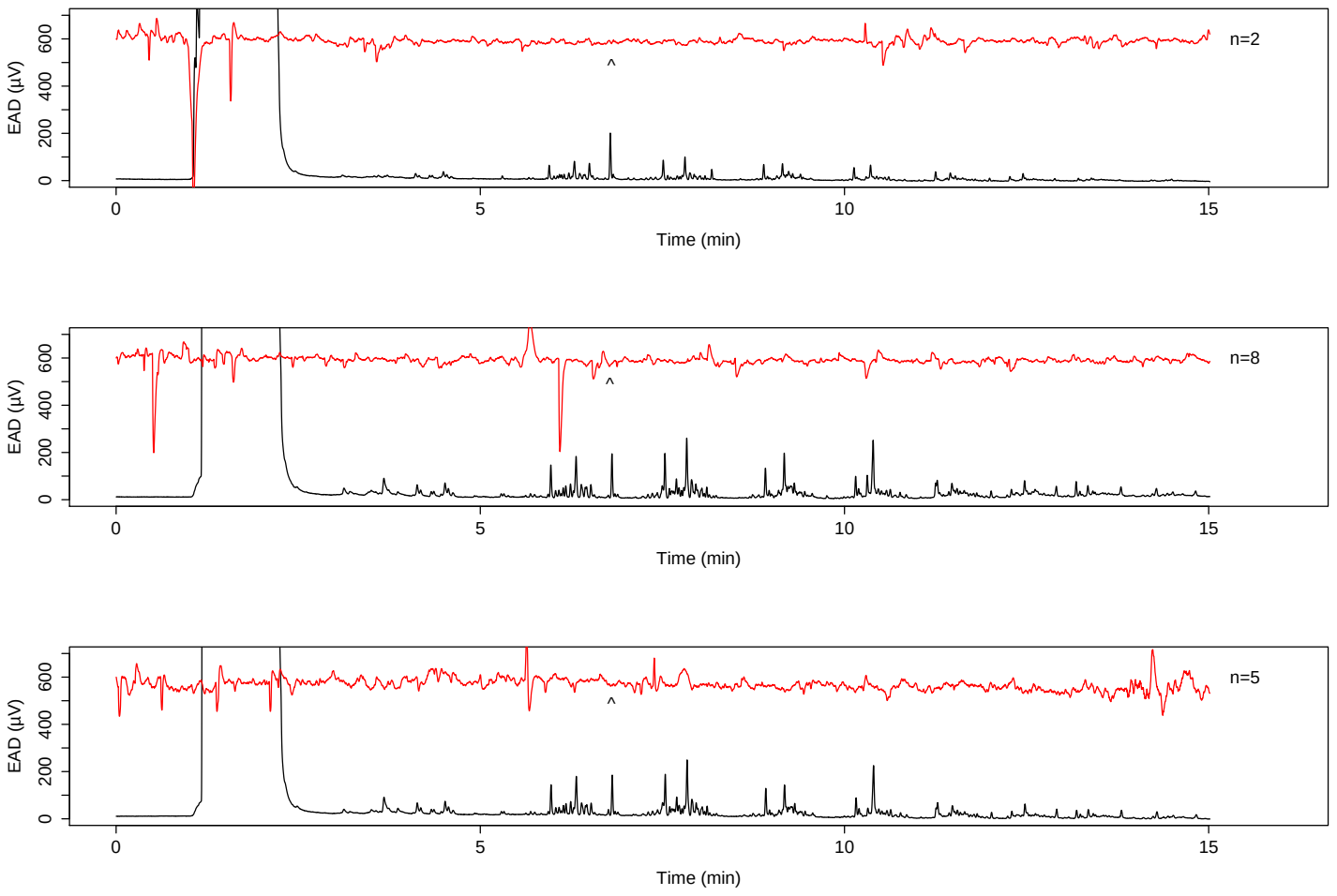

FIGURE 4.3: Averaged GC-EAD response of *Ibalia leucospoides* males (Top), *Sirex noctilio* females (Middle) and *Sirex noctilio* males (Bottom) to *Amylostereum areolatum* volatiles. Notice the absence of an EAD response to the same peak at 6.79 minutes.

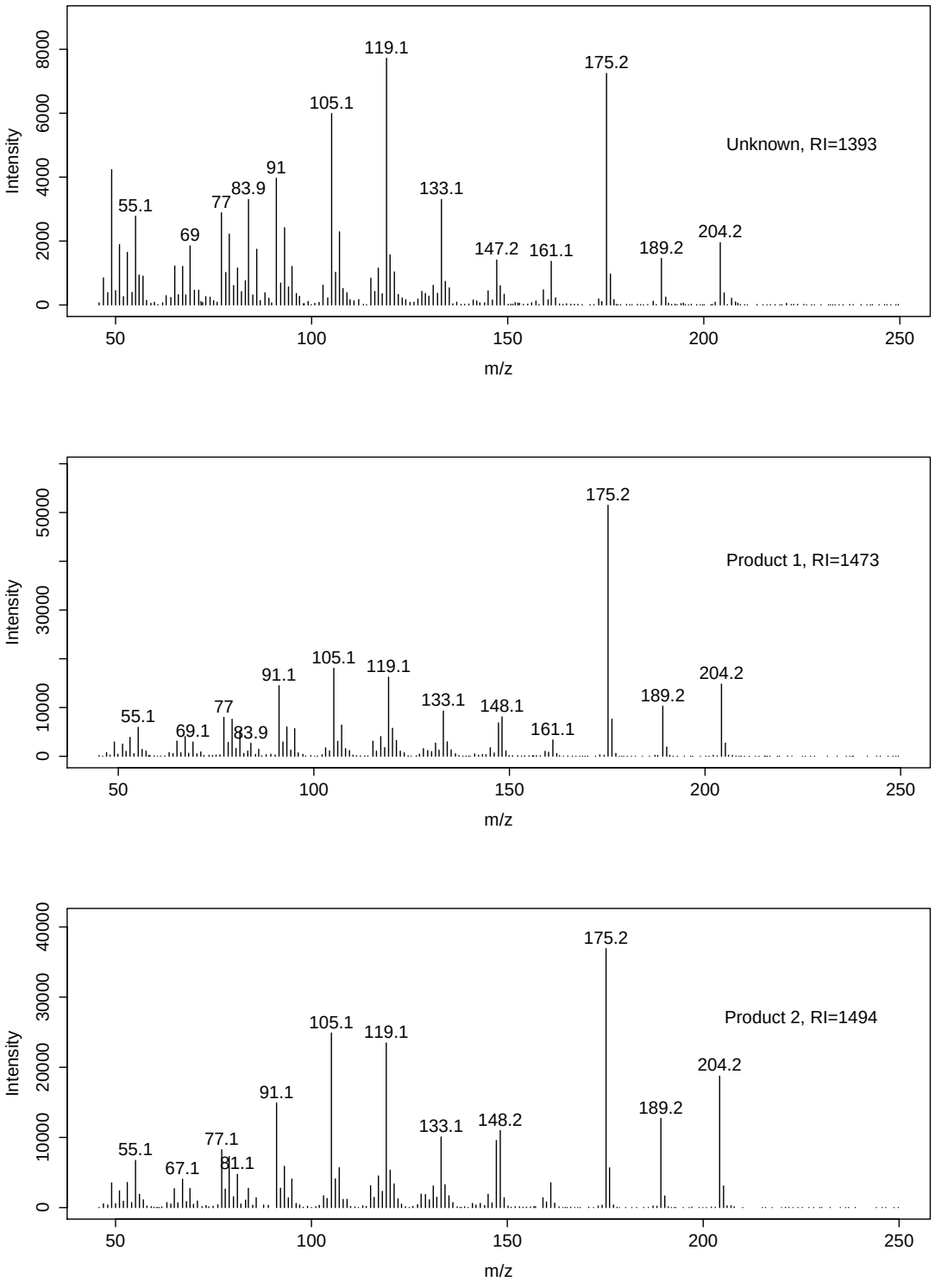

FIGURE 4.4: Mass spectrum of the unknown peak (Top). Mass spectrum of the first product (Middle). Mass spectrum of the second product (Bottom). Kovats retention indices are indicated. Please note, the first decimal place after the comma is not accurate

Chapter 5

Potential semiochemicals from the egg clutches of *Thaumastocoris peregrinus* (Heteroptera: Thaumastocoridae)

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5.1 Abstract

Thaumastocoris peregrinus is a *Eucalyptus* leaf-feeding insect that was recently introduced into South Africa. This insect is regarded as serious pest because it can cause severe discolouration and defoliation of plantation trees. The egg parasitoid, *Cleurocoides noackae*, has been imported from Australia into South Africa as a biological control agent for *T. peregrinus*. These parasitic wasps locate and oviposit inside the eggs of *T. peregrinus*, which are typically laid in clusters on *Eucalyptus* leaves. In this study the volatile profile of the egg clutches of *T. peregrinus* were analysed. Volatiles were sampled through dynamic headspace sampling and the samples were analyzed by GC-MS. Results revealed over 21 different compounds present in the airspace around these egg clutches. Six of the prominent compounds, namely sulcatol, n-decanal, n-octanal, n-nonanal, cuminaldehyde and 2-undecanone were confirmed with reference standards and quantified. These compounds were released from the sampled eggs at an average rate of 0.26 ± 0.23 ng per minute. The possible behavioural functions of these compounds were investigated for female *T. peregrinus* and *C. noackae* insects in a Y-tube olfactometer. While none of the compounds had a significant behavioural effect on the females of either of the two species they are still expected to function as semiochemicals, perhaps within specific blends, for either *T. peregrinus* or *C. noackae*.

5.2 Introduction

The bronze bug, *Thaumastocoris peregrinus*, was accidentally introduced into South Africa, probably from Australia, where it was first reported in 2003 (Jacobs and Neser, 2005; Nadel *et al.*, 2010). *T. peregrinus* is considered a pest in South Africa because it feeds on the leaves of Eucalyptus trees commonly grown in commercially managed plantations and reached extremely high population numbers in the apparent absence of natural enemies. Both the nymphs and adults feed on leaf sap and can cause leaf discoloration, especially during periods of high populations. This may lead to defoliation, stunted growth and tree death in severe cases (Jacobs and Neser, 2005). This insect pest has lately also been introduced into South America and other parts of Africa, where it continues to spread and cause concern to forestry industries (Carpintero and Dellapé, 2006; Nadel *et al.*, 2010).

The life cycle of *T. peregrinus* was studied by Noack *et al.* (2007) at temperatures between 17 and 22 C. In these conditions the eggs take approximately six days to hatch followed by four nymphal stages that last approximately 15 days. Adults live for 40 days and females can produce at least 60 eggs during their life cycle (Noack *et al.*, 2007). The

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egg laying behaviour of *T. peregrinus* females is unusual with one female laying at least one egg per day (Noack *et al.*, 2007; Wilcken *et al.*, 2010), which is typically placed on a rough spot or crevice on the leaf surface (Soliman *et al.*, 2012). In the field clusters are found that can contain more than a hundred individual eggs. The females are thus able to located other eggs of their own species on the leaf surfaces and add eggs to already existing clusters. The manner in which females locate eggs of their own species has not been investigated.

A potential biological control agent, the parasitic wasp *Cleurocoides noakae*, has recently been imported from Australia into South Africa as well as various South American countries (Jacobs and Neser, 2005; Mutitu *et al.*, 2013). Females of this species parasitize the *T. peregrinus* eggs by laying their eggs into the eggs of *T. peregrinus*. The resulting larvae then feed on and kill the *T. peregrinus* egg contents. It is not known how *C. noakae* locates the eggs of *T. peregrinus*.

The involvement of semiochemicals in the behaviour of these two insects has been studied in greater detail for *T. peregrinus* than for *C. naockae*. This is due to its more recent discovery and the small size of the parasitoid, which makes it very difficult to handle. *Thaumastocoris peregrinus* individuals are known to form large swarming masses at times and this behaviour has lead to the suspicion of the involvement of a possible aggregation pheromone (Noack, personal communication). A male aggregation pheromone (3-methyl-2-butenyl butanoate) was identified in two separately published studies (González *et al.*, 2012; Martins *et al.*, 2012). It was also shown that mated males produce this compound in greater amounts when compared to unmated males and females. Other host specific semiochemicals have also been identified for *T. peregrinus*. These compounds include aromadendrene, globulol and a-pinene, which were shown to be released in greater quantities from *E. benthamii* leaves after *T. peregrinus* herbivory (Martins and Zarbin, 2013). These compounds were shown to repel mated *T. peregrinus* females. It was postulated that females use these volatile cues to avoid plants that are already infested in order to avoid competition and to escape parasitism (Martins and Zarbin, 2013).

In this study we identified volatile organic molecules released from the egg clutches of *T. peregrinus*. The release rates of some of these volatiles were measured in laboratory conditions. The volatiles isolated from the eggs are hypothesized to function as semiochemicals for both *T. peregrinus* females and the biological control agent *C. noakae*. Y-tube olfactometer tests were conducted on *T. peregrinus* and *C. noackae* females with volatiles from the eggs of confirmed identity.

5.3 Materials and Methods

5.3.1 Collection of egg clutches

A total of 80.6 mg of un-hatched eggs of *Thaumastocoris peregrinus* were obtained by carefully removing them from leaves that were sampled from a single Eucalyptus tree on the experimental farm of the University of Pretoria. Removal of the eggs from the leaf surfaces was achieved using a sterilized surgical blade or in some cases by bending the leaves slightly. This method was used to collect eggs because eggs obtained from reared individuals were not in clusters and were often in small numbers.

5.3.2 Sampling of volatiles

Eggs were placed inside conditioned (300°C, 15 min, 50 ml/min, He) glass tubes (178 x 6 mm, 4 mm ID) between two pieces (0.5 mg) of conditioned (300°C for 15 min, He 50 ml/min) silane treated glass wool (Supelco). Sample blanks were prepared in the same way without any eggs or glass wool. The eggs were split into two groups of 23.0 mg and 57.6 mg which were sampled separately (n = 5 and n = 4, respectively for each group of eggs) at 22°C. Samples were also taken after heating the 57.6 mg egg chamber to 35°C for a 30 minute period (n = 4). After sampling the traps were capped with Teflon sealed glass stoppers to avoid contamination.

Dynamic headspace sampling was done by filtering laboratory air through two activated charcoal traps (150 mg, ORBO^{TM}, Supelco) coupled in series upstream from the sample tube. Conditioned Gerstel sampler traps (178 x 6 mm, 4 mm ID, Tenax^{TM} TA 60/80, 35 m2/g) were connected down stream of the material being sampled. Samples were taken with an analytical sampling pump (S/Kick ATEX NIMH pump, SKC) for a 60 minute period at a flow rate of 36.84 ± 0.76 ml/min for the egg samples and 38.02 ± 0.39 ml/min for the blank samples (Samples n = 13 ± SD, Blanks n=13). The total sampling volume was 2280 ± 23 ml (mean ± SD) for blanks and 2210 ± 45 ml (mean ± SD) for egg samples. The sampling volume was below the breakthrough volume of chloroform (26800 cm^3/g, 20°C) for the Tennax TA traps (Kroupa *et al.*, 2004) and below the breakthrough volume of acetone (9380 cm^3/g, 100°C) for the charcoal filters (Wartelle *et al.*, 2000).

5.3.3 Gas chromatography coupled to mass spectrometry

Traps were desorbed (25 - 300°C at 60°C/min, 50 ml/min, He, 10 min, splitless) in a Gerstel thermal desorption system (TDS) coupled (transfer line, 350°C) to a cooled

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injection system (CIS, solvent vent mode) containing a Tenax TA inlet liner. Sample traps were removed and replaced with an empty conditioned glass tube before CIS injection. The CIS was cryogenically cooled with liquid nitrogen to −10°C for the entire desorption time and was heated to 300°C at a rate of 12°C/sec for 10 minutes st the start of the analysis (7 psi, He, splitless, vent time 3 min).

Separation and analysis were done on a Agilent 6890A gas chromatograph containing a HP 5 capillary column (30 m, 0.32 mm ID, 0.25 μm film, J & W Scientific, Agilent) coupled to a 5975C inert mass selective detector (Agilent, MSD, EI:70 eV, m/z 45-545). The GC oven was cryogenically cooled to 10°C for three minutes ramped to 300°C for three minutes at a rate of 10°C per minute.

Conditioned blank and sample blank traps (traps sampled from tubes not containing eggs) were analysed and compared to establish the presence of any volatiles that may be present inside the tubes. Blanks and egg samples for each day of sampling were analysed and compared to find peaks that were not present in blank samples. Peaks were checked for purity by comparing sequential mass spectra across the peak and were tentatively identified based on Kovats retention indexes and mass spectral comparisons to the NIST (2008) library. Confirmation of the tentative identity was obtained by comparing both retention times/Kovats retention indices and mass spectra to those of authentic reference standards.

5.3.4 Quantification of egg-specific chromatographic peaks

External calibration was used to set up calibration curves for quantification in a range of 0.5, 1.0, 5.0, 10.0 and 50.0 ng. Quantification was done by measuring single ion chromatographic peak areas and errors in quantification were calculated through the standard deviation about regression method. A one-way Anova was conducted with Tukey HSD as a post hoc test. Statistical parameters were calculated in R version 3.1.0.

5.3.5 Bioassay

Behavioural tests were conducted in a glass Y-tube olfactometer (7 mm OD, 3 mm ID, 90° angle between Y arms) housed in a closed box to limit uneven light stimulation (based on a similar design by de Kogel *et al.* (1999). The olfactometer and box were kept at an angle of 20° to the horizontal plane (arms of Y at higher point). Air was drawn sequentially through activated carbon and distilled water in two separately linked wash bottles before entering the olfactometer at a rate of 46 ml/min (total flow). Individual female insects were identified under a microscope (for *T. peregrinus* a symmetrical

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abdominal tip (Martins *et al.*, 2012), and for *C. noackae* a club shaped antenna) and placed inside the lower arm of the olfactometer with a fine paint brush. A dark cloth was placed over the apparatus and the box was closed for a period of 3 minutes to allow each insect to make a choice. After 3 minutes, the box was opened and the individual was located. A choice was assigned when the individual insect moved at least half way up one of the olfactometer arms. No choice results were discarded. Each test was done with a different individual female. These tests were repeated 10 times before the apparatus was inverted for 10 additional tests. This was done to minimize any positional bias that might be present in the system. A two-sided binomial test was used to calculate associated p values (R version 3.1.0).

Stimuli consisted of a 100 ppm solution of a compound made up in dichloromethane with only the solvent serving as a non-stimulus. A volume of 1 μl was placed on a piece of Whatman 1 filter paper (0.5 by 1.5 cm). The filter paper was inserted into identical chambers at each end of the Y arms. A mixture of the six tested compounds was made in relative proportions similar to chromatographic results. This mixture (100 ppm total) was made up in a ratio of 16:18:30:13:13:10 for 6-methyl-5-hepten-2-ol: n-octanal: n-nonanal: n-decanal: cuminaldehyde: 2-undecanone and was also tested (volume 10 μl). The compound with lowest concentration in the mixture was 2-undecanone, which corresponded to a treatment of 100 ng on the filter paper. One hundred *T. peregrinus* eggs, carefully removed from tissue paper with a scalpel blade, were used as a positive control for behavioural tests with *C. noackae*. There was an insufficient number of *T. peregrinus* eggs laid on filter paper for the behavioural tests with *T. peregrinus* females.

5.4 Results

Gas chromatographic investigation of the egg headspace samples revealed twenty-one unique peaks that were not present in blank samples (Figure 5.1). Tentative identities of these peaks were made based on mass spectral data and retention indices that were compared to published values (Table 5.1). The identities of six of these peaks that were prominent in the chromatograms were confirmed and these compounds were quantified with reference standards using regression equations in Table 5.2.

The average amounts released for all samples under the different sampling conditions were calculated (Figure 5.2, 5.3). N-nonanal was released at highest rate based on the average for all measurements, but this was not significantly different from the release rates of n-octanal, n-decanal and cuminaldehyde (Table 5.3). 2-Undecanone was released at the same rate as n-nonanal, n-decanal and cuminaldehyde (Table 5.3). Sulcatol was

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released at a lower rate than the other compounds. In these sampling conditions, an average release rate was estimated to be in the pico-gram per minute range.

Behavioural tests revealed that neither *T. peregrinus* nor *C. noackae* females were significantly attracted or repelled by any of the compounds at the tested dose levels (Figure 5.4). *C. noackae* females were also not attracted to *T. peregrinus* eggs which were laid on tissue paper. A mixture of the six compounds with confirmed identity also did not attract or repel females of either species.

5.5 Discussion

This is the first study undertaken to identify and measure the release rate of volatiles from *T. peregrinus* egg clutches. Results showed that twenty-one different volatiles can be isolated from the egg clutches after removal from *Eucalyptus* leaves. These included straight chain aldehydes of various chain lengths, as well as other aromatic aldehydes and alcohols such as cuminaldehyde and sulcatol. The identities of six of these compounds were confirmed and the average release rate was estimated at two different sampling temperatures in the laboratory. Quantified compounds were released from the eggs at a rate of between one and seven nano-grams per hour.

None of the identified compounds showed any behavioural activity in bioassays using either *T. peregrinus* or *C. noackae* females. Neither could the attraction between *C. noackae* females and *T. peregrinus* eggs (laid on tissue paper) be confirmed in the laboratory study. The original hypothesis that semiochemicals might be involved in egg laying patterns and the parasitism of egg clusters emerged from field observations, and suggestions of such influences in the published literature. However, the results of the present study suggests that: a) the semiochemical signal may be more complex than initially thought; b) behaviour is significantly affected by the laboratory situation and/or handling of the insects, or; c) that there is no involvement of semiochemicals in the egg laying and parasitism behaviour in this system. We discuss these possibilities below, and consider the potential of the identified chemicals to influence insect behaviour, based on comparison with studies in other systems.

It is known that certain predaceous true bugs such as *Podisus maculiventris* (Hemiptera: Pentatomidae) use terpenes such as α-terpineol, terpinen-4-ol and linalool as attractants and some of these terpenes are part of their male produced pheromone (Aldrich *et al.*, 1984). Two of these terpenes, namely α-terpeneol and terpenen-4-ol, were found in the *T. peregrinus* egg samples and could, potentially, be part of an egg-laying cue for *T. peregrinus*. Another possibility is that they may also function as semiochemicals for *C.*

noakae females to locate *T. peregrinus* eggs. It is known that certain egg parasitoids, such as *Oomyzuz gallerucae* (Hymenoptera: Eulophidae) are attracted by compounds that are produced by the plant, which are induced by the eggs of their host (Meiners and Hilker, 2000). 2-Undecanone is a compound that is commonly found as part of mandibular gland secretions of ants (Longhurst *et al.*, 1980) and this compound attracts female mosquitoes to oviposition sites when it is present in a blend with other chemicals (Du and Millar, 1999). 2-Undecanone may, therefore, be part of the oviposition queue for either *T. peregrinus* and *C. noackae* females, but it is likely that it functions only when present at a certain concentration within a specific blend of chemicals.

Sulcatol is one of the potentially significant compounds identified in the present study. This compound is chiral and a mixture of the enantiomers is known to cause aggregation in the ambrosia beetle *Gnathotrichus sulcatus* (Coleoptera: Curculionidae) (Borden *et al.*, 1976). This compound was also later identified as a potential pheromone of *Gnathotrichus materiarius*, a related beetle (Flechtmann and Berisford, 2003). *Platypus mutates* (Coleoptera: Curculinidae) beetles produce this compound in their galleries together with sulcatone (corresponding ketone) and both these compounds are attractive to female beetles (Audino *et al.*, 2005). Sulcatone is also a component of the aggregation pheromone of the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae), which resides in a closely related family of *T. peregrinus* (Siljander *et al.*, 2008). Furthermore, sulcatol is known as a repellent for the aphid, *Rhopalosiphum padi* (Hemiptera: Aphididae) and it is thought of as a spacing pheromone for that species (Quiroz *et al.*, 1997). These authors show that the ratio between the enantiomers is critically important for the repellent behaviour to occur (Quiroz and Niemeyer, 1998). These comparisons suggest that this compound or its corresponding aldehyde could play a role in the egg clustering behaviour observed for *T. peregrinus*.

Three aldehydes were identified in this study, including n-octanal, n-nonanal and n-decanal. Both octanal and decanal were found previously in the exuvial extracts made from all five instars of *T. peregrinus* (Martins *et al.*, 2012). All three of these aldehydes are also essential components of the aggregation pheromone of the bed bug *Cimex lectularus* (Siljander *et al.*, 2008). Electroantennographic studies on true bugs are limited, but it has been shown that the tarnished plant bugs, *Lygus lineolaris* (Hemiptera: Miridae) are sensitive to aldehydes and specifically n-nonanal, which was found in highest quantities in this study (Chinta *et al.*, 1994). These studies suggest that the aldehydes found in this study have the potential to affect the behaviour of *T. peregrinus* and *C. noackae*.

Although bioassay tests conducted here could not show significant behavioural effects on the females of either *T. peregrinus* or *C. noakae*, these compounds remain candidate

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semiochemicals for the two species. Stress created when handling these insects may have had a profound effect on their behaviour. For example, the egg laying behaviour of *T. peregrinus* is significantly altered when stressed in laboratory conditions (see Noack *et al.* (2007)). Handling small insects such as *C. noakae* is a difficult task and these insects may have been in a stressed state when they were moved into the Y-tube olfactometer. It is also possible that these compounds function synergistically. Synergism implies that the presence and/or absence of even one compound, and even the ratio between each of the different compounds, may be crucial for behavioural function (see for example: Quiroz *et al.* (1997); Quiroz and Niemeyer (1998); Weber *et al.* (2014)). The ratios tested in this study may also have been incorrect and, therefore, not behaviourally active for *T. peregrinus* nor *C. noackae* females. These stress factors and synergistic effects must be considered in future trials.

An important consideration in interpreting results of the experiments conducted in this study is the fact that the eggs were removed from leaf material. This makes it impossible to distinguish between the origin of the compounds. One assumes that a certain set of these volatiles originated from the leaf particles and another set originated from the eggs. The eggs that were sampled were present in much larger quantities than the small leaf particles and it is therefore, logical to focus on obvious peaks in the chromatograms and also those that were not of terpenic origin. Terpenes may have originated from the *Eucalyptus* leaves on which the eggs were laid. For example, it is known that terpenes such as linalool, β-pinene, 9-epi-(E)-caryophylene and viridifloral are released from *E. benthamii* plants after *T. peregrinus* feeding has occurred (Martins and Zarbin 2013). Two of these terpenes (β-pinene and caryophylene) were detected in these samples together with aromadendrene, which was also found as one of the most abundant compounds in the study by Martins *et al.* (2012). These compounds are most likely produced by the host. Further studies on eggs which are laid on clean surfaces should give a clear indication of which compounds originate from the eggs only and those compounds that are induced by the eggs on the host. All of these compounds (excluding those found in blank samples), nevertheless, form part of the bouquet that exists around the egg clusters of *T. peregrinus* which are normally found on *Eucalyptus* leaves.

It is known that *T. peregrinus* females lay their eggs on leaf irregularities such as crevices close to leaf veins and the edges of the leaves (Jacobs and Neser, 2005). It is also known that stressed individuals lay eggs preferentially on rough spots created for rearing in the laboratory (Noack *et al.*, 2007). These observations raise the possibility that cues other than semiochemicals could influence egg laying behaviour of this insect. One hypothesis may be that female *T. peregrinus* insects lay their eggs next to other eggs of their own species because this would facilitate the removal of the relatively large egg

from the bodies. *Thaumastocoris peregrinus* females have large eggs in comparison to their body size (Noack *et al.*, 2007) as is true for other species, *Discocoris drakei*, in this family (Couturier *et al.*, 2002). *Discocoris drakei* also has the characteristic behaviour of attaching their eggs to crevices at the bases of the pistils of the flowers of their host plant, *Oenocarpus mapora* (Couturier *et al.*, 2002). The egg laying cue could thus be a combination of both olfactory and visual cues. Observations made during the bioassay suggest that these insects run along visual lines such as the lines created by the screw fittings at the end of the Y-tube olfactometer that was used. If this is true then females might run along the leaf vein pattern and find by chance other eggs along this path. Close range olfactory cues may then stimulate the egg laying process. This behaviour may result in the observed egg clusters.

5.6 Acknowledgements

We thank Mrs. Marlene Harney and Ms. Samantha Bush for their help in collecting field samples and assisting with behavioural trials, as well as Dr. Yvette Naudé of the Department of Chemistry, University of Pretoria for assistance in analyzing samples. Members of the Tree Protection Co-operative Programme (TPCP), the THRIP initiative of the Department of Trade and Industry and the DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB) are acknowledged for financial support.

TABLE 5.1: Retention times and Kovats retention indexes of peaks found in egg headspace samples of *T. peregrinus*

No	Rt (min)	KI	Lit KI	Name	Cas no
1	9.309	974.6	974	β-Pinene	127-91-3
2	9.767	997.3	997	($\pm$)-6-methyl-5-Hepten-2-ol (Sulcatol)	1569-60-4
3	9.910	1005.4	1006	Octanal	124-13-0
4	10.089	1016.3	1016	α-Terpinene	99-86-5
5	10.237	1025.3	1025	p-Isopropyltoluene	99-87-6
6	10.698	1053.3	1052	Ocimene	502-99-8
7	11.630	1110.1	1110	Nonanal	124-19-6
8	11.897	1126.3	1126	4-Isopropyl-1-methyl-2-cyclohexen-1-ol	29803-82-5
9	12.781	1180.2	1180	1-Terpinen-4-ol	562-74-3
10	12.844	1184.0	1182.6	Naphthalene	91-20-3
11	12.926	1189.0	1188	4-Isopropyl-2-cyclohexen-1-one (Cryptone)	500-02-7
12	13.000	1193.5	1193	α-Terpineol	98-55-5
13	13.218	1207.8	1207	Decanal	112-31-2
14	13.754	1245.2	1245	p-Isopropyl-benzaldehyde (Cuminaldehyde)	122-03-2
15	14.528	1299.4	1300	2-Undecanone	112-12-9
16	15.970	1400.2	1400	Tetradecane	629-59-4
17	16.283	1424.7	1424	β-Caryophyllene	87-44-5
18	16.836	1468.0	1468	Aromadendrene	109119-91-7
19	18.344	1586.1	1586	Spathulenol	6750-60-3
20	18.417	1591.8	1592	Caryophyllene-oxide	1139-30-6
21	18.526	1600.4	1600	Hexadecane	544-76-3

TABLE 5.2: Regression equations used to quantify the confirmed compounds from *T. peregrinus* egg clutches

Compound	Equation	R^2
6-methyl-5-Hepten-2-ol (Sulcatol)	$y = 2307066x - 3068668$	0.9957
Octanal	$y = 719225x + 486412$	0.9914
Nonanal	$y = 787997x - 1071066$	0.9953
Decanal	$y = 1172153x - 1637909$	0.9950
p-Isopropyl-benzaldehyde (Cuminaldehyde)	$y = 1891830x - 2828621$	0.9947
2-Undecanone	$y = 1646777x - 2285761$	0.9953

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TABLE 5.3: Averaged quantities released from eggs in 60 minutes for all measurements

n	Compound	Ion	ng	SD about regression	SD between measurements	Letters*
13	(±)-6-methyl-5-Hepten-2-ol (Sulcatol)	95	2.58	1.85	1.63	A
13	n-Octanal	57	3.68	1.42	1.26	B
13	n-Nonanal	57	6.39	1.59	3.76	BC
13	n-Decanal	57	2.69	1.75	1.72	BC
13	p-Isopropyl-benzaldehyde (Cuminaldehyde)	133	2.19	2.00	0.85	BC
13	2-Undecanone	58	1.47	1.67	0.16	C

*Tukey HSD, $p<0.05$

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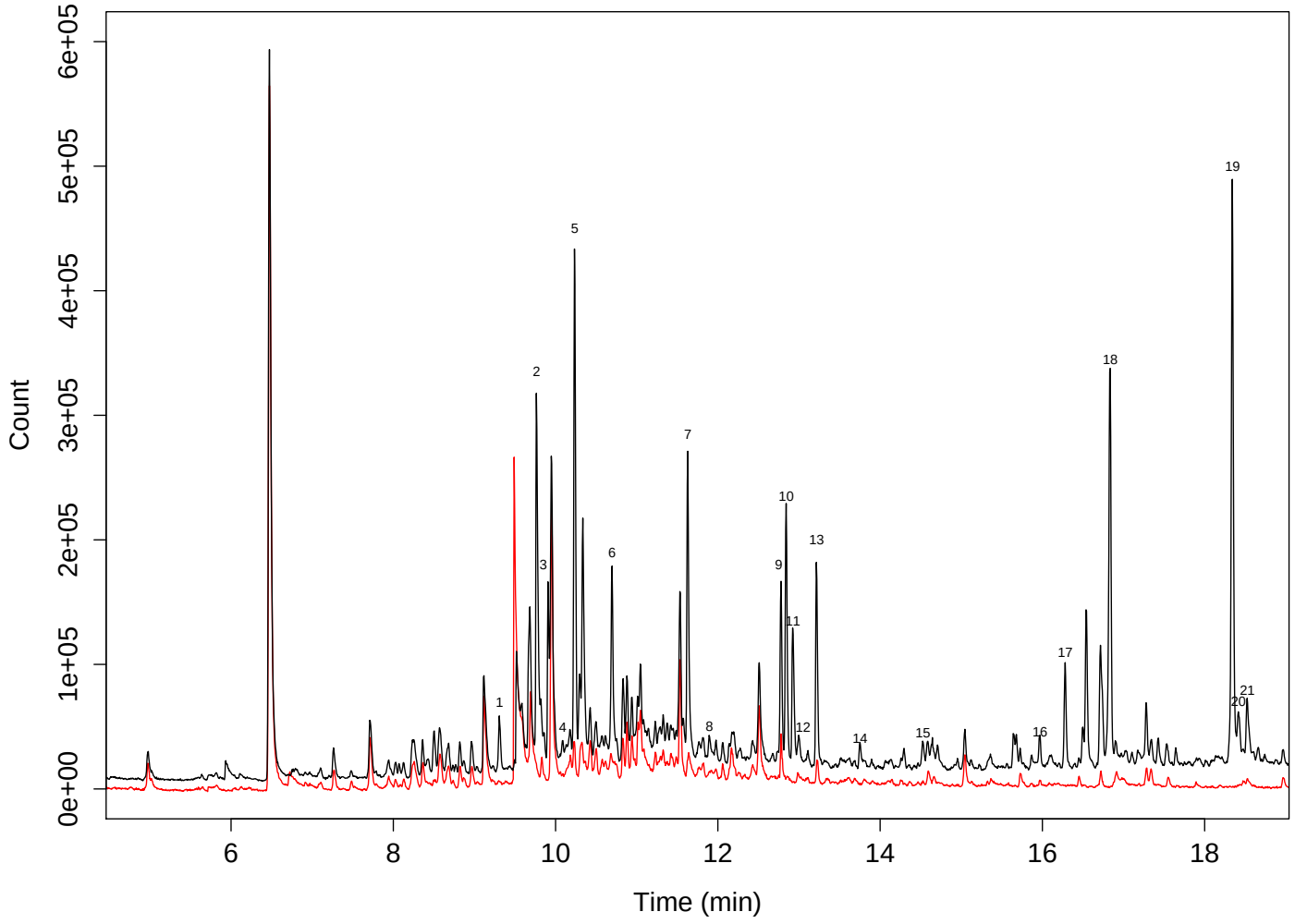

FIGURE 5.1: Representative total ion chromatogram of the headspace samples obtained from *Thaumastocoris peregrinus* eggs overlaid with a blank sample. Peaks with numbers refer to Table 5.1.

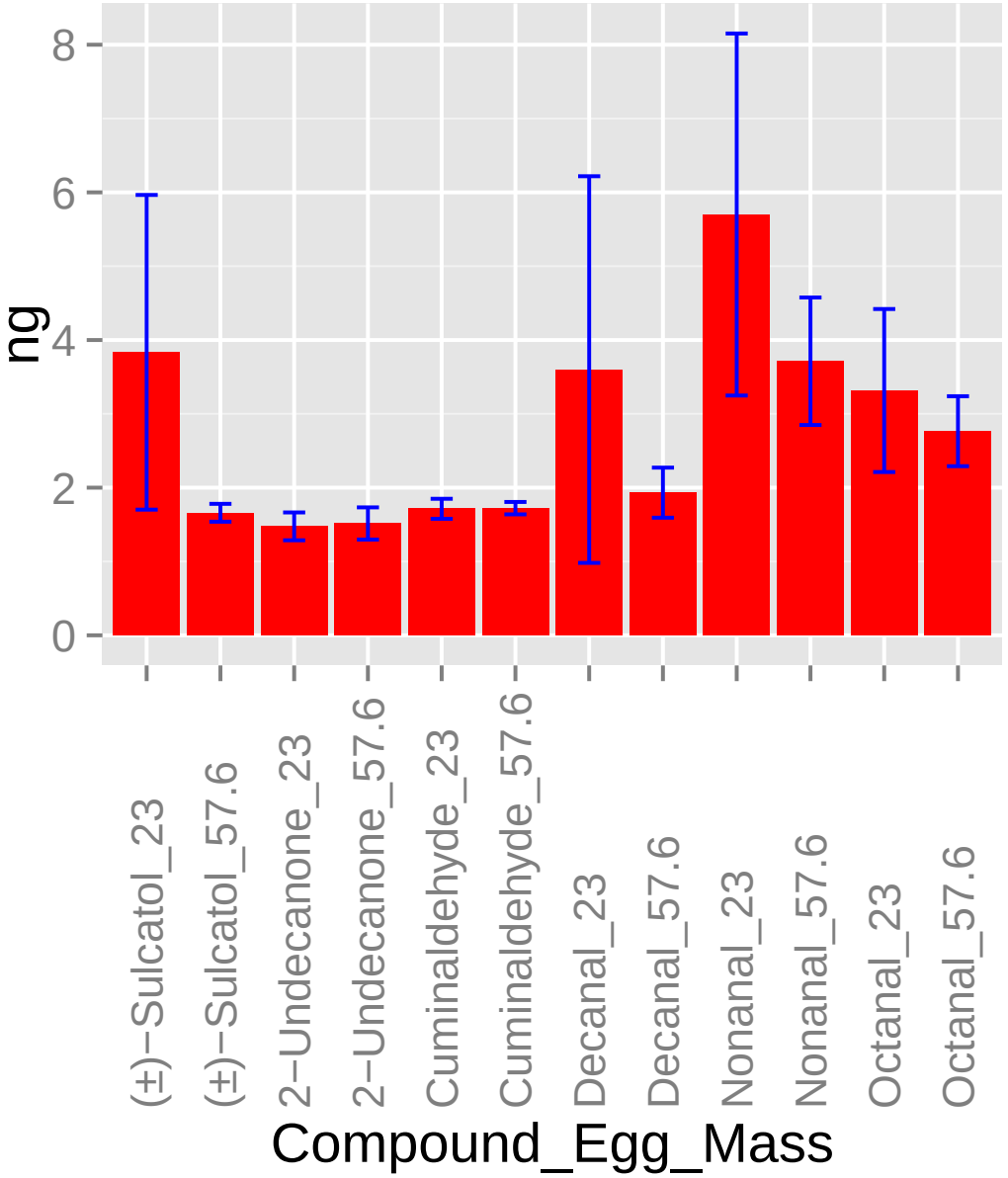

FIGURE 5.2: The amounts of egg-specific volatiles measured after 60 minutes of sampling relative to the sampling condition in terms of egg mass (mg). (n=5 for 22 mg, n = 4 for 57.6 mg, mean ± SD)

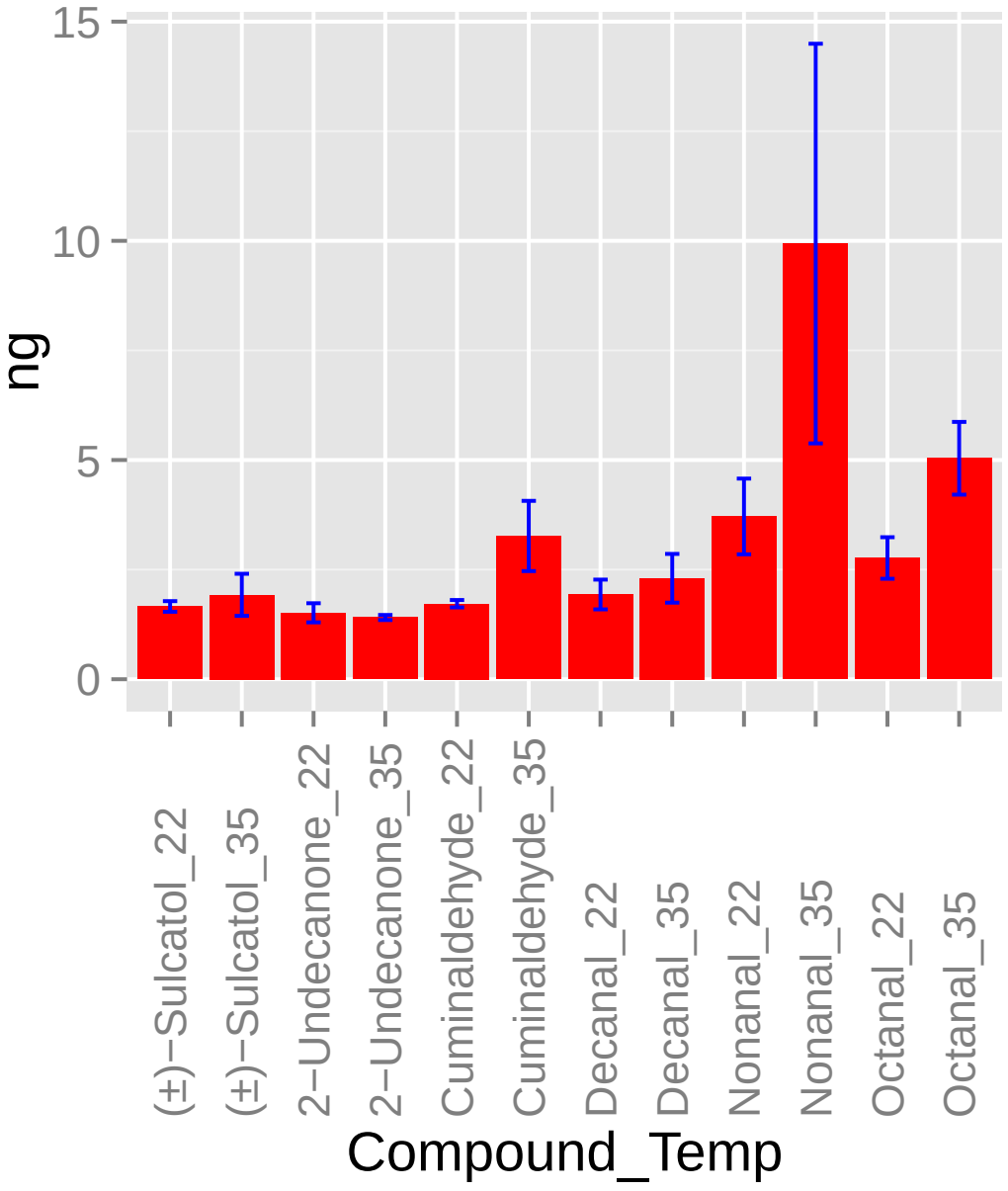

FIGURE 5.3: The amounts of egg-specific volatiles measured after 60 minutes of sampling relative to the sampling condition in terms of temperature (°C). (n=4, mean ± SD, 57.6 mg of eggs)

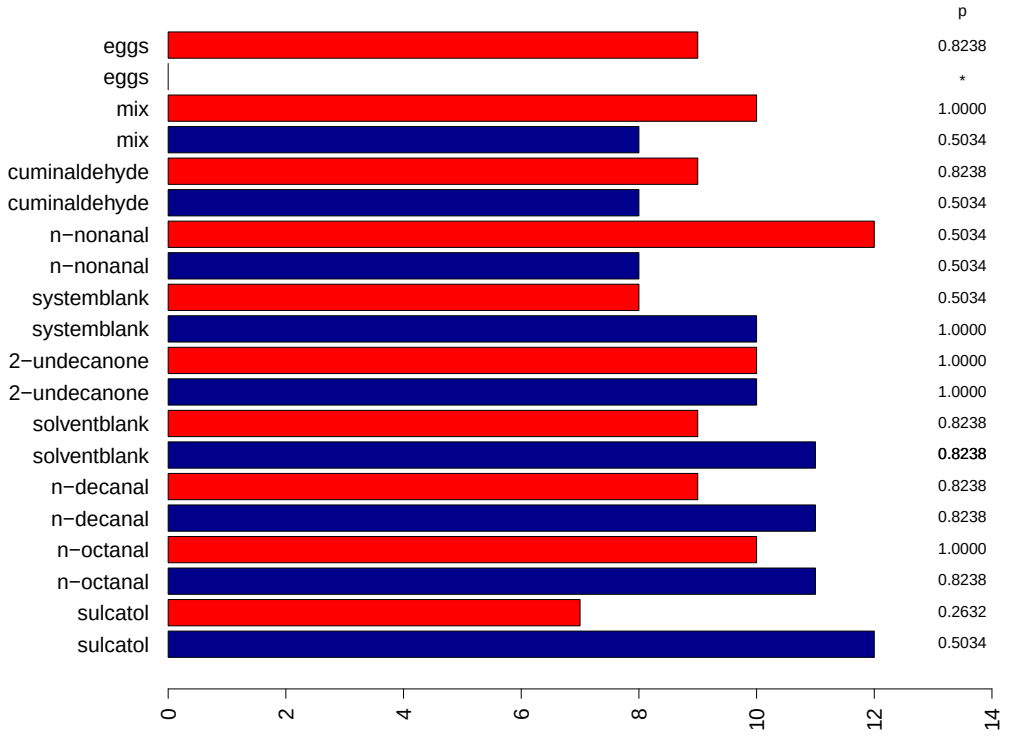

FIGURE 5.4: Bio-assay results for *Thaumastocoris peregrinus* (blue) and *Cleurocoides noackae* (red). Bars indicate the number of female insects that chose the stimulus side out of twenty individuals. Associated p values are indicated on the right-hand side (two sided binomial test). * not tested for *T. peregrinus*

Chapter 6

Summary

The results in this thesis made a substantial contribution to our knowledge regarding the chemical ecology of the species that were investigated. The general aim of discovering and characterizing semiochemical signals was achieved using analysis techniques such as gas chromatography coupled to electro-antennography (GC-EAD) and gas chromatography coupled to mass spectrometry (GC-MS).

A GC-EAD instrument was used to link chemical interactions to chromatographic peaks. This technique was used mainly as a screening tool to reduce the number of chromatographic peaks investigated to only those that were detected by the specific insect species under investigation. The GC-MS instrument was used to obtain mass spectral information of each peak that was indicated as stimulating a response through the GC-EAD analysis. Mass spectral data was used to confirm peak purity and for assigning tentative identities to specific peaks. Proving the tentative identity was achieved by matching retention characteristics, mass spectral patterns and electroantennographic responses to obtained reference standards. These confirmation procedures were limited by the simultaneous availability of commercial reference standards and live insects. The behavioural function of the identified chemicals was tested either in laboratory bioassays or field trials where possible.

Species of economic importance to the South African forestry industry were studied and included the Eucalyptus Weevil, *Gonipterus* sp 2 (Chapter 2), the Cossid Moth, *Coryphodema tristis* (Chapter 3), the Sirex Woodwasp, *Sirex noctilio* (Chapter 4) and the Eucalyptus Bronze Bug, *Thaumasticoris peregrinus* (Chapter 5). Different aspects of the chemical ecology of each insect species were investigated. Exploring these interactions was achieved by identifying what was known about the specific insects behaviour

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from personal observation or from literature. This information was used to determine where possible semiochemical signals may exist for each of the studied species.

It was shown that female *Gonipterus* sp 2 beetles detect volatiles from their *Eucalyptus* hosts especially when the leaves were freshly damaged. This type of interaction was further shown to be very complex because many host volatiles were detected by these beetles. These volatiles included general green leaf volatiles and terpenes of which thirteen were identified and confirmed to be detected by females. While many of the volatile compounds overlapped between the hosts, the ratios in which they occurred relative to each other varied between the different *Eucalyptus* species. These differences were speculated to help female beetles differentiate different host species and possibly stress levels among different trees, as female antennae responded more strongly to the unique blends from known preferred hosts and volatiles from damaged leaves. The identified volatile chemicals are expected to have a behavioural function for *G. scutellatus* and these behavioural functions need to be tested in a bioassay or field trial in future.

The sex pheromone of the cossid moth, *C. tristis*, was isolated from both the female sex pheromone gland and from the air around virgin female moths. Analyses of pheromone gland samples showed that the pheromone was possibly a combination of at least *Z*9-14:OAc and 14:OAc. The analysis of the headspace samples confirmed the existence of another component, *Z*9-14:OH, which was not detected in gland extract samples. All of these compounds were shown to be electro-physiologically active for male antennae that were morphologically different from female antennae. The biologically active ratio was tested in laboratory conditions through experiments in a wind tunnel. No consistent behaviour could be induced in the wind tunnel. Through these and other experiments and it was shown that these moths are highly susceptible to factors that induce stress when in captivity.

Two field trials were used to investigate the biological activity and active ratios of the identified pheromones. The first field trial relied on a natural rubber septum as a pheromone dispenser and the identity of compounds found in pheromone gland extract samples. No male *C. tristis* months were caught with the artificial lures, but female moths used in controls could attract males to traps. The inclusion of *Z*9-14:OH in lures in a second trial attracted *C. tristis* males through a synergistic effect with *Z*9-14:OAc. Biological activity occurred when these two compounds were dispensed in a ratio of between 2.5 – 6 % for *Z*9-14:OH and 94 – 95 % for *Z*9-14:OAc. Additional synergistic effects with other minor pheromone compounds were also possible and it was shown that addition of a small fraction of 14:OAc (2.5 %) to this mixture enhanced the luring effect, albeit very little. *Z*9-14:OAc was proven to be the major component in the pheromone blend, because the reversed ratio did not attract males in the field. The biologically

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active range of *Z*9-14:OH and *Z*9-14:OAc was patented for *C. tristis* in a provisional patent.

Two different pheromone dispensers that relied on two different pheromone releasing mechanisms were used during the two field trials. Natural rubber septa released pheromones through a diffusion process that was thought to be inadequate during the first field trial. In the second field trial pheromones were dispensed in pheromone permeation devices that consisted of glass capillaries connected to methyl silicone rubber tubes. These permeation dispensers allowed for dispensing compounds without a carrier solvent which eliminated the possible effect that it may have had during the first field trial. The new dispensers were shown to lure males. These dispensers were also much easier to load in the field and limited possible cross contamination. The design of these dispensers was registered in a provisional patent.

The pheromone release rate of both dispenser types were measured gravimetrically. This method allowed for an estimation of, at least, the upper limit of the release rates of different compounds. Such an estimate allowed for a comparison of the release rates between the two different dispenser types. Pheromone compounds with the acetate functionality were released at a rate of between 44 and 102 ng/hour for both dispenser types. This finding suggested that the released ratio may be closer to the loaded volumetric ratio but only for compounds with similar functionality. The degree of polarity of the dispensed pheromone or pheromone formulation was shown to have an effect on the recorded data, but this effect was only evident after blank correction was done and only for the permeation dispensers. It was observed that the presence of *Z*9-14:OH caused the permeation dispensers to gain mass and this phenomenon was attributed to the more hydrophilic nature of the polar alcohol functionality when compared to the acetate functionality. Water absorption from the atmosphere was the suspected cause.

It was hypothesized that female *S. noctilio* wasps might be able to detect volatiles from their obligate fungal symbiont, *A. areolatum*, as is known for some Siricids and their parasitoids. The volatile profile of the *Amylostereum* spp. was, therefore, characterised through GC-EAD and GC-MS. A prominent, unique chromatographic peak was identified and shown to be a sesquiterpnene of unknown structure. Subsequent electroantennographic experiments with both *S. noctilio* and its parasitoid wasp, *I. leucospoides*, showed that only the females of the parasitic wasp detect this peak. This finding confirmed the mechanism through which *I. leucospoides* wasps detect *A. areolatum*, and in the process also their *S. noctilio* prey. Future work will be aimed at characterizing this potentially unique sesquiterpene from *Amylostereum* species and investigating other volatiles that are potentially detected by *S. noctilio* females. Such volatiles have potential for enhancing trap efficiencies in future.

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The volatile profile of the egg clutches of *T. peregrinus* was investigated. These experiments were performend in order to determine the identity of compounds that may be involved in the egg laying behaviour of both these and the *C. noackae* parasitoid females. Analysis of the egg headspace samples revealed complex chromatographic profiles, with twenty-one tentatively identified compounds that were not found in blank samples. Compounds with confirmed identity included (±)-sulcatol, n-octanal, n-nonanal, n-decanal, cumnaldehyde and 2-undecanone. These compounds were released from the eggs at an average rate of 0.26 ± 0.23 ng/min.

The behavioural functions of these compounds were investigated in Y-tube olfactometer tests. These tests were done with single compounds and a mixture of these compounds. Results were not conclusive for both *T. peregrinus* and *C. noackae* females. Both species behaved erratically which was possibly the result of being stressed through handling them in the laboratory. It was concluded that the results may have been influenced by other factors besides the volatiles that were tested. Factors included unfavourable environmental conditions, unknown synergistic effects with other untested compounds and difficulties in handling small insects with short life cycles. It was also possible that *T. peregrinus* and *C. noackae* females did not rely on only the volatiles that were tested and the chemical signal may be more complex. Clearly further work is needed to elucidate the role of volatile compounds in this system. The enatiomeric ratio of sulcatol, which was not determined, could also play a significant role here. The results of this study provided a foundation for future work that should include electroantennographic experiments that may give a better indication of which of these compounds should be the focus on for future behavioural experiments.

Semiochemical signals between organisms have different levels of complexity. These levels are based on three main parameters that are defined by the presence of a volatile chemical, the concentration of that chemical and the presence or absence of other chemicals. The ratios between the chemicals seem to play important roles because they are involved in synergisms that occur for each species. The scope of the study only allowed the unraveling of these synergistic effects to a certain level. The information provided here, however, will provide the foundation to continue the complex task of studying the interaction between these volatiles and the behavioural response of the insects.

The work presented here took the first steps in the elucidation of semiochemical interactions for insect pests in the South African commercial forestry industry. Both the identity of volatile chemicals and their behavioural proof was desired. Such behavioural proof was difficult to obtain and could only be confirmed for the *C. trisitis* pheromone communication system. Other semiochemical interactions such as those between different species were shown to be more complex especially in cases where the interactions

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were not specific. These complexities arise after finding that samples contain a multitude of volatile chemicals from which a selection needs to be made for behavioural trials. This selection process was simplified through electrophysiological methods but only in some cases. Such complexities made the task of identifying an attractant formulation difficult. Success seems to rely on the thorough understanding of both entomological ecology and analytical chemistry. More intensive behavioural studies on the chemicals that were identified here are needed and requires intensive collaboration between behavioural ecologists and chemists. Such collaborations will result in converting more research results into applied science in future.

Appendix A

Optimization and synchronization of GC-EAD and GC-MS

A.1 Theory and experimental results

Optimization of a chromatographic instrument is an essential process that needs to be conducted before any separation is done. After optimization, a specific column head pressure or average linear flow rate can be chosen at a specific temperature. The choice depends on if one desires to work in constant pressure or constant flow rate. This allows separation to be done under set chromatographic conditions that gives the best efficiency (number of plates, N, in a given length, l, of column) at a specific temperature. In open tubular columns the number of plates in a column can be maximized when the plate height, H, is approximately equal to the column internal diameter. Plate height is simply defined as a chromatographic peaks' distribution variance (σ^2) per unit length (l) of the column (A.1).

$$H = \frac{\sigma^2}{l} \tag{A.1}$$

Minimizing plate height can be achieved by setting up a calibration curve known as the Van Deemter curve. Such a plot relates the average linear flow rate (distance travelled l, in cm per time taken t_r in seconds) to the plate height (H in cm) at a specific temperature, however, the plate height can not be measured from a chromatogram directly, rather it is calculated from the width at the base of a peak in a chromatogram. This can be done by firstly assuming a Gaussian distribution for a pure non-overloaded chromatographic peak. The width at the base (W) of such a peak, in time units, is equal to four times the standard deviation (4τ) in time units. The standard deviation in time

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units, τ, obtained from the width of a peak in chromatogram, is related to the standard deviation that occurs in distance σ inside the column through the inverse migration rate which is measured as distance moved (l) per unit time (t_r) (A.2).

$$\tau = \sigma \frac{t_r}{l} \tag{A.2}$$

and

$$W = 4\tau = 4\sigma \frac{t_r}{l} \tag{A.3}$$

therefore

$$\sigma = \frac{Wl}{4t_r} \tag{A.4}$$

substituting into equation (A.1) gives:

$$H = \frac{lW^2}{16t_r^2} \tag{A.5}$$

The number of plates (N) in a column of length (L) is the length of the column divided by the length of one plate (H) (A.6). This number is also related to the width of a chromatographic peak and therefore plate height.

$$N = \frac{L}{H} = 16\frac{t_r^2}{W^2} \tag{A.6}$$

The width at the base of a chromatographic peak is often more difficult to measure than the width at half the height and the number of plates can be approximated with another equation (A.7).

$$N = 5.54(\frac{t_r}{W_{1/2}})^2 \tag{A.7}$$

Equation (A.7) was used to set up the following Van Deemter plots for the GC-EAD and GC-MS instruments. Retention time and width at half height values were obtained by injecting an n- alkane solution at 80 °C at different column head pressures. Butane was used to determine average linear flow rate of the mobile phase (helium gas).

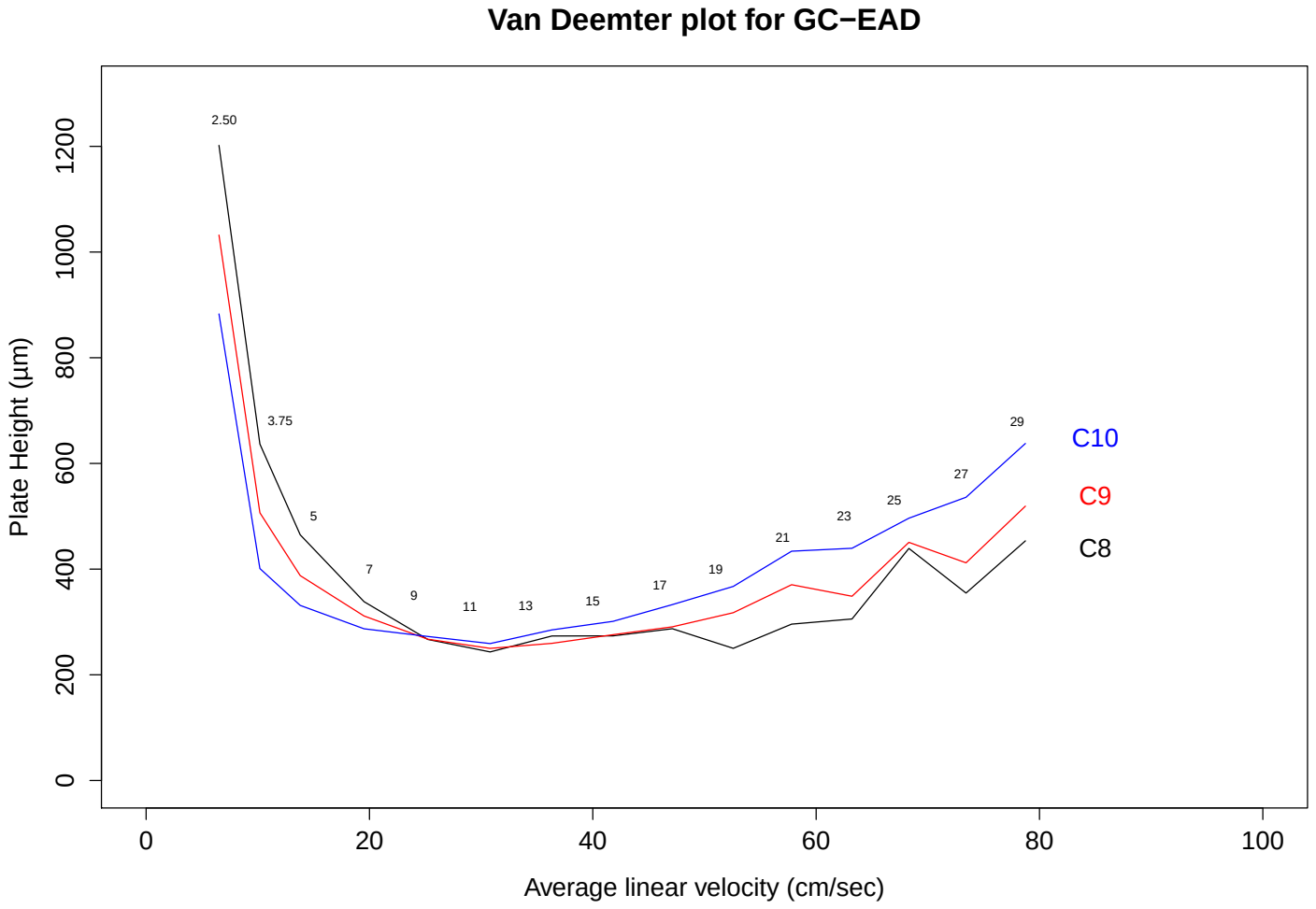

FIGURE A.1: Van Deemter curve for GC-EAD (Isothermal 80 °C, numbers refer to pressure in psi).

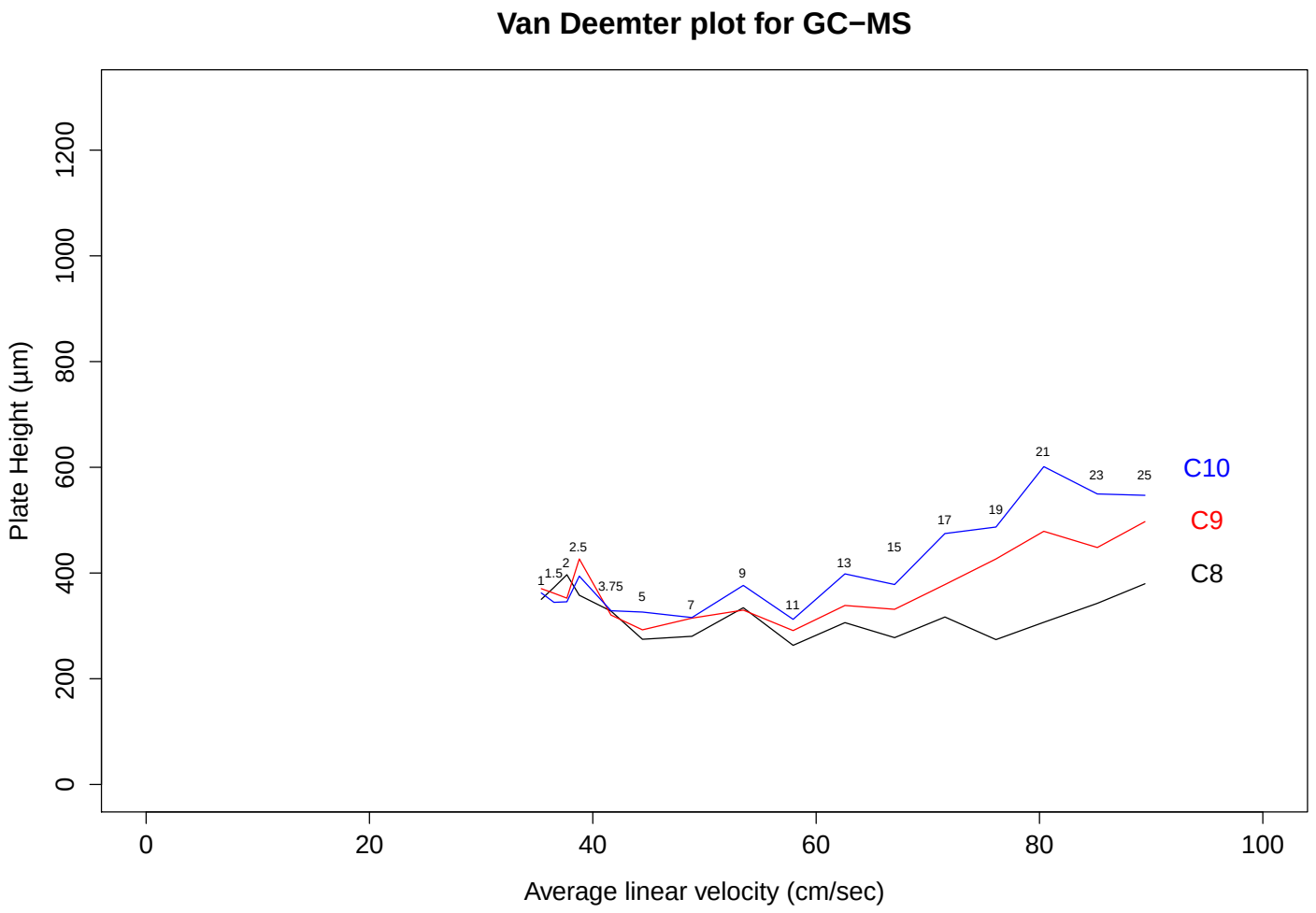

FIGURE A.2: Van Deemter curve for GC-MS (Isothermal 80 °C, numbers refer to pressure in psi).

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From figure A.1 and A.2 it was decided to use a constant column head pressure of 16 psi for the GC-EAD and 7 psi for the GC-MS system. These pressures roughly correspond to 48 cm/sec average linear velocity at 80 °C on both systems (Figure A.3). The column head pressure used in these experiments was different for the two systems due to the difference in the pressure at the column outlet. Optimization in this way ensured that both systems operated under the same resolution capabilities and allowed for accurate matches between retention times/indexes which was critical when peaks were located in unknown samples analyzed on both systems with the same column type.

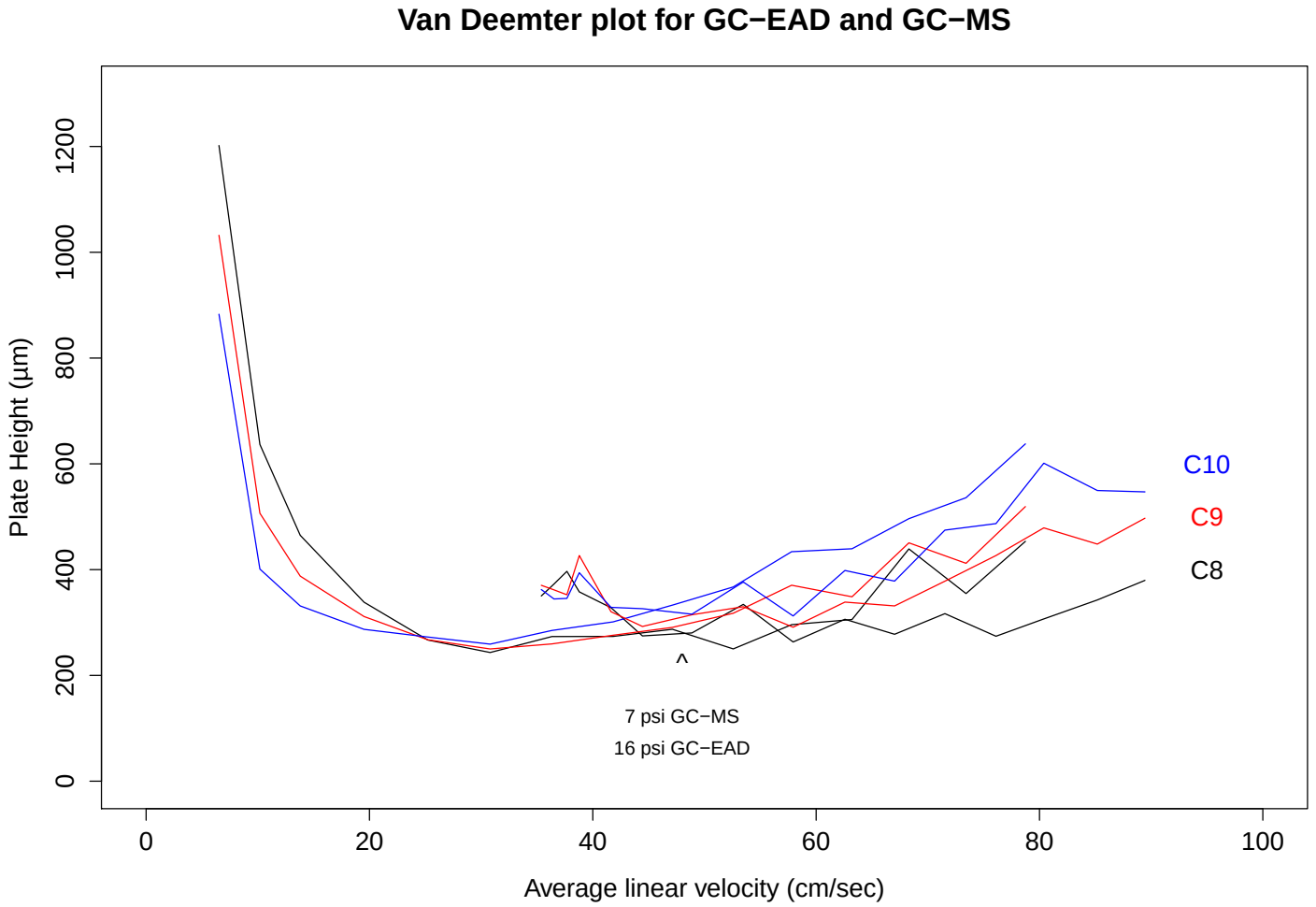

FIGURE A.3: Combined Van Deemter curves for the GC-EAD and GC-MS (Isothermal 80 °C, arrow indicates the chosen pressure for both instruments).

Appendix B

Construction of wind tunnel for behavioural experiments

B.1 Wind tunnel construction

A wind tunnel is a common tool that is employed in chemical ecology laboratories in order to investigate insect behaviour. These tunnels may have a variety of different shapes, but certainly the most common type is simply a rectangular box of certain diameters with a fan on one side to provide air flow. A simple design was published by Miller and Roelofs (1978). Miller and Roelofs (1978)'s tunnel was constructed from clear Plexiglass sheets and had a length of 2.44 m and was 0.89 m wide and 0.96 m high (Figure B.1).

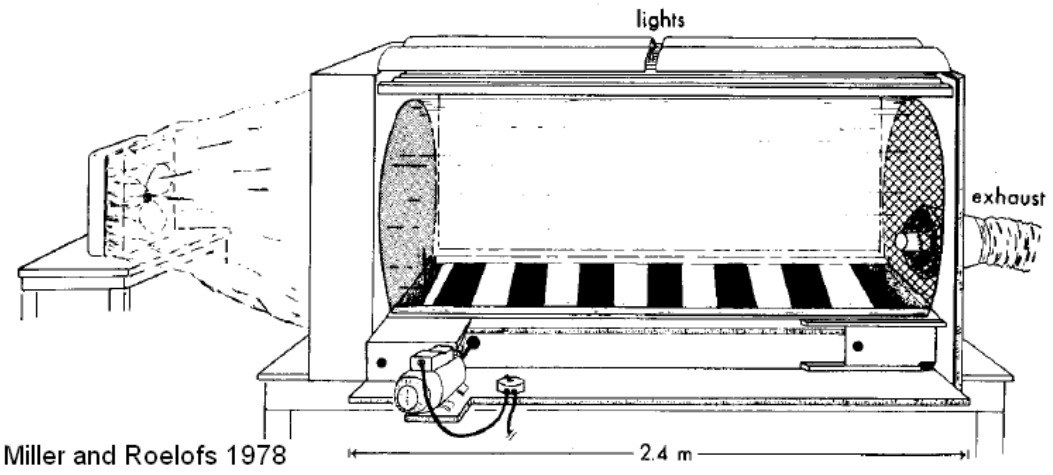

FIGURE B.1: A flight tunnel design by Miller and Roelofs (1978).

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Turbulence in the air flow from the fan is limited by a turbulence damper. Such a device is constructed through layering a series of mesh grills with different pore sizes. Two layers of cheese cloth (9 mesh/cm) tightly stretched over the opening inside the tunnel functions as a damping screen (Miller and Roelofs, 1978). Flow rates within the tunnel are typically in the range of 50 cm/sec. For example Miller and Roelofs (1978) set their flow to approximately 47 - 65 cm/sec. Pheromone plumes were vented from the tunnel to outside through a 30 cm diameter exhaust coupled flexible tubing and another fan Miller and Roelofs (1978). This exhaust was placed directly opposite and downstream of the pheromone plume that exited the tunnel.

Light within the tunnel was provided by 12 1.2 m florescent bulbs 14 cm above the tunnel. The light was allowed to pass through a light diffuser that was constructed from corrugated fiberglass (Miller and Roelofs, 1978). Light intensities could be varied depending on the type of insect being tested. For daytime flying insects the light intensity was set between 500 and 700 lux (Miller and Roelofs, 1978). Light intensity as low as 0.05 lux could be used for night flying insects (Miller and Roelofs, 1978).

B.1.1 Wind tunnel development

The wind tunnel that was manufactured for our labouratory was heavily influenced by the designs of Erling Jirle from Lund University (Figures B.2, B.3, B.4).

Our tunnel was constructed from 3.01 X 0.480 X 0.008 m perspex sheets within a galvanized steal frame (Figure B.5). This tunnel had approximately the same dimensions as the primary fan (AMS, HXM 350 model, 465 X 390 mm) that was used to blow air into the tunnel. Overlapping sliding doors were also manufactured from the same perspex sheets and were installed on one side of the tunnel. Fine steel mesh grids were installed at both ends of the tunnel. A flow damper, made from doubly folded cheese cloth wrapped around a steel frame, was installed in the front (near fan) end of the tunnel.

The light source was made from another perspex sheet with slightly larger dimensions than the tunnel itself. This sheet was housed inside another metal frame with natural white LEDs (LED lighting SA, Seol Semiconductor) that were fixed between the frame and the perspex sheet edge. The LED's were spaced 15 mm apart on each LED strip. This setup was intended to function as a fiber optic (internal reflection throughout optically dense perspex sheet) light source that provides even intensity light throughout the tunnel.

In order to get rid of tested odours a steel funnel with approximately the same dimensions as the tunnel was placed over the downstream end of the tunnel. This funnel was linked to an exhaust fan, positioned outside the room, through flexible hose tubing.

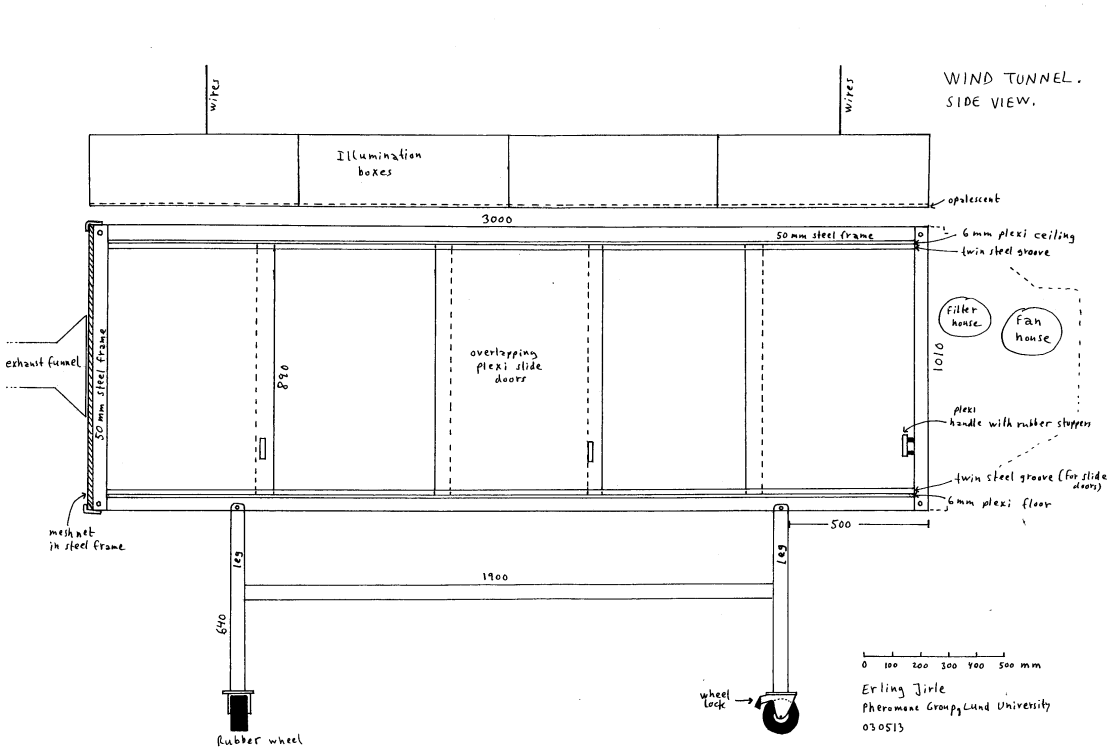

FIGURE B.2: Flight tunnel design 1 by Erling Jirle

B.2 Wind tunnel standardization

The wind tunnel was standardized based on two variable parameters: light intensity and wind speed. Light intensity was recorded throughout the tunnel as shown in Figure B.6 and wind speed was recorded at two positions (one meter from the middle of the tunnel) in the center of the tunnel. Temperature inside the room was controlled to 25 °C with two air conditioners.

B.2.1 Wind tunnel light

Light intensity was controlled with a 12 V dimmer and it was set to one lux as measured (TM-209 digital light meter) at the base of the tunnel. A one lux light intensity corresponds roughly with that of the light intensity of the full moon at night and it was used

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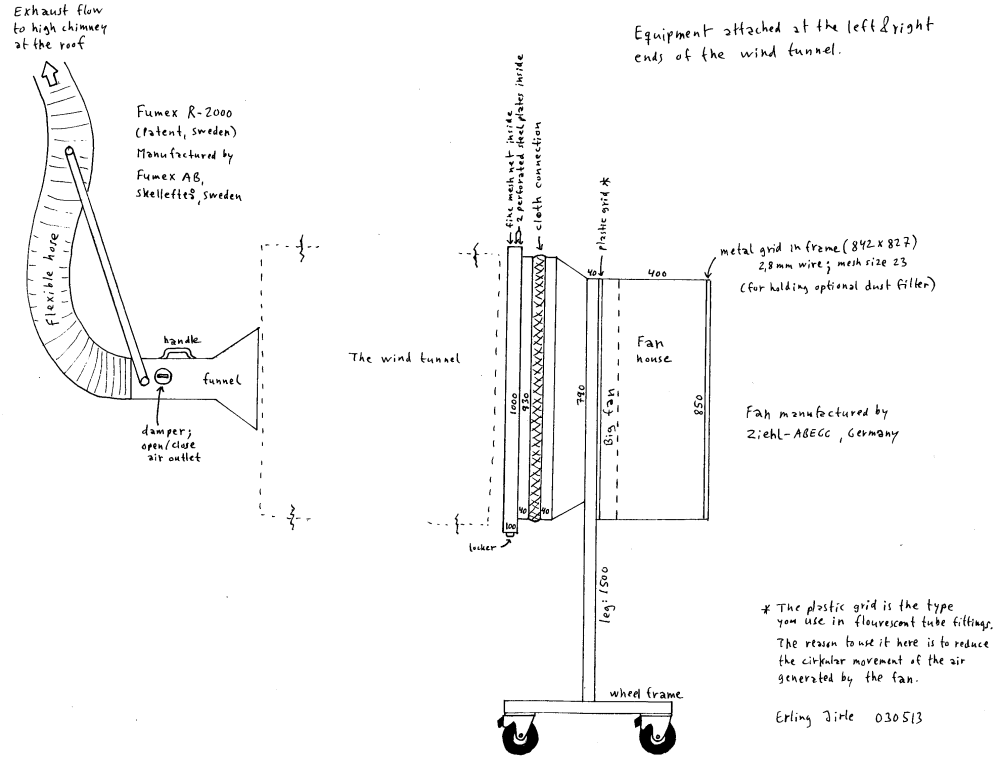

FIGURE B.3: Flight tunnel design 2 by Erling Jirle

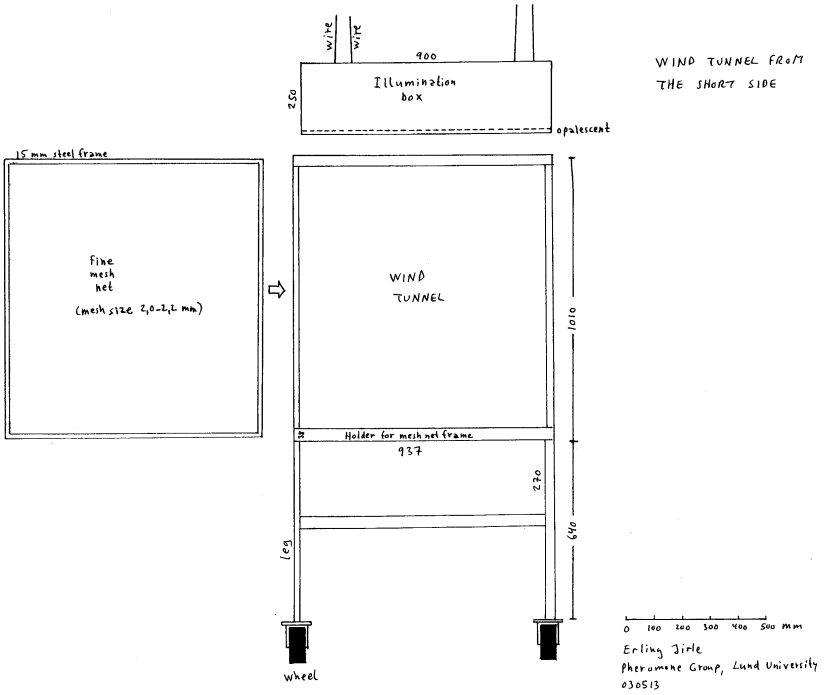

FIGURE B.4: Flight tunnel design 3 by Erling Jirle

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FIGURE B.5: Custom made wind tunnel at the FABI experimental farm.

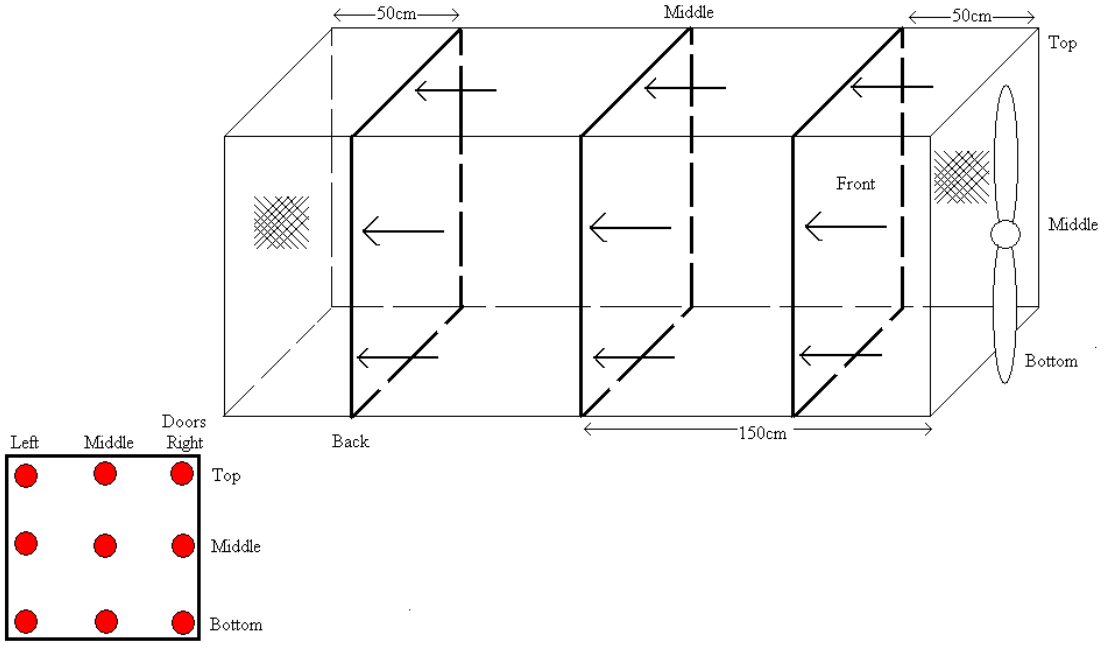

FIGURE B.6: Flight tunnel schematic that indicates how the tunnel was split up into sections for recording of light intensity and wind speed.

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as a baseline parameter for night flying insects. Recordings were taken at three levels vertically and horizontally within the tunnel (Figure: B.6, B.7).

Light intensity was relatively stable at the bottom and middle of the tunnel especially when comparing the measured recordings at a set height. Light intensity was significantly higher close to the sides at the top of the tunnel when compared to the recordings taken from the middle top position. This was expected since it was the closest position to the light source. Light intensity varied between one and two lux from the bottom to the top of the tunnel.

B.2.2 Wind tunnel air speed

Air speed was controlled with two auto-transformers (Variacs) each linked to either the front or exhaust end fans. The fan speed of these two fans could therefore be controlled separately.

The exhaust fan was set to 70 % of maximum output in the first calibration and then to 80 % in the second calibration. The main fan speed was then varied between the range of 180 and 240 V. Recordings were taken with a vane probe anemometer at two different positions within the tunnel. The first position (front) was located one meter upwind and the second position (back) was located one meter down wind from the mid point along the length of the tunnel (Figure B.8).

A flow rate of approximately 30 cm/sec was desired for these experiments. According to these calibrations this flow rate could be selected only when the back Variac was set at 70 % and the front Variac was set at 180 V. These calibrations also show that the flow rate varied non-linearly as the main fan speed was increased. The increase in flow rate seemed to decrease at higher voltages of the main fan. This non-linearity in the flow rate was expected.

A difference in flow rate (at a certain setting) between the two positions in the tunnel was also observed. The difference in flow rate was ascribed to the smaller diameter of the outlet (when compared to the main tunnel) part of the tunnel. This diameter difference caused a decrease in the internal volume at the end of the tunnel and therefore increased flow rate for the same volume of air. This difference was however within acceptable limits for our purposes.

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B.3 Results

Many tests were conducted in the flight tunnel especially for the *C. tristis* pheromone formulations that were suggested by GC-EAD and GC-MS analysis. Most of these tests had varying results. A number of reasons for the varying results were suggested.

Male *C. tristis* moths were observed to show some behaviour that indicated that they detected the presence of pheromone chemicals. This type of behaviour was associated with wing fanning and antennal grooming and sometimes flight toward the stimulus. These behavioural observations were often only observed during the first exposure to pheromone chemicals. It was suggested that such observations indicate that males experienced some form of habituation of pheromone perception. Other possible factors included the fact that the first formulation tested in a specific experiment may not have been the correct formulation. This first formulation may have included pheromone compounds that inhibit attraction. These types of factors had effects that linger in time and could have influenced subsequent tests especially if the same male moths were tested more than once.

The state of the male moths being tested may have varied between individuals. Factors that influenced the state of the moths included unforeseen stresses that may have been created through laboratory rearing and handling. Differences in the age of different males may have also confounded some results. It was observed that only certain males of a group of males that were tested *C. tristis* took flight in the direction of the stimulus being tested. These males never landed on the pheromone source and it was possible that the pheromone plume structure and frequency (see 1.8.1.3) were not correct.

Due to these factors it was concluded that individual formulations needed to be tested singly each night on insects with the same internal state. In future, male moths should be tested one at a time and they should be acclimatized to conditions within the tunnel before any tests are conducted.

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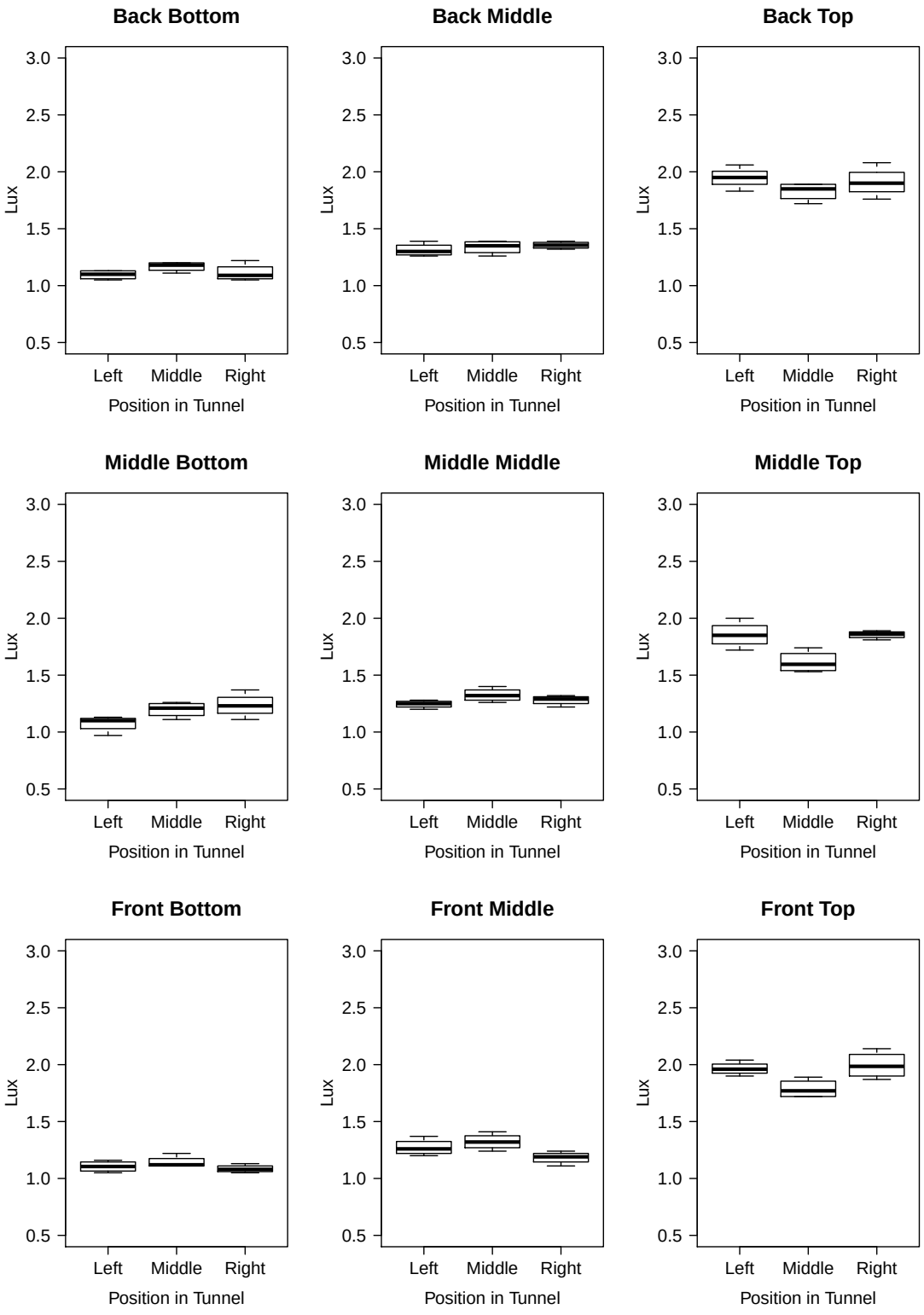

FIGURE B.7: Box plots of flight tunnel light intensity measurements at different positions in the tunnel (n = 4)

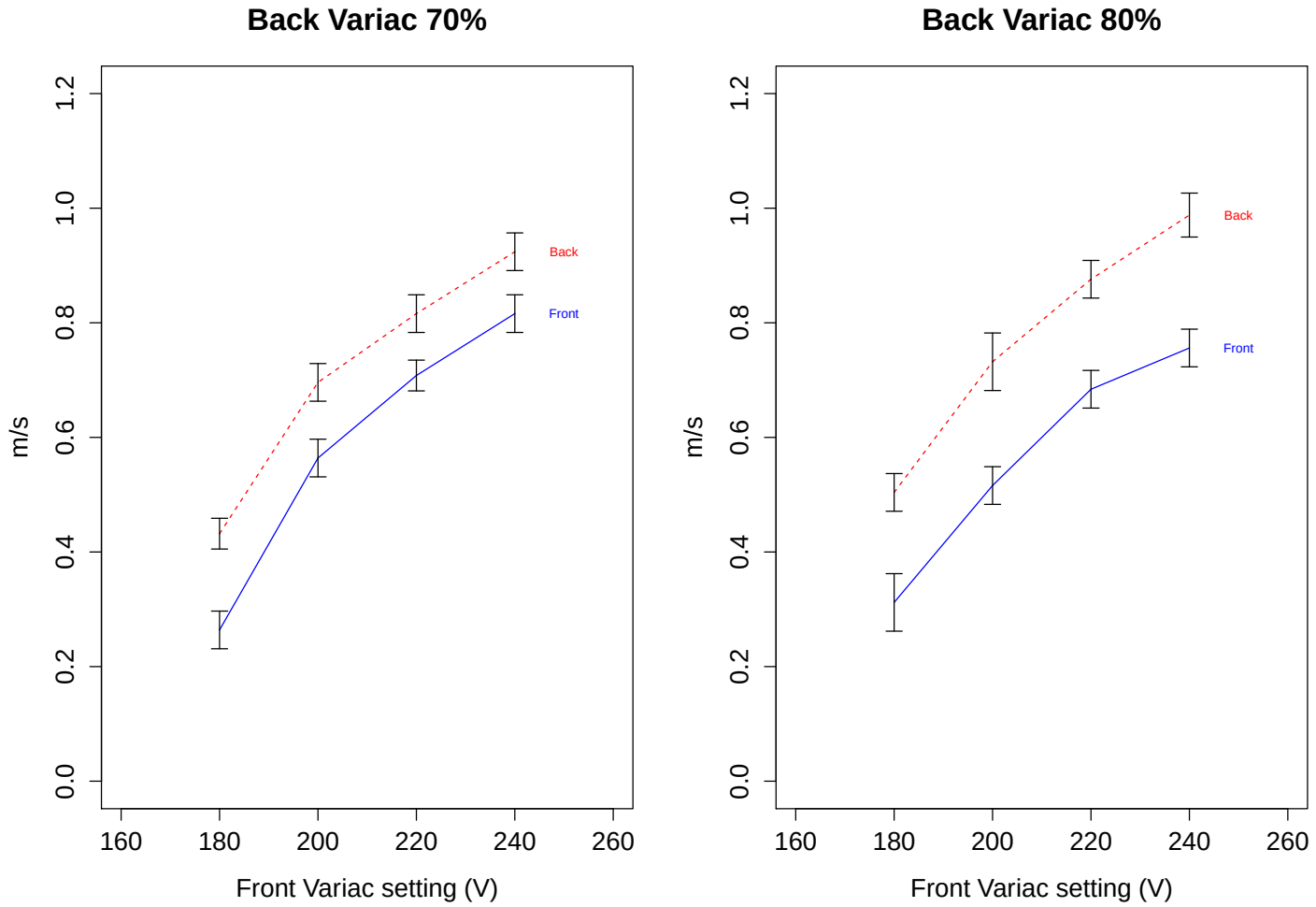

FIGURE B.8: Air velocity measurements taken in the middle position 1 m upwind (Front) and downwind (Back) from the middle of the tunnel (n = 5, SD)

Appendix C

Pheromone release rate study of natural red rubber septa and methyl silicone rubber permeation devices as artificial pheromone dispensers for *Coryphodema tristis*

C.1 Introduction

During 2011 a field trial was conducted with three of the identified potential pheromone components for *Coryphodema tristis* (Chapter 3). Red rubber septa were used as pheromone dispensers. Results from the field trial were not encouraging because only two male insects were caught with the pheromone dispensers and one male was caught in a blank dispenser (only n-hexane) trap. These negative results led to a gravimetric release rate study because it was not known if the pheromones were being released from the septa and at what rate they were being released.

A different pheromone dispenser was used in a subsequent field trial during 2013. These dispensers were custom made from glass capillaries and methyl-silicone rubber tubes. This new dispenser design was used for two reasons. Firstly, to eliminate the presence of solvent that was needed when loading the natural red rubber dispensers. A second aim was to use permeation as a mechanism to create a zero order release rate of the

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pheromone. Here we investigate how pheromone compounds are released from these two dispenser types gravimetrically.

C.2 Materials and methods

C.2.1 Natural red rubber septa

Five replicate dispensers (red rubber septa, Insect Science in Tzaneen) were prepared in the laboratory for each pheromone component namely: *Z*9-tetradecenyl acetate, *E*9-tetradecenyl acetate, tetradecyl acetate and blanks. These septa were impregnated with 1 μl (870 μg for *Z*9-14:OAc, 870 μg for *E*9-14:OAc, 865 μg for 14:OAc) dispensed in 20 μl of n-hexane as a solvent. Blank septa were loaded with the same volume of the n-hexane solvent only. These septa were weighed before pheromone was loaded and at regular intervals spanning a period of 2350 hours after loading the septa (Figure C.1, C.2). A Mettler Toledo XP6 analytical mass balance with an accuracy of 1 μg was used during the experiment. The septa were kept in a temperature controlled room at 25 $\pm$ 1.9 °C (mean $\pm$ stdev, n = 22).

Correction for both the septum degradation and evaporation of n-hexane was done by subtracting the masses of the blank septa from the masses of the septa that contained the pheromone components. This correction was made on the basis of two assumptions; firstly, that the n-hexane should evaporate approximately equally between pheromone impregnated septa and blank septa; secondly, that the mass difference between blank septa and pheromone loaded septa represented the approximate mass of the pheromone compounds that were loaded into each septum (note that septa were chosen with approximately the same starting mass to minimize errors in this correction procedure). The large variation in mass that occurs before $\pm$70 hours was eliminated by removing the first three data points of each septum. Linear regression lines were fitted through these data points using R version 3.1.0. The release rates were estimated from the regression line slopes.

C.2.2 Methyl silicone permeation devices

Dispensers were made from glass capillary tubes (10 mm by 1.5 outer diameter by 0.8 mm Id, $\pm$5 μl internal volume) with one 30 mm long methyl silicone rubber tube (2.16 outer diameter by 1.02 mm i.d, Carlin Medical Extrusions) attached to both ends to form a seal. The glass capillary served as a reservoir for the pure pheromones and mixtures and the silicone served as a membrane through which the pheromones would permeate.

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A zero partial pressure of the pheromone compound outside the dispenser membrane was assumed and each silicone membrane of each dispenser was assumed to be of equal thickness. These assumptions are of course not true, but they are relatively safe given the fact that dispensers were kept in an air conditioned room and that they were made from the same batch of silicone rubber.

This study included four pure pheromone compounds, *Z*9-14:OAc, *E*9-14:OAc, 14:OAc, *Z*9-tetradecenol and a 1:1:1 mixture of *Z*9-14:OAc, 14:OAc and *Z*9-14:OH (by volume). The mixture represented one of the successful treatments during the 2013 field trial (treatment 7). Five replicates of each dispenser type containing 1 μl of pheromone was made including blank (empty) treatments. One μl of each pheromone corresponds to 870 μg for *Z*9-14:OAc, 870 μg for *E*9-14:OAc, 865 μg for 14:OAc and 846 μg for *Z*9-14:OH by mass. The mixture contained 290 μg for *Z*9-14:OAc, 282 μg for 14:OAc and 288 μg for *Z*9-14:OH (total 860 μg). The data was accumulated over a period of 2475 hours and measurements were done in the same manner as for the natural red rubber septa. The dispensers were kept in a temperature controlled room at 21.5 $\pm$ 1.6 °C (mean $\pm$ stdev, n = 48). Blank correction was conducted in the same manner as for the red rubber septa (Figure C.3, C.4. Regression equations and associated statistical parameters for each fit was determined in R version 3.1.0.

C.3 Results

C.3.1 Natural red rubber septa

The mass of the pheromone component and n-hexane solvent in each septum could be obtained by subtracting the mass of the unloaded septum from the loaded septum. This procedure was also completed for blank septa and this result shows the mass of the n-hexane carrier solvent only. The plots of these masses show the expected exponential release rate that was characterized by the decreasing negative slope as time progressed (Figures C.1, C.2). The plot of the blank revealed evidence of septum degradation because the mass reached a point in time (below 0 mass) where it was less than the original unloaded mass of the septum (Figure C.2). This point occured at approximately a 140 hours ($\approx$ 6 days) after loading the septa and it was expected that the n-hexane carrier solvent should have evaporated to near completion after that time, although it was not directly measured. The release rate was estimated to be 44 $\pm$ 36, 65 $\pm$ 27 and 52 $\pm$ 26 ng/hour for *E*9-14:OAc, *Z*9-14:OAc and 14:OAc respectively using this method (Table C.1).

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C.3.2 Methyl silicone permeation devices

A steady mass loss of all dispensers was observed before blank correction (Figures C.3, C.4). This mass loss was partially due to degradation of the permeation disperser itself, evidenced by the mass loss observed for the blank dataset (Figure C.4). After blank correction mass loss was evident only for *E*9-14:OAc, *Z*9-14:OAc and 14-Ac (Figure C.3) whereas the alcohol, *Z*9-14:OH and the mixture showed an unexpected steady mass gain (Figure C.4).

These curves showed that the change in mass for the dispensers was mostly constant over time (apart from the very first few data points). The release rate was estimated to be 102 ± 9, 62 ± 9 and 49 ± 16 ng/hour for *E*9-14:OAc, *Z*9-14:OAc and 14:OAc respectively when using this method (Table C.1). The mass gain of the alcohol, *Z*9-14:OH, and mixture was estimated at 38 ± 18 and 59 ± 6 ng/hour (Table C.1).

C.4 Discussion

The release rates of four different pheromone compounds and one mixture were measured for methyl-silicone rubber permeation dispensers. The release rates of three pheromone compounds were measured for natural red rubber dispensers. These rates were determined through a gravimetric method. The release rates of the pure pheromone components were below 100 ng/hour for all three compounds tested with the natural red rubber septa. The release rates of the same compounds were in the same order magnitude for the methyl silicone rubber permeation devices, however, a mass gain was noted for devices containing the alcohol, *Z*9-14:OH pheromone compound and the mixture of the two acetates, *Z*9-14:OAc and 14:OAc, and the same alcohol compound. This alcohol and mixture was not tested with the natural red rubber septa.

A first order release rate was expected for the compounds that were tested with the natural red rubber septa. For these septa the release rate of a compound, at a set temperature, should be defined by both the amount of compound that was loaded and the solubility of the compound in the rubber. These curves were therefore expected to have a first order release rate. This first order shape of the curve was evident especially before the blank correction procedure and it was attributed mostly to the release of the large quantities of solvent used to load the septa. Blank correction removed most of this effect, nevertheless it was still evident at early time points. A linear approximation was used to estimate the release rate of the pheromone compounds, yet, this methodology approximates the true value more accurately only when the curves become more linear. Linearity was observed due to the fact that small quantities were released relative to

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the amount which was loaded in the dispenser. It is expected that a longer time scale of measurement should reveal the expected first order release rate. A moving average of the slope of these lines may give a more accurate representation of the release rate and how the release rate changes in time.

For the permeation dispensers it was expected that the release rate of a compound, at a set temperature, should be defined by its vapour pressure and its ability to permeate through the membrane barrier. Vapour pressure inside the dispenser should be at equilibrium at a set temperature making it relatively constant. The permeation flux of the compound across the membrane barrier then should become a function of the compound permeability constant for a specific membrane polymer. Factors that influence the flux are the membrane thickness and the difference between the partial pressure across the membrane barrier (Von Wroblewski, 1879). At equilibrium vapour pressure the release rate of a compound should not be a function of the amount of substance loaded into the dispenser. The release rate should remain constant until the vapour pressure difference across the membrane barrier changes. It was expected that compounds with similar vapour pressures and permeability should be released at similar rates from the same dispensers types (meaning same membrane polymer). This was seen from the very similar slopes of each of the acetate replicate dispensers, see for example *Z*9-14:OAc and *E*9-14:OAc.

This permeation effect could have occured both ways across the membrane and it may explain why a mass gain was observed for the alcohol compound and mixture that also contained the alcohol. It was possible that the hydrophilic nature of the alcohol, *Z*9-14:OH, caused it to absorb water vapour inside the permeation tube. This may have caused water molecules to permeate to the inside of the dispenser. This absorption of water may have occurred at a faster rate than the rate at which pheromone was lost to the outer atmosphere. It may also be that the positive slope was artificially created by our blank correction procedure, but it was not observed for the other acetate treatments. Whatever the real cause this effect diminished the ability to measure the release rate of *Z*9-14:OH and the mixture.

The release rates measured in this study are based on mass loss only and although corrections for mass loss that occurs due to effects such as solvent loss and degradation of the rubber polymers was done, actual release rate values of the pheromone compounds are expected to be lower than what was measured. Compound degradation and changes in polymer chemistry may have affected how the pheromones dissolved in the polymers and this phenomenon could not be accounted for in this experiment. Nevertheless, our values do give an upper limit to the actual release rate.

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A study such as this coupled with a headspace sampling procedure and chromatographic quantification of each compound from the dispensers would give more accurate release rate values for each compound. Such a study would also show discrepancies between the gravimetric method and the chromatographic method. The chromatographic method would also confirm if the released ratio of a mixture of pheromone compounds was mirrored between that which was loaded in the dispensers and that which was released from the dispensers.

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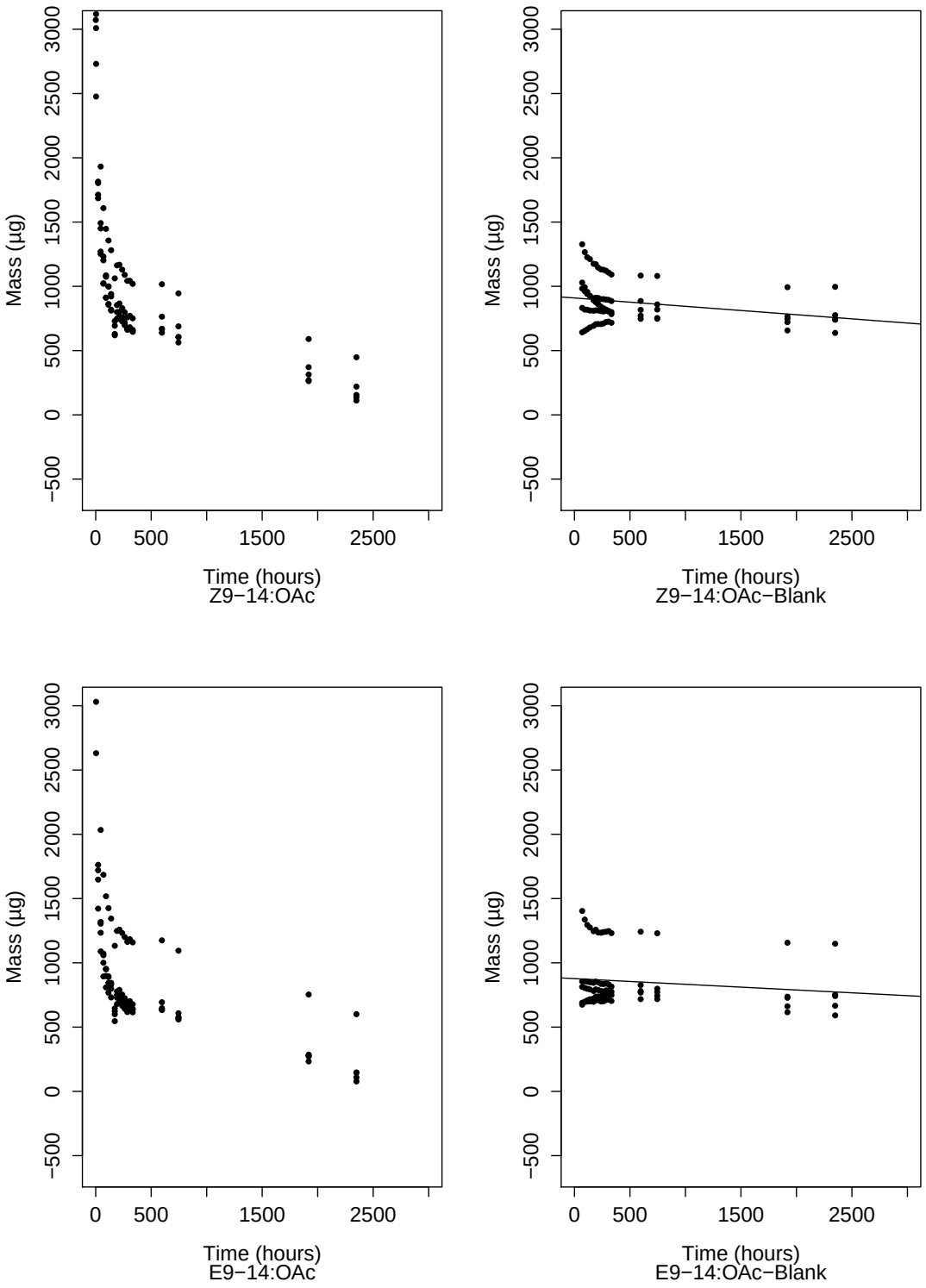

FIGURE C.1: Estimated mass of pheromone compounds in red rubber septa with fitted regression lines. Each device was impregnated with 1 μl of each pheromone treatment dispensed in 20 μl n-hexane as solvent. Blank corrected data is shown on the right-hand side (N = 5)

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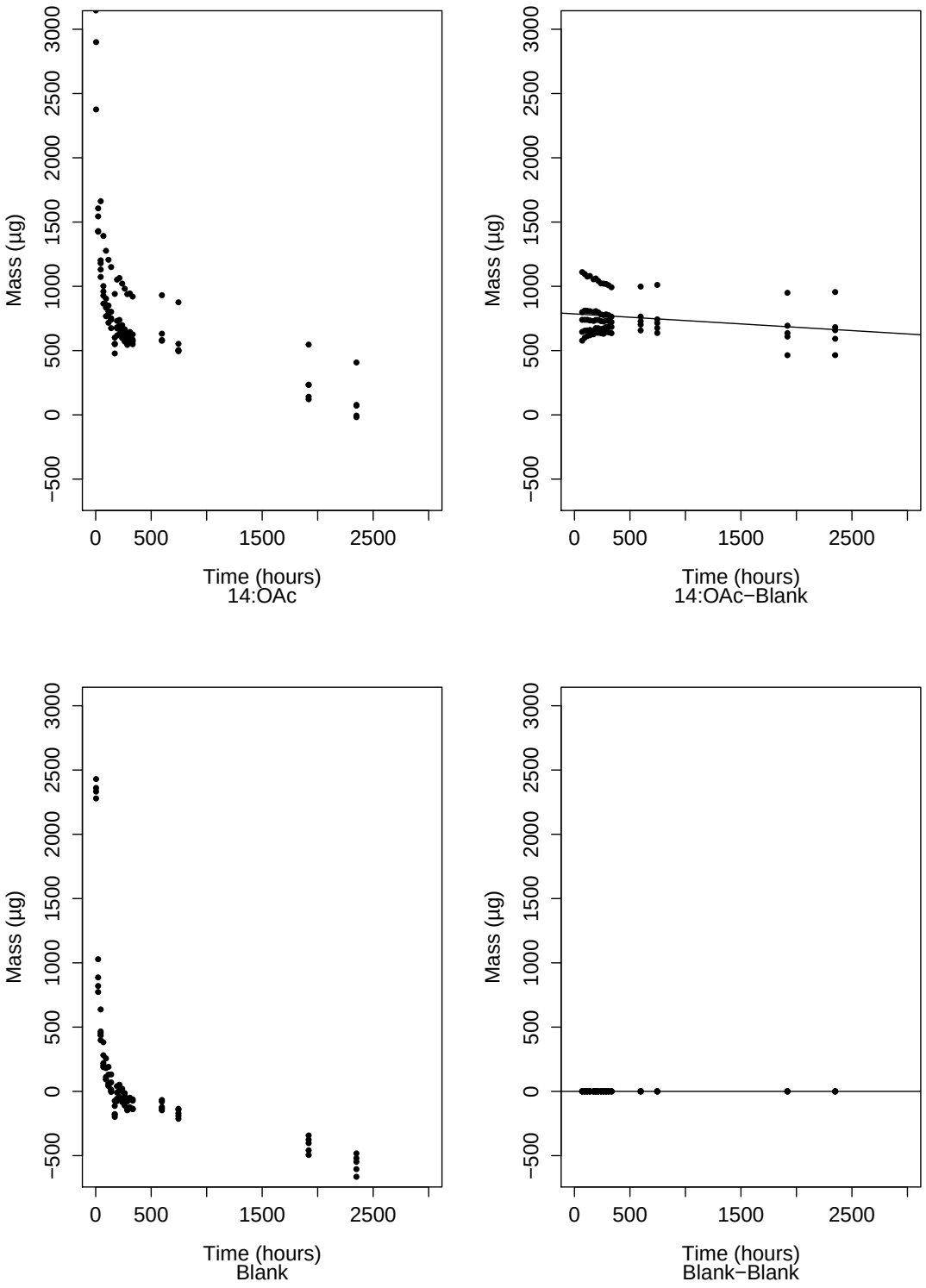

FIGURE C.2: Estimated mass of pheromone compounds in red rubber septa with fitted regression lines. Each device was impregnated with 1 μl of each pheromone treatment dispensed in 20 μl n-hexane as solvent. Blank corrected data is shown on the right-hand side (N = 5)

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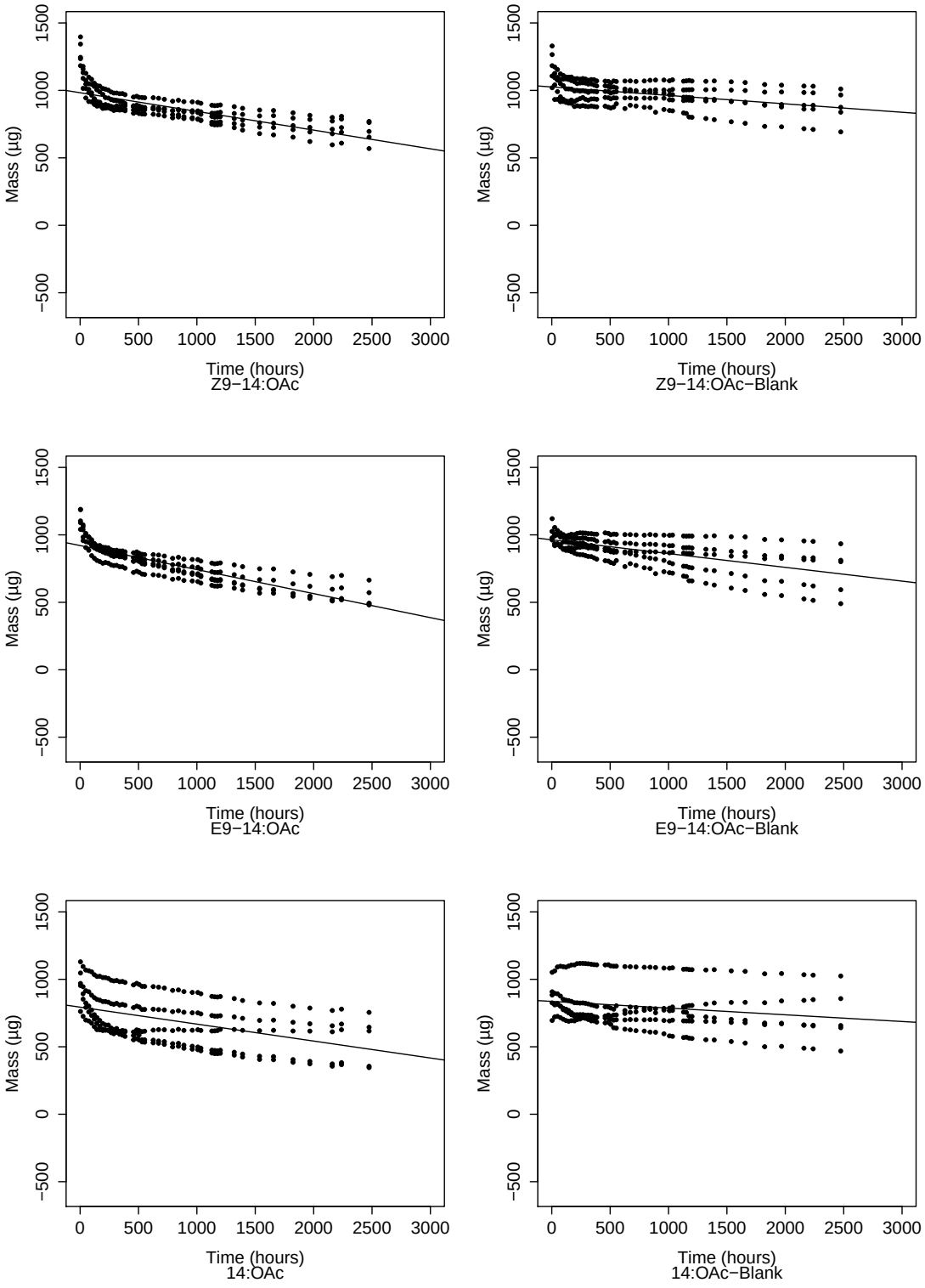

FIGURE C.3: Estimated mass of pheromone compounds in silicone permeation devices with fitted regression lines. Each device was impregnated with 1 μl of each pheromone treatment. Blank corrected data is shown on the right-hand side (N = 5)

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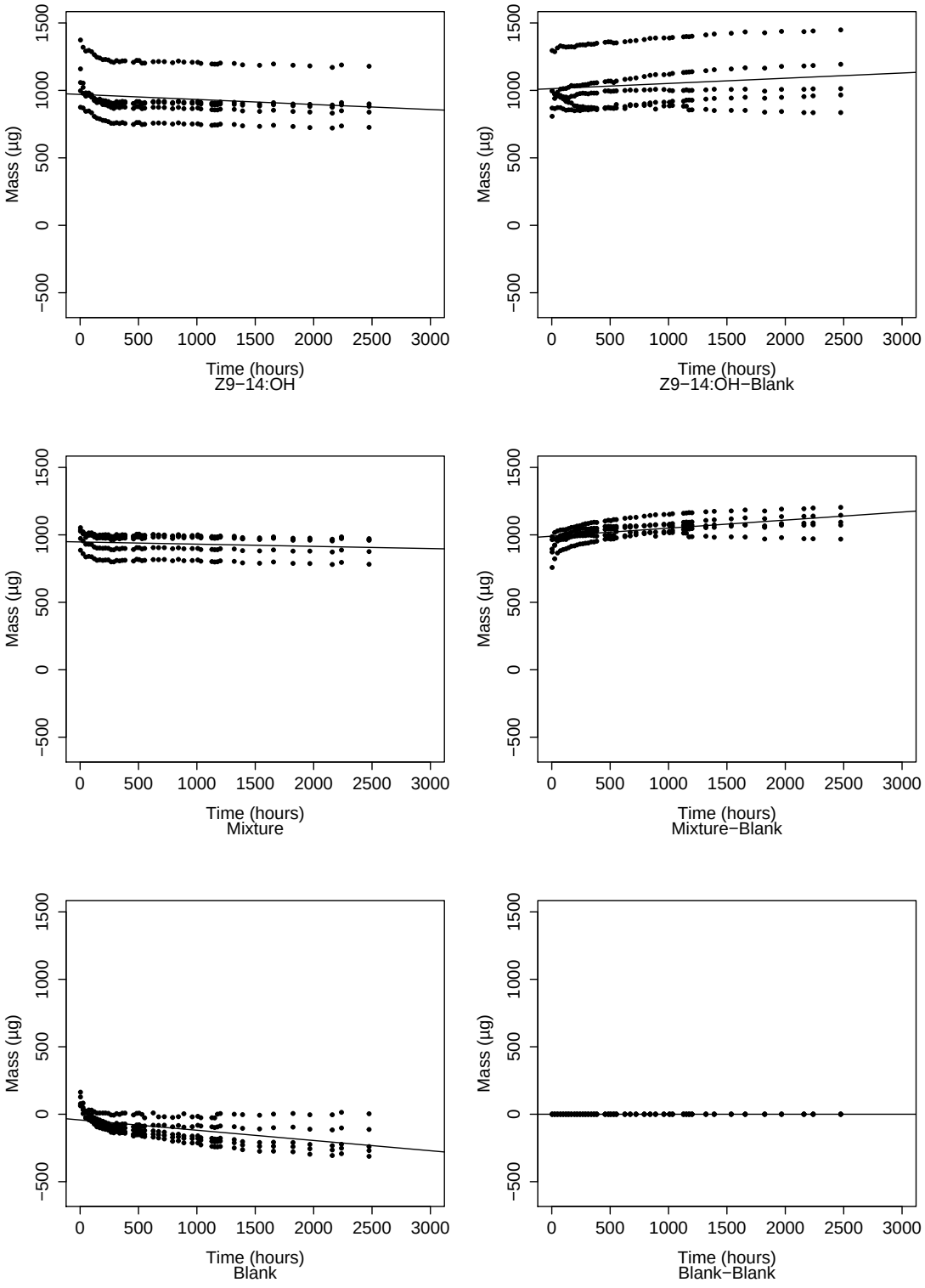

FIGURE C.4: Estimated mass of pheromone compounds in silicone permeation devices with fitted regression lines. Each device was impregnated with 1 μl of each pheromone treatment. Blank corrected data is shown on the right-hand side (Mean ± SD, N = 5)

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TABLE C.1: Estimated release rates from the blank corrected data set for each pheromone compound in the two different dispenser types. The mixture was made from *Z*9-14:OAc, 14:OAc and *Z*9-14:OH (1:1:1 by volume).

			Intercept (μg)				Slope (μg/hour)				Fit parameters	
Compound	Type*	R^2 (adj)	Estimate	Std. Error	t value	P value	Estimate	Std. Error	t value	P value	F ratio	P value
*E*9-14:OAc	RR	0.0063	877.11	29.44	29.80	<0.001	-0.0442	0.0360	-1.23	0.224	1.503	0.224
*Z*9-14:OAc	RR	0.0560	909.33	22.26	40.84	<0.001	-0.0650	0.0273	-2.39	0.0195	5.688	0.020
14:OAc	RR	0.0362	785.46	21.34	36.80	<0.001	-0.0520	0.0261	-1.99	0.05	3.963	0.050
*E*9-14:OAc	SPD	0.3373	963.32	9.83	98.02	<0.001	-0.1021	0.0096	-10.61	<0.001	112.5	<0.001
*Z*9-14:OAc	SPD	0.1788	1025.00	9.15	112.06	<0.001	-0.0625	0.0090	-6.98	<0.001	48.69	<0.001
14:OAc	SPD	0.0348	836.43	17.00	49.21	<0.001	-0.0497	0.0167	-2.98	0.003	8.897	0.003
*Z*9-14:OH	SPD	0.0144	1013.00	19.30	52.49	<0.001	0.0387	0.0189	2.05	0.042	4.191	0.042
Mixture	SPD	0.2907	990.22	6.39	154.93	<0.001	0.0596	0.0063	9.53	<0.001	90.77	<0.001

* RR refers to Red Rubber Septa and SPD refers to Silicone Permeation Device
Adjusted R^2 values calculated for the average of all data points
and are therefore lower than individual R^2 values

Appendix D

Electron microscopy of *Coryphodema tristis* antennae

D.1 Introduction

Scanning electron microscopy (SEM) was conducted on removed antennae of both males and females of *Coryphodema tristis*. This work was conducted in order to obtain a better understanding of the differences in antennal morphology between males and females of this species.

D.2 Methodology

Antennae of both males and females (n = 5) were removed by cutting at the base of the antenna with a surgical blade. These antennae were air dried and mounted on a glass microscopy slide with Japan Gold size glue (Winston & Newton). A gold plasma (25 mA, 0.1 Torr, Argon, Emitech K550X) was used to coat them twice. The antenna of *C. tristis* is quite large (up to 10 mm) and sections of the antenna were visualized at a time.

D.3 Results

Scanning electron microscopy images of antennae of females and males show clear differences in their morphology. Females and males have 60 + 1 (tip) segments on their antennae, but male segments are split into two separate rod like structures located on

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both sides of the antenna like a fan (Figure D.1, D.2). The larger male segments occur in the middle of the antenna and are approximately 600 μm long. Female segments are approximately 200 μm long.

Both male and female antennae have scales on the main branch of the antenna. There are at least two different types of sensilla present on both the male and female antenna. In males the most abundant sensilla occur all over each segment with a different type of sensillum (thicker) at the midpoint and tip of each segment branch. Females have similar sensilla types, however the two branches (of the fan structure) are fused and the thicker sensilla occur 4 times on each segment. Two of these occur along the midpoint and sides and one on the two corners of each segment. A larger number of the thicker sensilla occur on the last segment of both male and female antennae.

The function of the different sensilla is unknown at this stage but is suspected that the most abundant sensilla on male antennae are probably involved in pheromone perception. The most abundant sensilla on female antennae are most likely involved in detecting volatiles originating from potential host plants. The thicker sensilla are suspected to function as mechano-receptors.

These morphological differences between male and female antennae possibly contribute to the differences in their relative sensitivity to pheromone compounds.

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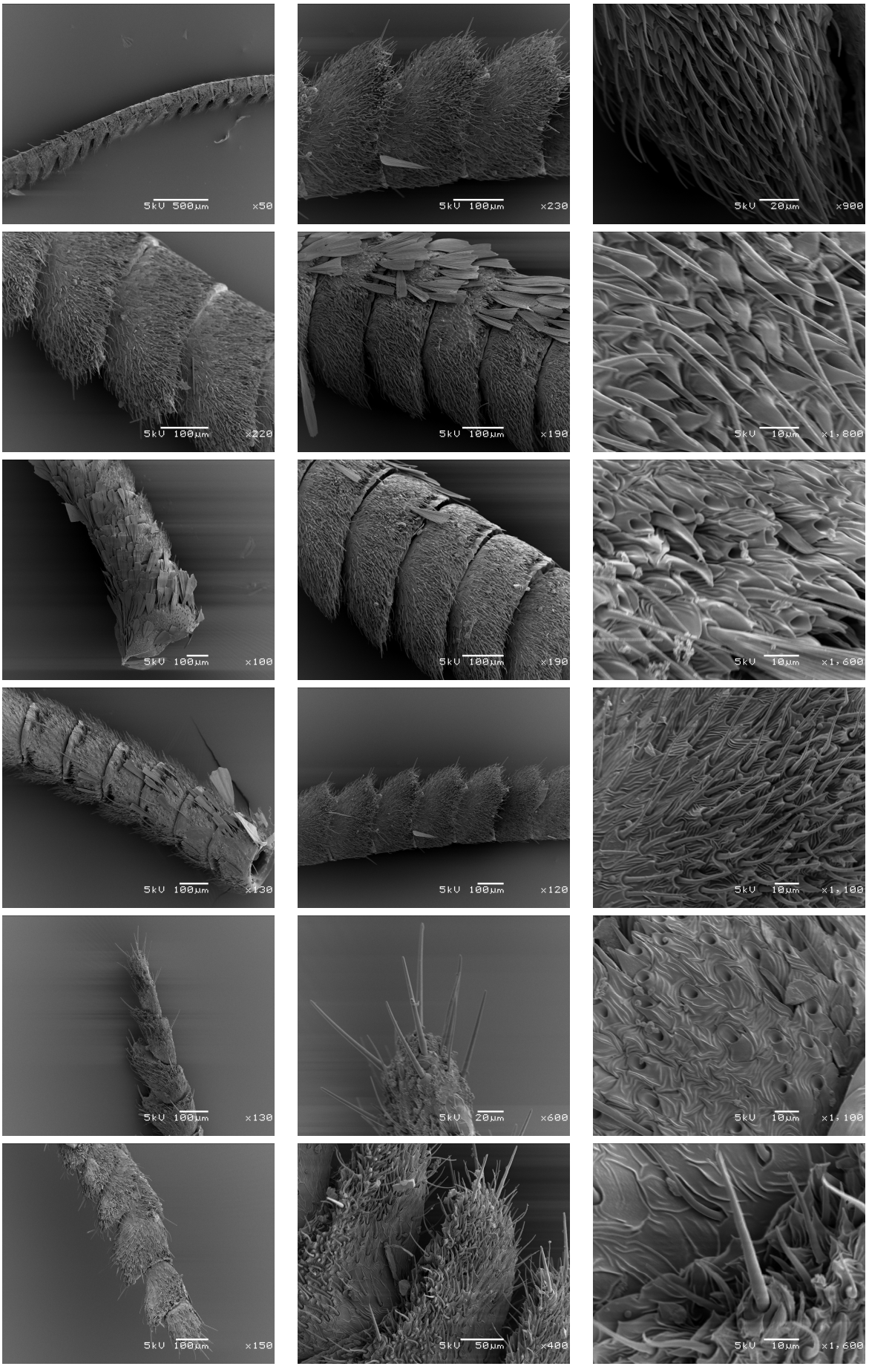

FIGURE D.1: Scanning electron microscopy images of *C.tristis* female antenna

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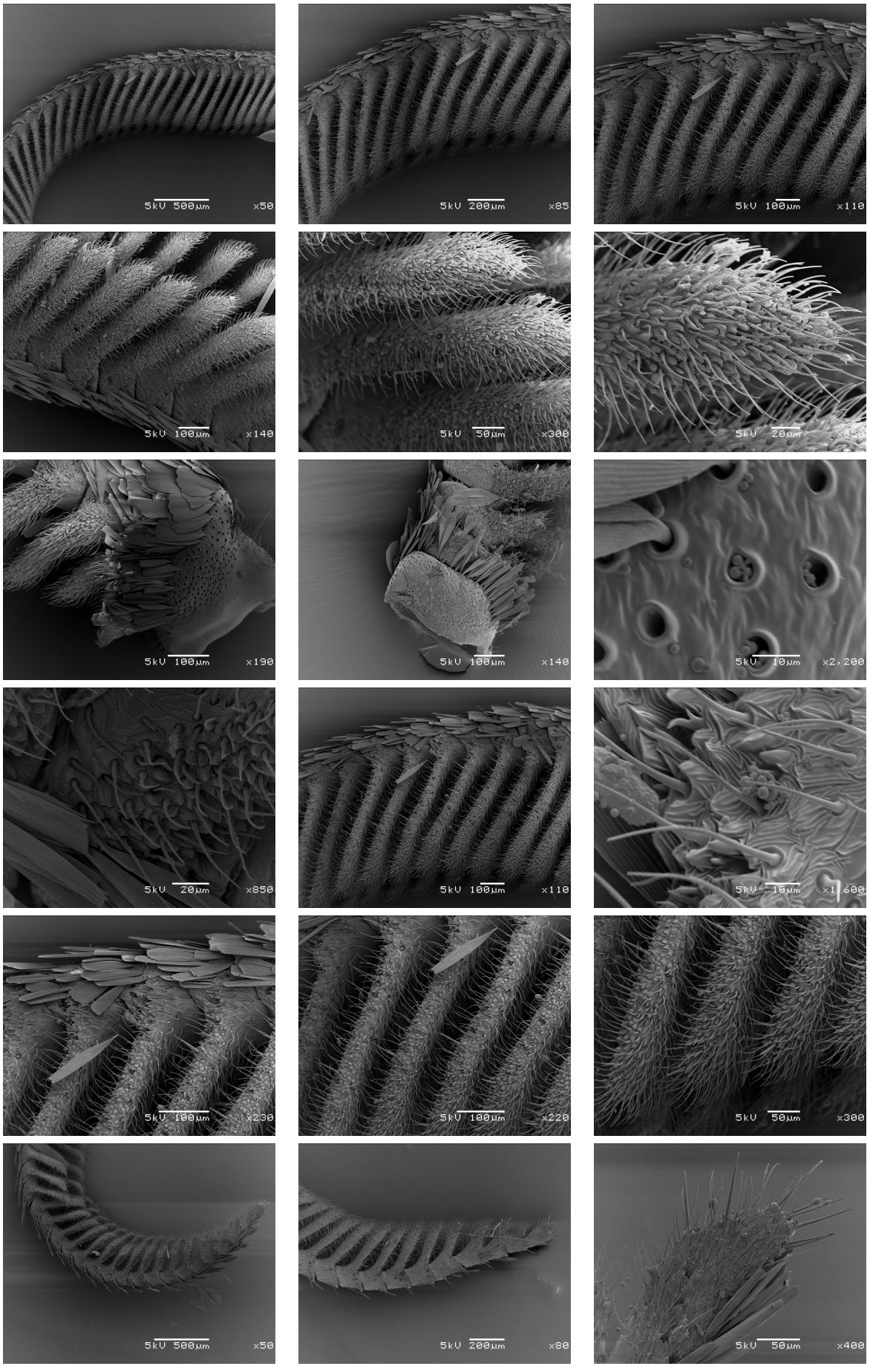

FIGURE D.2: Scanning electron microscopy images of *C.tristis* male antenna

Appendix E

Headspace time trial for *Coryphodema tristis*

E.1 Introduction

Analysis of pheromone gland extracts of *Coryphodema tristis* females suggested that the pheromone is a combination of *Z*9-14:OAc and 14:OAc. Field trials conducted with these two compounds confirmed only minimal biological activity. The results of the 2011 field trial clearly suggested that the identity of the pheromone was not only these two compounds, because no moths were caught with treatment five (see Table F.1). The 2013 field trial showed that these compounds do have some biological activity because nine male moths were caught with treatment two and three during the 2013 field trial (see Table 3.3).

Close observation of moths in captivity revealed that males often position themselves close to females, but mating was never observed. This observation suggested that the females possibly produce other compounds that adsorb onto the netting that was used in the cages. These compounds were unknown before 2013 and it led to headspace investigation of female moths during 2012 and 2013. A time trial was also conducted because it was not known when the females produced these additional compounds. It was expected that this experiment should give some idea of the ratios between the pheromone compounds that are released by the female moths. We report the results of the time trial here.

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E.2 Materials and methods

E.2.1 Female moths

Eucalyptus nitens billets were collected from Lothair Mpumalanga during 2013. These logs were stored in the FABI insectary and 15 newly emerged females were removed from the cages just after they emerged from their pupae.

E.2.2 Sampling

Each female was placed inside a glass chamber that contained a pre-weighed amount (Table E.1) of silane treated glass wool (Supelco, USA). Blank samples contained the glass wool without a female moth. The glass chambers were covered with foil to minimize light disturbances and left overnight. The glass wool was extracted the following morning with two 500 μl aliquots of analytical grade n-hexane (Fluka). This procedure was repeated for the first five nights after emergence of each female moth. It should be noted that not all moths survived through all five nights and that the experiment proceeded for seven nights because not all females were the same age at a particular point in time.

E.2.3 GC-EAD analysis

GC-EAD analysis of samples was performed on an Agilent 6890N gas chromatography system (Chemetrix, Midrand, South Africa) coupled to an EAD system (Syntech, Hilversum, The Netherlands). One μl of each sample was injected split-less (inlet vent time at 1 minute) onto a HP 5 open tubular capillary column (J & W scientific, 0.32 mm ID, 0.25 μm). The column effluent was split at the end of the column with a Y-quartz splitter (Agilent, PN:5181-3398). Half of the effluent was directed to the EAD preparation and the other half to the FID. The oven was programmed as follows: 120 °C for 1 minute to 300 °C for 2 minutes at 20 °C/minute.

Male moths were wrapped in cotton wool and dental wax (Utility wax strips white, Wright Millners) to limit insect movement during the recordings. Glass capillary microelectrodes made from silver wire immersed in Beadle-Ephrussi-Ringer solution (Bjostad, 1998) were connected to the tip and base of the antenna of each male. Samples collected each day were run the same day on one individual male insect (n = 7). Males were placed in front of a stimulus delivery tube with a purified and humidified air flow of 180 ml/min. Direct current recordings were made and the baseline drift and noise in the recordings were removed as in Slone and Sullivan (2007). Averages and standard

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deviations of recordings made for each female age class were calculated and plotted in R version 3.1.0. Response sizes were measured from direct current raw data in GCEAD32 V4.3 software (Syntech, Hilversum, The Netherlands).

E.3 Results

These results clearly showed two repeatable responses that were not observed in blank recordings (Figures E.1, E.2, E.3, E.4, E.5, E.6). These responses coincided with the elution times of *Z*9-14:OH and *Z*9-14:OAc. Both these responses were not detected for 11 out of the 54 samples and only one of either response was detected for 18 out of the 54 samples, 25 of the samples had two detectable responses. These two responses could be detected for all 5 nights that the females survived indicating that virgin females produce these compounds each night of their lifetime (Table E.2).

A statistically significant difference between the two response sizes could not be detected. This was mainly due to the large variation in response size for different samples and the differences observed between different males that were tested. The same chromatographic starting temperature and temperature rate was used as in Chapter 3. This allowed for a comparison of the electro-antennographic response size of the males with the dose response curves that were determined for that chapter. Comparing the average response sizes to those dose response curves might give an indication of the ratio of the two pheromone compounds in these samples. A ratio $96 : 4 \pm 12$ for *Z*9-14:OH: *Z*9-14:OAc was suggested by doing such a comparison but this ratio did not catch males in the field trial of 2013 (see Table 3.3). The reversed ratio caught more moths and it was suspected that the absorption efficiency of the glass wool was higher for the alcohol component than for the acetate.

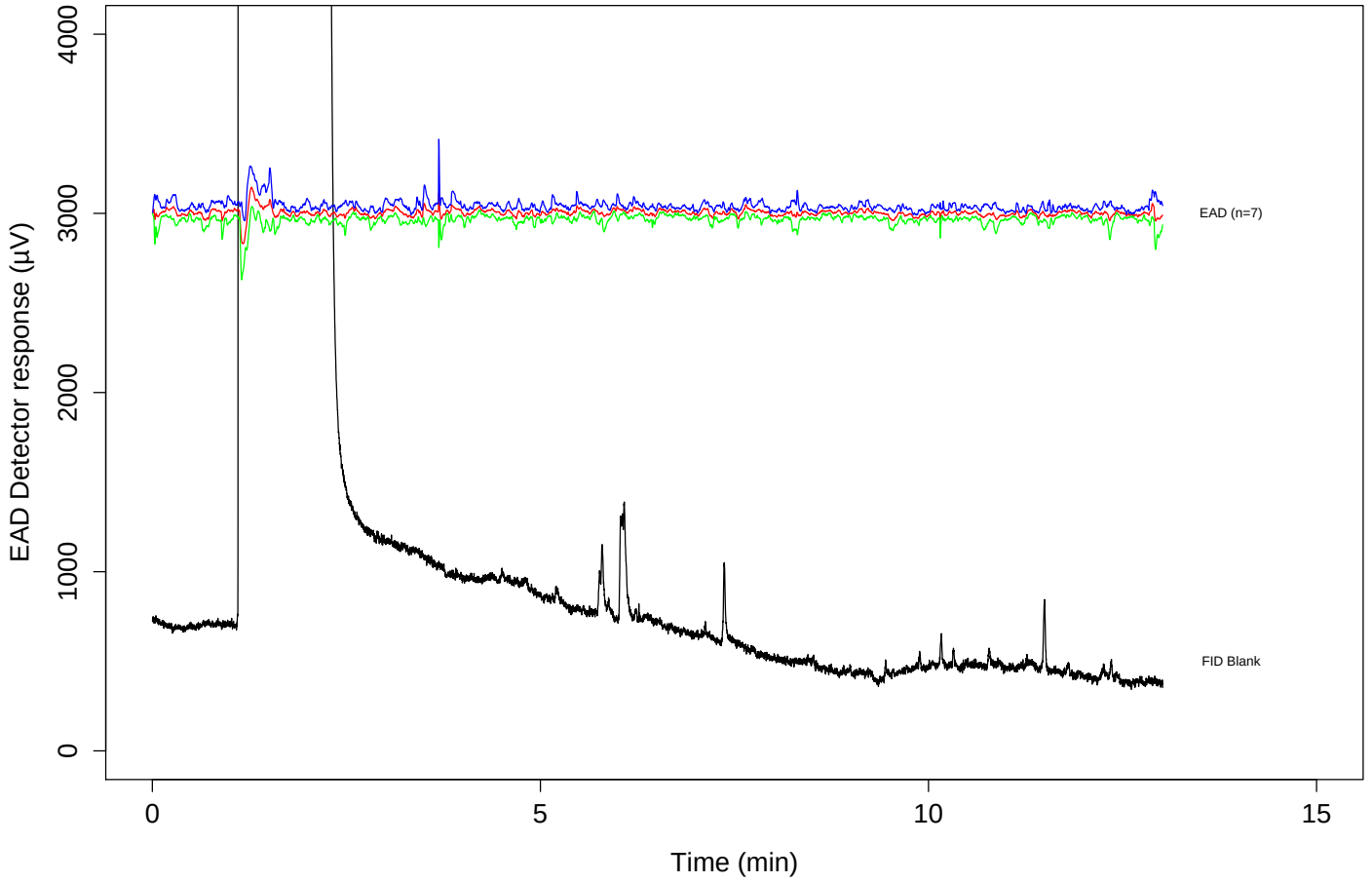

FIGURE E.1: Electro-antennogram response of live males to empty headspace extract sample (Mean ± SD (blue & green), N = 7).

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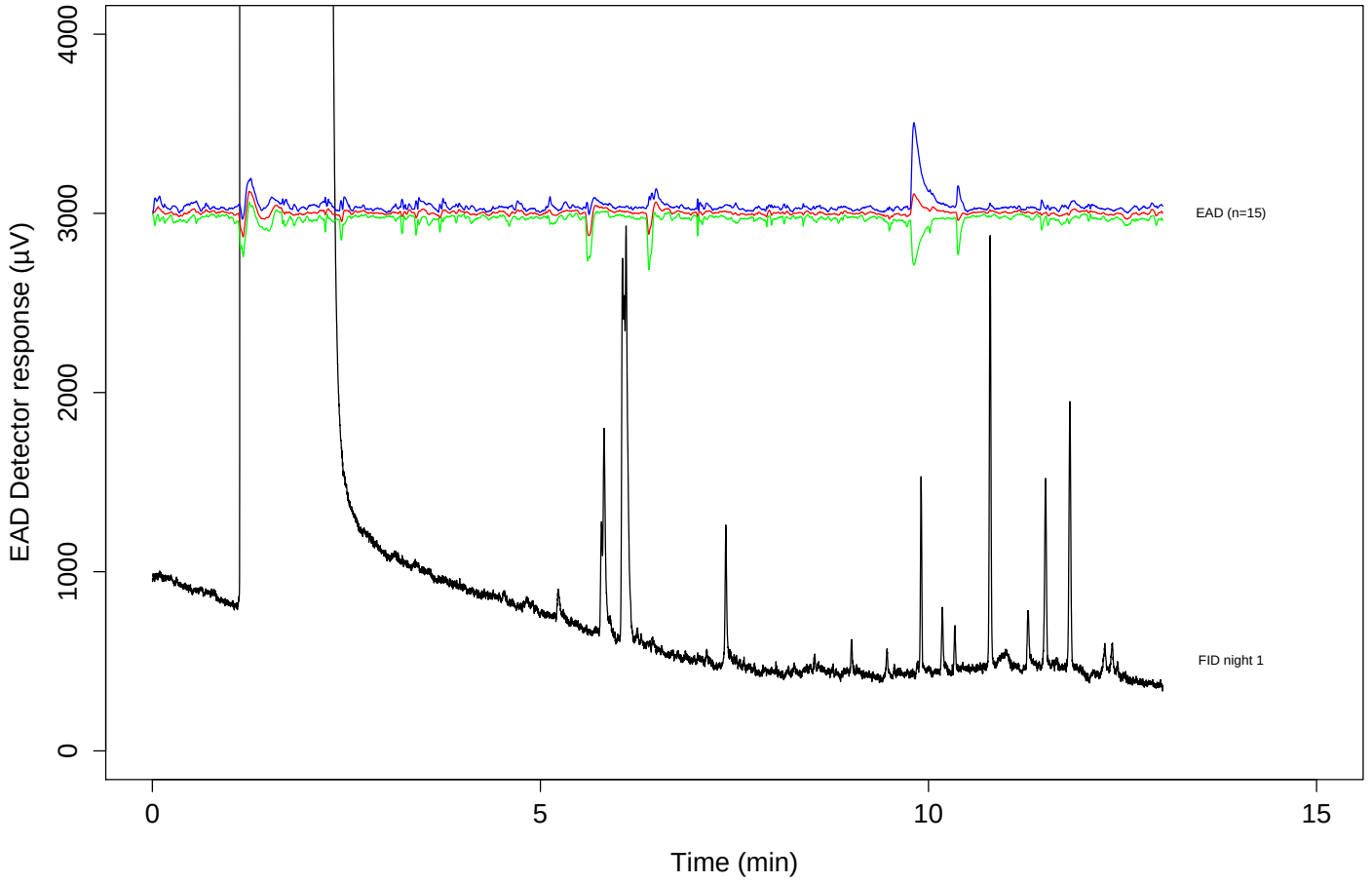

FIGURE E.2: Electro-antennogram response of live males to female *C. tristis* headspace extract samples after night 1 (Mean ± SD (blue & green), N = 15).

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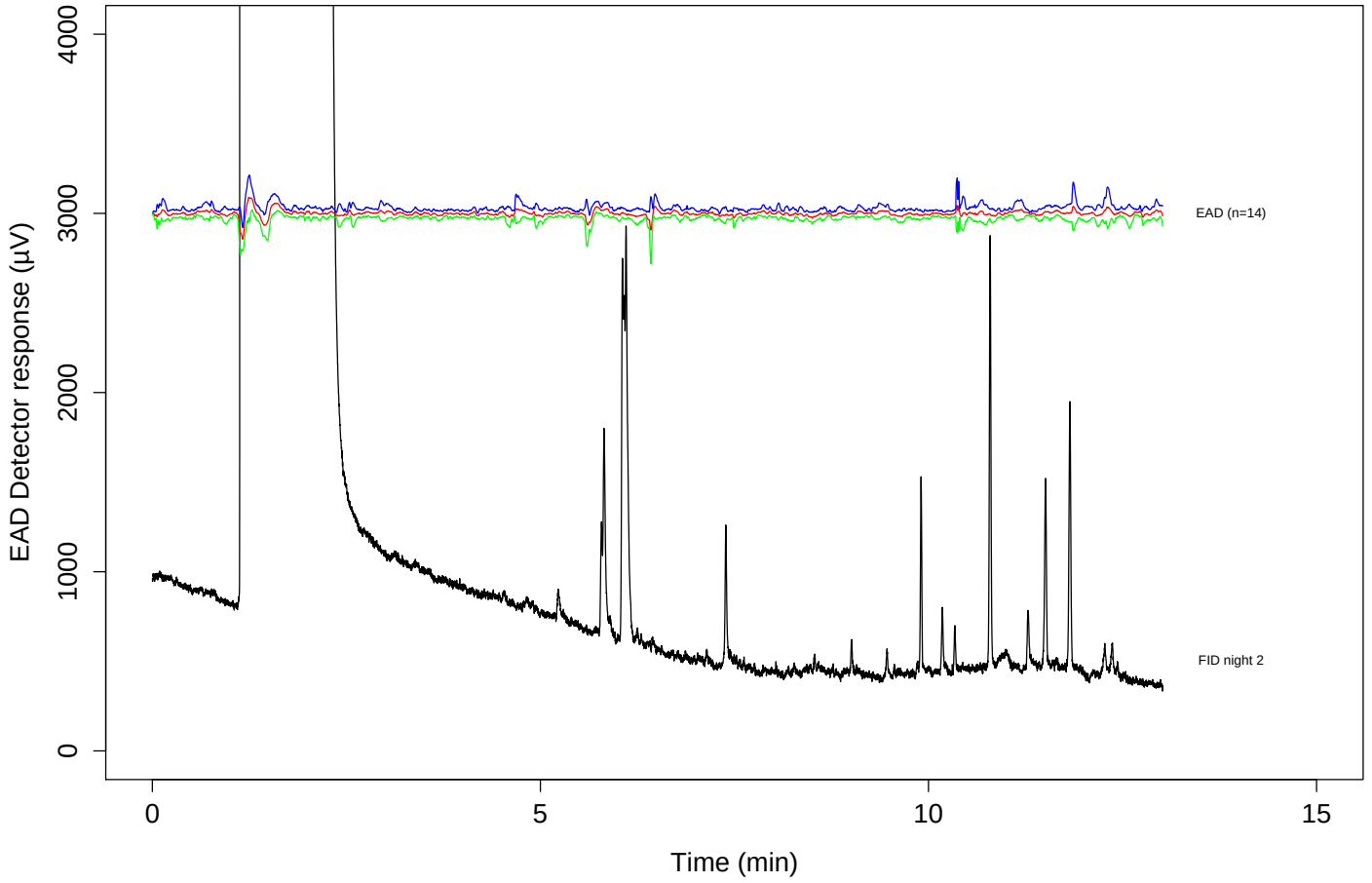

FIGURE E.3: Electro-antennogram response of live males to female *C. tristis* headspace extract samples after night 2 (Mean ± SD (blue & green), N = 14).

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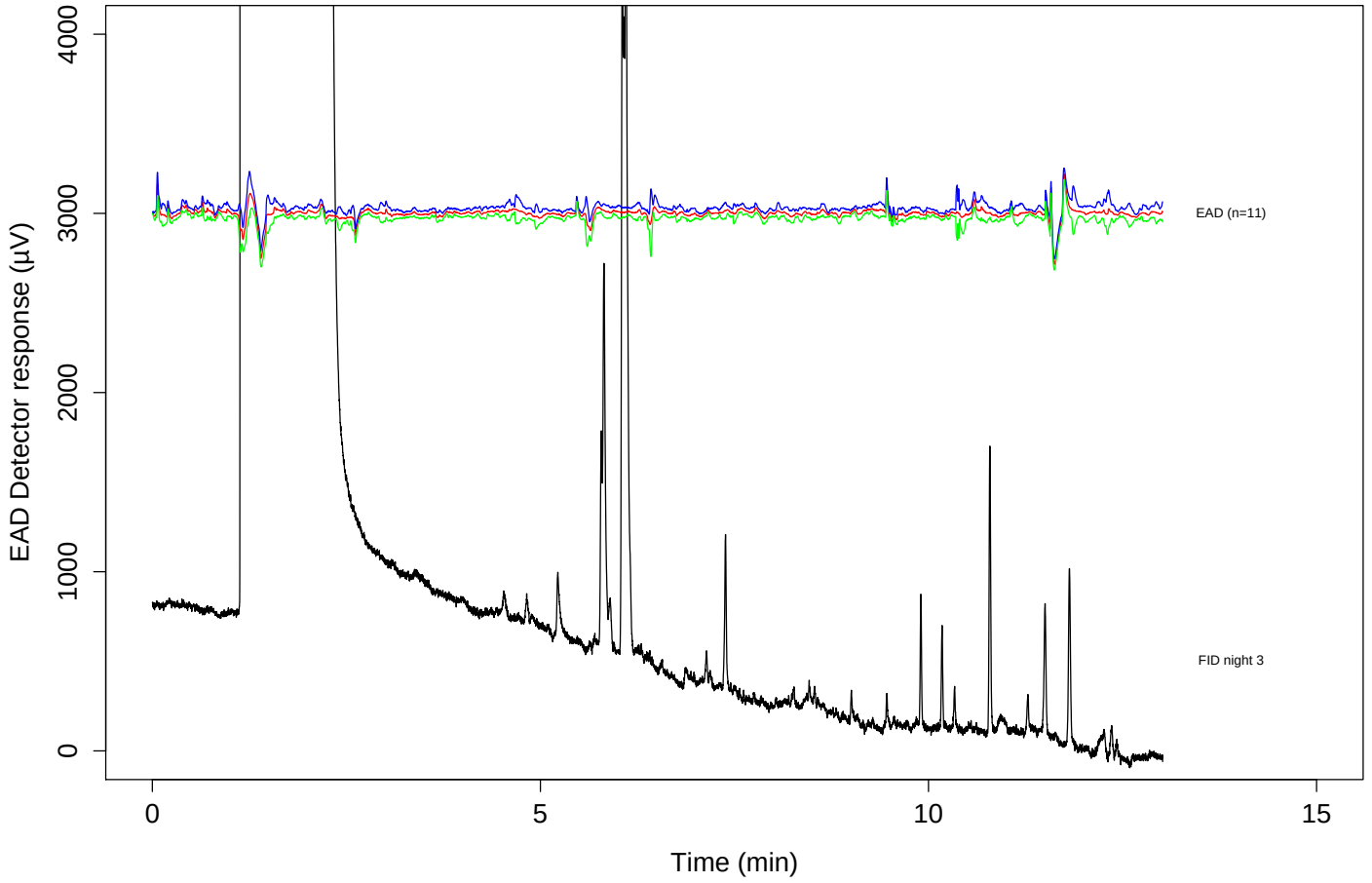

FIGURE E.4: Electro-antennogram response of live males to female *C. tristis* headspace extract samples after night 3 (Mean ± SD (blue & green), N = 11).

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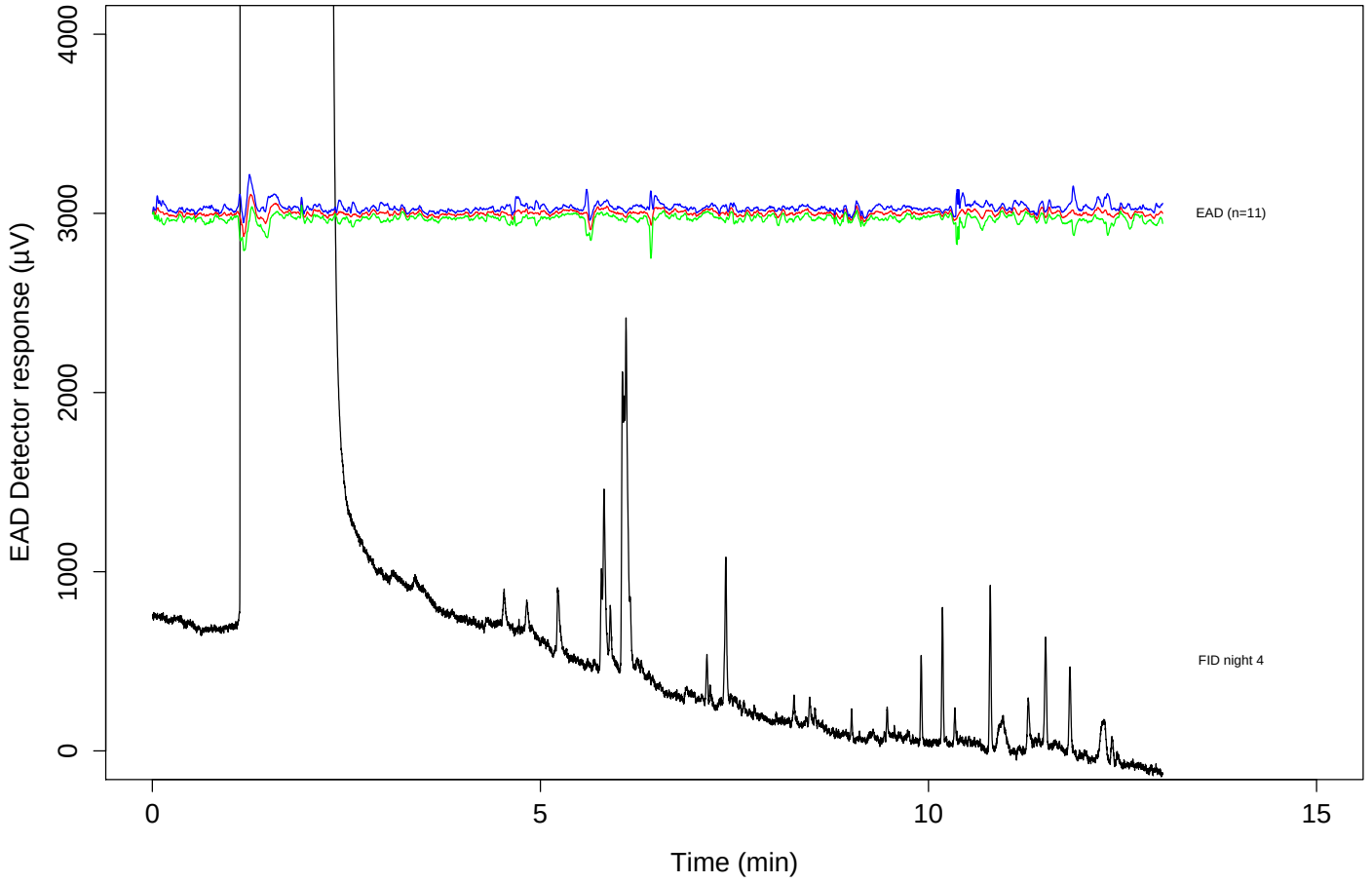

FIGURE E.5: Electro-antennogram response of live males to female *C. tristis* headspace extract samples after night 4 (Mean ± SD (blue & green), N = 11).

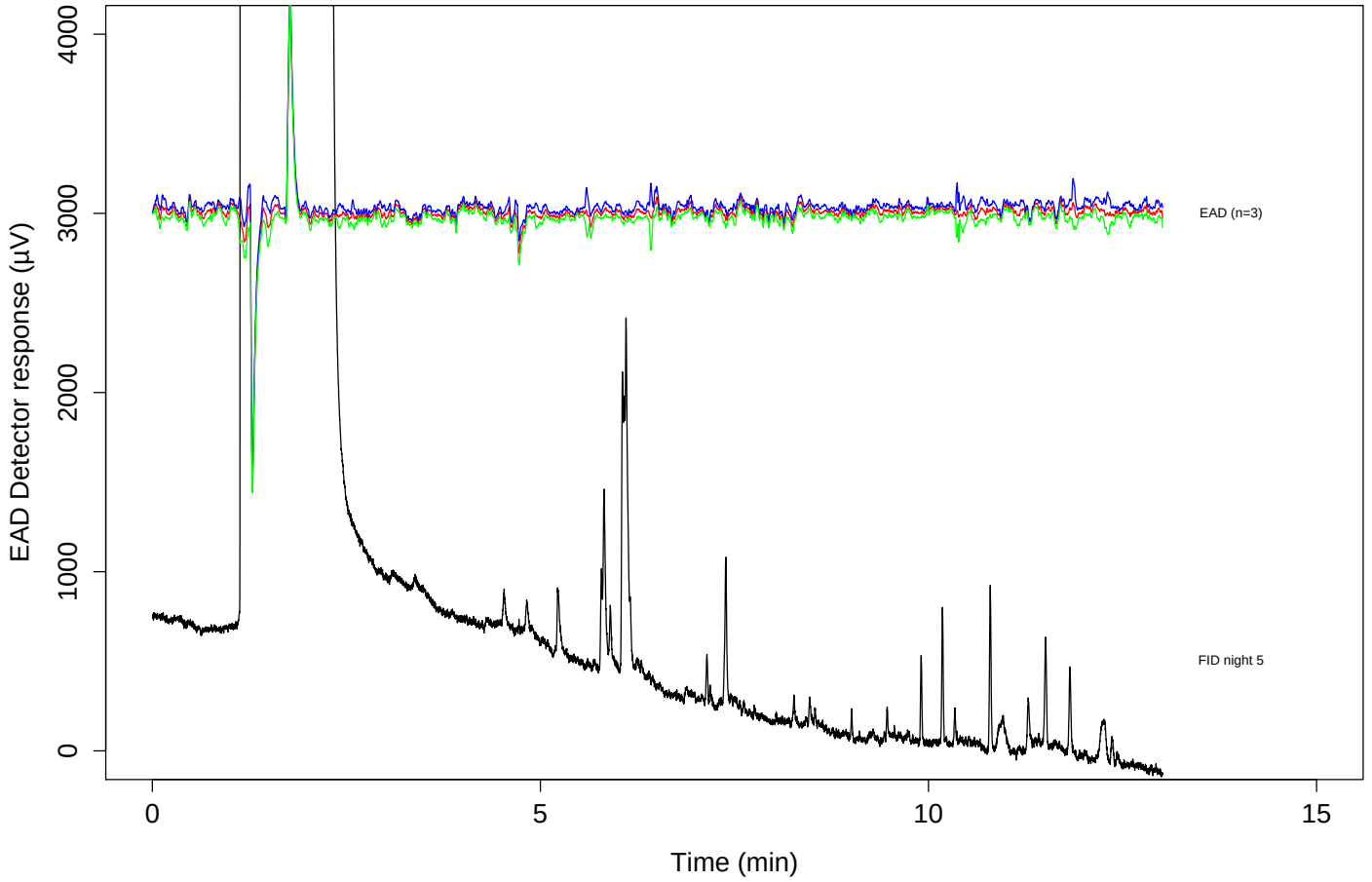

FIGURE E.6: Electro-antennogram response of live males to female *C. tristis* headspace extract samples after night 5 (Mean ± SD (blue & green), N = 3).

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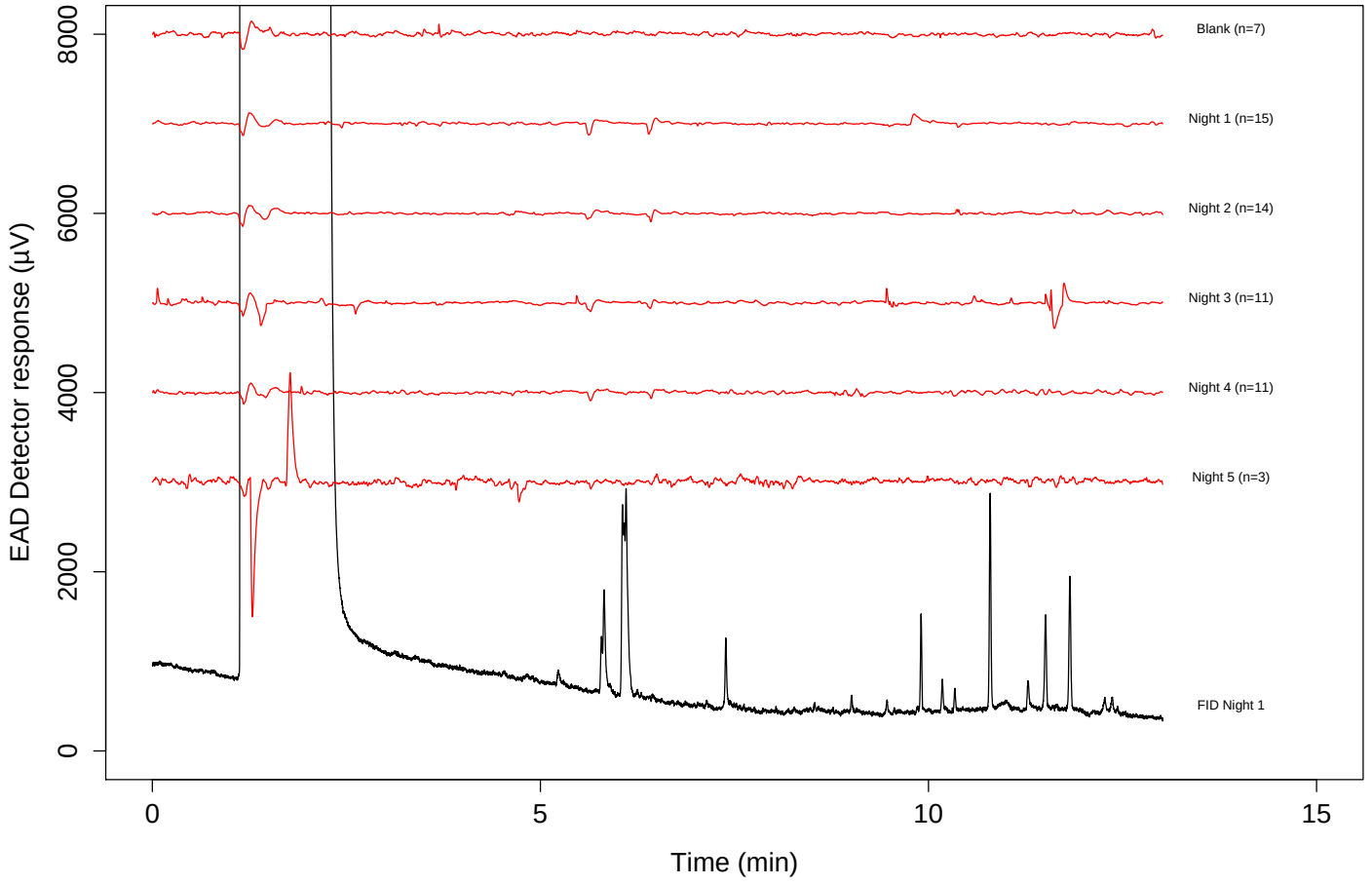

FIGURE E.7: Average electro-antennogram response of live *C. tristis* males to female *C. tristis* headspace extract samples for 5 nights after female emergence.

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TABLE E.1: Glass wool mass used for extractions for the time trial of *Coryphodema tristis*.

	Night 1 mass (g)	Night 2 mass (g)	Night 3 mass (g)	Night 4 mass (g)	Night 5 mass (g)
Blank	0.1005	0.0959	0.0682	0.0514	0.0492
1	0.0678	*	*	*	*
2	0.0689	0.1059	0.1266	0.8440	0.0616
3	0.0654	0.0690	*	*	*
4	0.0722	0.0705	*	*	*
5	0.0732	0.0454	*	*	*
6	0.0638	0.0729	0.0544	0.0608	0.0667
7	0.0794	0.0788	0.0754	0.0567	0.0552
8	0.0720	0.0757	0.0966	0.0712	*
9	0.0631	0.0536	0.0483	0.0651	*
10	0.0601	0.0597	0.1088	0.0581	*
11	0.0925	0.0441	0.0615	0.0879	*
12	0.0509	0.0752	0.0886	0.0865	*
13	0.0850	0.0762	0.0852	0.0972	*
14	0.0548	0.0620	0.0641	0.0741	*
15	0.0533	0.0993	0.0717	0.0699	*

* denotes the absence of a sample due to death of female moth

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TABLE E.2: Electro-antennographic response size of male *Coryphodema tristis* to glass wool extracts for each night of the female lifetime.

Night	n	*Z*9-14:OH (μV)	stdev (μV)	*Z*9-14:OAc (μV)	stdev (μV)
1	15	336	292	316	365
2	14	199	202	187	318
3	11	267	259	162	326
4	11	227	207	120	166
5	3	33	58	53	92

Appendix F

Field trial tests of pheromones for *Coryphodema tristis*

F.1 Introduction

Two field trials were conducted to test for possible biological activity of the potential pheromone components of *Coryphodema tristis*. These trials were used to confirm both the identity and active ratios of the pheromones.

F.2 Methods

F.2.1 Field trial 2011

Three possible pheromone compounds were identified from preliminary investigations of female *C. tristis* gland extracts. These included *Z*9-14:OAc, *E*9-14:OAc and 14:OAc. Compounds were purchased from Insect Science^{TM}(South Africa, Tzaneen). Each compound or mixture was made up in a carrier solution of analytical grade n-hexane (Fluka). A total of 20 μl of these solutions were used to load natural red rubber septa (Insect Science^{TM}(South Africa, Tzaneen)) that were then placed into the pheromone cage of yellow bucket funnel traps (Insect Science^{TM}, South Africa, Tzaneen).

A total of 48 traps were deployed in a stratified random block design at two sites near the original sampling area where these insects were collected. This trial consisted of eight treatments (Table F.1) each with three replicates at each site.

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TABLE F.1: Composition of the field trial treatments during the 2011 field trial and the number of males caught.

n	Treatment	*Z*9-14:OAc	*E*9-14O:Ac	14:OAc	Results
6	1	1	0	0	1
6	2	0	1	0	0
6	3	0	0	1	0
6	4	0.96	0	0.04	0
6	5	0.95	0.03	0.02	1
6	6	0.81	0.17	0.02	0
6	7 (Blank septa)	0	0	0	1
6	8 (Female)	0	0	0	12

* Volumetric ratios made up as 1 μl in 20 μl n-hexane

F.2.2 Field trial 2013

The additional pheromone component (*Z*9-14:OH) that was found in the headspace of female *C. tristis* prompted an additional field trial during 2013. Additional GC-MS analyses also excluded the *E*9-14:OAc as a possible pheromone constituent. Pheromone dispensers made from glass capillaries and methyl silicone rubber tubes were used as pheromone permeation devices. This field trial was reported in Chapter 3.3.4.

Appendix G

Posters

Identification of pheromone compounds in Cossid moth *Coryphodema tristis*, a serious pest of *Eucalyptus* in South Africa

Marc C. Bouwer[1,2], Bernard Slippers[3], Mike J. Wingfield[1] and Egmont Rohwer[2]
Forestry and Agricultural Biotechnology Institute[1], Department of Chemistry[2], Department of Genetics[3], University of Pretoria, South Africa

Introduction

- *Coryphodema tristis* causes significant damage to *Eucalyptus nitens* plantations in South Africa (Fig 1)
- The larvae tunnel through and feed on the xylem inside infested trees for up to eighteen months
- Adult moths emerge in spring and fly at night
- The female moths expose their ovipositors to the outer atmosphere and fan their wings to attract mates
- The male moths have highly filamentous antennae that are exquisitely sensitive to the pheromone compounds released by the females
- The males fly toward the calling females as soon as the pheromone is detected
- The chemical make-up of this pheromone message is unknown and is potentially very useful to enhance monitoring traps for this pest

Aim

To identify the pheromone compounds released by the female moth to lure the male moth

Figure1 : Adult *Coryphodema tristis* and the damage caused by the larvae

Confirmation of the antennal responses in male moths

Experiment 1

- Standard compounds were purchased and the retention time, mass spectrum and electrophysiological response was confirmed by repeating the GC-EAD and GC-MS analysis
- The Kovats retention index of the main active peak in the gland extracts was calculated to be 1798 and the standards were calculated as 1797 (Z9-14Ac) and 1799 (E9-14Ac) respectively (DB 5 column)
- The male moth antennae were more sensitive as compared to the female moth antennae toward the identified compounds (Fig 5)

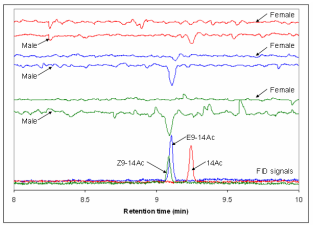

Figure 5: Male vs. Female EAD response comparison observed for the three identified compounds. Responses to specific compounds are colour coded

Experiment 2

- Dose response curves were determined for the three identified compounds and a 1:1:1 mixture of these compounds (Fig 6)
- This was done by puffing known concentrations of the reference standard compounds onto the antennal preparation
- The voltage deflection was measured and a relative response was calculated as a percentage response as compared to that observed for 1ul 1000ppm phenyl ethyl acetate (PEA)

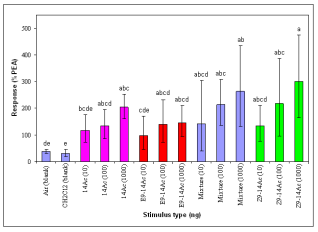

Figure 6: The relative response of the male antenna toward the identified compounds and a mixture of the three compounds as compared to an air blank and a solvent blank. Letters of significance were determined with the Tukey-Kramer honestly significant difference test. (*ANOVA*, $P < 0.05$, $N = 5$, ± CI(95%).

Searching for the pheromone compounds

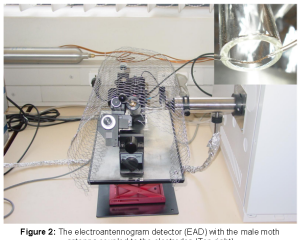

Figure 2: The electroantennogram detector (EAD) with the male moth antenna coupled to the electrodes (Top right)

GC-EAD

- The female pheromone was extracted from the pheromone gland located near the ovipositor with organic solvents (n-hexane, acetone and CH2Cl2)
- Gland extracts were analysed on a gas chromatography system coupled to electroantennography detector (GC-EAD)
- The male antennae were used as detectors to indicate when electrophysiologically active peaks eluted from the column (Fig 3)
- A large repeatable electroantennographic response was observed and corresponded to a small peak eluting at 9.1 minutes during this experiment (Fig 3)
- The female antennae did not give a similar response to the same peak (Fig 3)

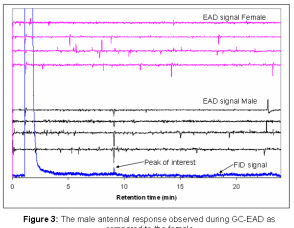

Figure 3: The male antennal response observed during GC-EAD as compared to the female

Mass Spectrometry

- Samples were analysed on a GC*GC-MS system in order to identify the compounds
- Three peaks were observed at the expected retention time window as compared with the GC-EAD results
- These peaks were tentatively identified as 14 carbon acetates by comparing their spectra to those found in the NIST library (Fig 4)
- Two of the peaks were confirmed to be unsaturated at one of the carbon-carbon bonds
- The ratio of the peaks were determined as 2.29 ± 0.90 : 2.58 ± 1.74 : 95.12 ± 2.28 (mean ± *SE*, *N* = 9) in the n-hexane extracts
- Double bond location of the unsaturated component of the pheromone was confirmed at the ninth carbon by reacting the samples with dimethyl disulphide (Fig 4)
- Standard compounds were purchased and the retention time, mass spectrum and electrophysiological response was confirmed

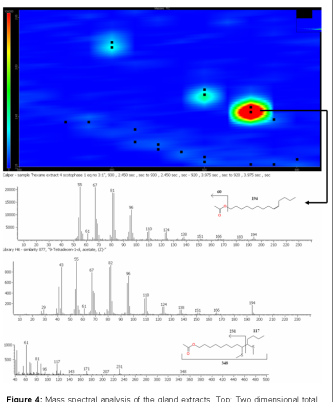

Figure 4: Mass spectral analysis of the gland extracts. Top: Two dimensional total ion chromatogram with three prominent peaks. Middle: Mass spectral pattern of the major component as compared to the NIST library spectrum. Bottom: Double bond location confirmation at the ninth carbon.

Conclusions

- Z9-14Ac, E9-14Ac and 14Ac were found in gland extracts of *C. tristis* females
- The male antennae showed repeatable electrophysiological responses at the same retention time as the peak that was observed in the gland extract chromatograms
- This peak has been shown to be a complex of two compounds: Z9-14Ac and E9-14Ac
- Z9-14Ac was identified as the major component in these extracts
- A third minor compound (14Ac) was also observed on the more sensitive GC*GC-MS system
- The male antennae did not appear to react synergistically to a 1:1:1 mixture of these compounds (see Fig 6); however all three these compounds were active on the male antennae and may be involved in the pheromone message of the female moth
- It is possible that the antennae may saturate very quickly under the tested concentration levels
- This saturation event may mask a possible synergism that may exist between the identified compounds
- The ratio of these compounds were determined for the n-hexane gland extracts as 2.29 ± 0.90 : 2.58 ± 1.74 : 95.12 ± 2.28 (mean ± SE, N = 9)
- This ratio was different in extracts made by using CH2Cl2 which may suggest that enzymes may have been co-extracted into these extracts
- These acetates are, therefore, potentially either the pheromone compounds themselves or precursor molecules of the true pheromone compounds
- N-hexane was also found to be active on the antenna and should be avoided as a solvent when doing electroantennography with this specific species
- Future work will include analyzing volatiles in the headspace around the calling female for comparison purposes and confirming the behavioural response of the males to these compounds
- The ultimate goal will be to develop a mixture of environmentally friendly chemicals that may be used to monitor and possibly control *C. tristis* infestations

Acknowledgements

- We thank Dr. B. P. Hurley and Mr. Dawit Degefu for providing insect samples. We also acknowledge the financial support of the Tree Protection Cooperative Programme (TPCP) and National Research Foundation (NRF)

FIGURE G.1: Poster presented at IUFRO 2011

IDENTIFICATION OF SEMIOCHEMICALS PRODUCED BY *AMYLOSTEREUM AREOLATUM*, THE FUNGAL SYMBIONT OF *SIREX NOCTILIO*

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Introduction

- *Sirex noctilio* is a major pest of Pinus spp. in South African plantations as well as elsewhere in the world
- The *Sirex noctilio* female insects introduce a mutualistic fungus, *Amylostereum areolatum*, into trees during oviposition
- The fungus contributes to the digestion of wood polymers on which the larvae feed
- The parasitoid wasp, *Ibalia leucospoides*, was introduced into South Africa for biological control of *S. noctilio*
- *Ibalia leucospoides* females lay their eggs into or close to the eggs and larvae of *S. noctilio*
- It is not known if *S. noctilio* females can detect the presence of their own symbiotic fungus in already infested trees (Fig 1) but *I. leucospoides* females have been shown to be attracted by *A. areolatum* fungal volatiles (Martinez et al 2006, Spadbery, 1974)
- It is thought that *I. leucospoides* females locate the *S. noctilio* oviposition channels based on the odour produced by the fungus (Fig 1)

Aim

To chemically investigate the volatile profile of *Amylostereum areolatum* in order to identify semiochemicals that may play a role in the oviposition behaviour of *S. noctilio* and *I. leucospoides*

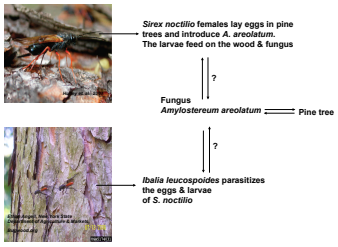

Figure 1: Potential semiochemical interactions between the *A. areolatum*, fungus its host *S. noctilio*, and the biological control agent *I. leucospoides*

Gas chromatography coupled to mass spectrometry

- Samples were injected into the liquid injector of a GC-MS system (HP 5973) coupled to an quadrapole mass selective detector (Agilent technologies 5975C inert MSD).
- Blank samples were compared to non blanks and an identical analytical column was used during this analysis (Fig 5)

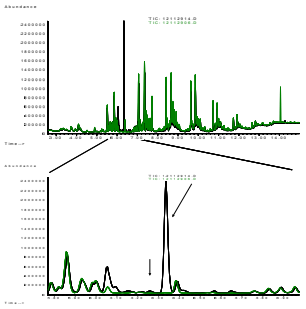

Figure 5: Total ion chromatograms of sample (black) compared to a blank (green). Whole chromatogram (top). Region with the electro-antennographically active peaks (bottom).

- The Kovats retention index of the main active peak in the fungal samples was calculated to be 1393.3
- The same peak that was observed on the GC-EAD system was found in these chromatograms (Fig 5)
- The mass spectra suggested that this active peak was a sequiterpene with a molecular mass of 204 atomic mass units
- The tentative identity was assigned as 1,3,4,5,6,7-hexahydro-2,5,5-trimethyl-2H-2,4a-Ethanonaphthalene (Fig 6)

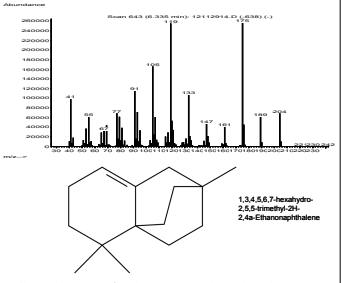

Figure 6: Mass spectra of the electro-antennographically active peak (top) with the suggested structure from the NIST, 2004 library (bottom).

Sampling and electro-antennal responses

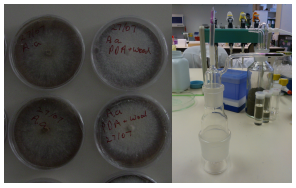

Figure 2: *Amylostereum areolatum* cultures on Petri dishes and the headspace sampling chamber

Sampling of fungal volatiles

- *Amylostereum areolatum* was cultured on potato dextrose agar (PDA)
- Headspace samples of both blanks (only PDA Petri dish) and fungal cultures (Petri dish with culture) were obtained by dynamic headspace sampling (Fig 2)
- Volatiles were trapped on standardized activated charcoal traps (ORBO™)
- The traps were eluted twice with 1ml analytical grade n-hexane solvent and concentrated to a few micro liters by evaporation under slow steam of nitrogen gas

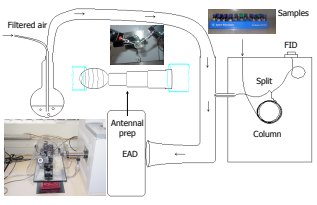

Figure 3: Schematic representation of gas chromatography coupled to electro-antennography. EAD = Electro-Antennogram Detector, FID = Flame Ionization Detector.

Gas chromatography coupled to electro-antennography

- Samples were analysed on a Agilent 6890N gas chromatograph coupled to a flame ionization and electro-antennogram detector (Fig 3)
- An HP5, 30 m, 0.25 µm FT, 0.32 mm ID open tubular column was used during the analysis
- The antennae of *I. leucospoides* and *S. noctilio* females were removed and coupled to the electrodes of the electro-antennogram detector
- The electrodes were made by using oxidized silver wire in Beadle-Ephruzi ringer electrolyte solution
- Electro-antennogram responses from *I. leucospoides* females, 125±64 µV (n = 5 ± sd), antennae were observed for two of the peaks that were not present in blank samples (Fig 4)
- *Sirex noctilio* female antennae did not show the same response to these two peaks (Fig 4)
- The Kovats retention index of the larger peak to which *I. leucospoides* female antennae respond was calculated to be 1394 on the GC-EAD system

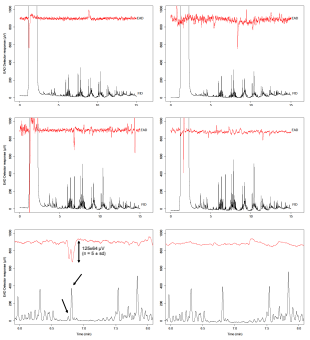

Figure 4: Antennal responses of *Ibalia leucospoides* (left) and *Sirex noctilio* (right) to blank Petri dish (top) and *Amylostereum areolatum* fungus (middle) samples. The bottom is a zoomed in picture of the response to the peaks not found in the blank samples.

Conclusions

- Results showed that there are specific chemicals released by *A. areolatum* that are detected by *I. leucospoides* females but not by *S. noctilio* females
- These analyses do not exclude the possibility that there may be certain chemicals that are produced by the symbiotic fungus that fall below our instrumental detection limits to which *S. noctilio* might respond
- Most of the chromatographic peaks in the chromatograms were also observed from blank samples and possibly originate from the activated carbon filters that were used during headspace sampling
- The tentative identity of the larger peak has been assigned as 1,3,4,5,6,7-hexahydro-2,5,5-trimethyl-2H-2,4a-Ethanonaphthalene
- This compound is a derivative of thujopsene, a sequiterpene that can be isolated from cedar wood oil
- The same fungal metabolite has been tentatively identified in other wood inhabiting fungi, for example, *Ophiostoma pluriannulatum*, which was found on the frass of the pine weevil, *Hylobius abietis* L. (Norin, 2011)
- Results from this study shows that *Ibalia leucospoides* females possess receptors on their antennae that can detect volatiles from the fungal symbiont of *S. noctilio*
- Future work will include synthesizing the tentatively identified compound from the sequiterpene (-)-thujopsene and confirming the identity by comparing both chromatographic and mass spectral data
- A proper mass spectrum for the smaller peak that also elicited a response from *I. leucospoides* female antennae must still be obtained for identification
- The ultimate goal will be to develop a mixture of environmentally friendly chemicals that may be used to monitor both *S. noctilio* and *I leucospoides* in the field

References

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- Spradbery J. P. 1974. The responses of *Ibalia* species (Hymenoptera: Ibalidae) to the fungal symbionts of siricid woodwasp hosts. Journal of Entomology (A) 48:217-222.

Acknowledgements

- We thank Mr Philip Croft and Mr. Theo van Zyl for providing insect samples. We thank Mr Hardus for providing the fungal cultures and pine logs. We also acknowledge the financial support of the Tree Protection Cooperative Programme (TPCP) and National Research Foundation (NRF)

FIGURE G.2: Poster presented at ESSA 2011

Headspace analysis reveals the pheromone identity of the Cossid moth *Coryphodema tristis*

Marc C. Bouwer[1,2], Bernard Slippers[3], Mike J. Wingfield[1] and Egmont Rohwer[2]
Forestry and Agricultural Biotechnology Institute[1], Department of Chemistry[2], Department of Genetics[3], University of Pretoria, South Africa

Introduction

- *Coryphodema tristis* causes significant damage to *Eucalyptus nitens* plantations in South Africa (Fig 1)
- The larvae tunnel through and feed on the xylem inside infested trees for up to eighteen months
- Adult moths emerge in spring and fly at night
- The female moths expose their ovipositors to the outer atmosphere and fan their wings to attract mates
- The male moths have highly filamentous antennae that are exquisitely sensitive to the pheromone compounds released by the females
- The males are directed towards the females by the pheromone
- The chemical make-up of the pheromone message has not been investigated yet

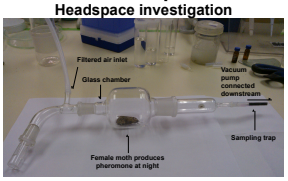
Aims

- To investigate the differences in male and female antennal morphology
- To identify the pheromone compounds present in the female pheromone gland
- To identify the pheromone compounds released by the female moth to lure the male moth

Figure 1: Adult *Coryphodema tristis* and the damage caused by the larvae (left). Highly invested trees can break during windy periods (right)

Antennal responses

Headspace investigation



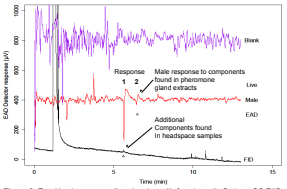
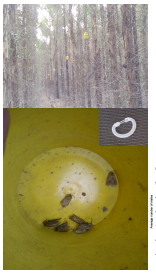


Figure 2: Top: Headspace sampling chamber with female moth. Bottom: GC-EAD responses of male insect antenna to the compounds found in the female headspace. Notice the two responses indicated by the arrows. The second response was also observed for samples obtained from female pheromone glands.

Field trial

- 90 Yellow bucket funnel traps were attached to *Eucalyptus nitens* trees four meters above ground level in two stratified random block designs in two plantation blocks near Lothair, Mpumalanga
- Synthetic pheromones and blends were dispensed in custom made pheromone permeation devices
- Seven artificial pheromone treatments were tested (n = 10) with empty dispensers as negative control and live females as a positive control



Treatment	# Males
1	2
2	6
3	3
4	0
5	39
6	1
7	63
8 (Blank)	0
9 (Female)	8
Total	122

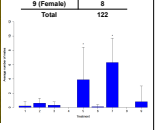
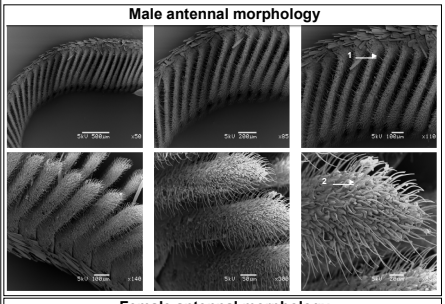


Figure 3: Top left: Yellow bucket traps in the field. Bottom left: Male moths caught in one of the traps with an example of a pheromone dispenser. Top right: Table showing the numbers of males caught for each treatment during October 2013. Bottom right: Histogram with average numbers caught showing the significant numbers caught in treatment 5 and 7 as compared to the blank (Steel-Dwass non-parametric, $P < 0.05$, $N = 10$, ± SD).

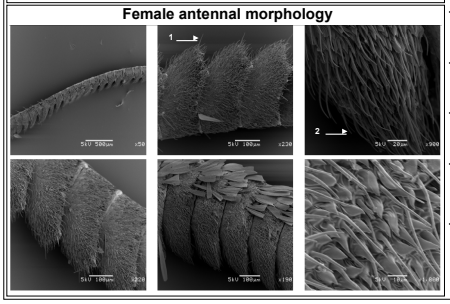
Scanning-electron microscopy of *C. tristis* antennae

Male antennal morphology



- The surface area of male antennae are at least 6 times larger than that of females
- The antennae of both males and females have 60 segments + 1 tip segment
- Males have two branches per segment while females have only one (bottom left)
- Many hairs, called sensilla, occur on each segment (bottom right)
- These sensillae house the olfactory neurons which are responsible for odor detection
- Two different sensillae were observed on both male and female antennae
  - 1: Long thick sensilla
  - 2: Shorter thin sensilla
- Sensilla type 1 only occurs a few times per segment while type 2 occurs more frequently

Female antennal morphology



- These morphological differences show that males have a highly ramified antenna compared to females
- Males use their antennae to detect pheromone compounds that are released by calling female moths
- Antennae of both males and females can be used as detectors to search for pheromone compounds
- Males antennae are expected to give stronger electro-physiological responses in the presence of pheromone when compared to female antennae
- Measuring electrophysiological responses of the antennae can be done using a technique called Gas Chromatography coupled to an Electro-antennogram detector (GC-EAD)

Conclusions

- A sex attractant pheromone for *C. tristis* has now been identified
- Over a 100 male moths were caught under field conditions in two very similarly baited artificial pheromone treatments
- Scanning electron microscopy revealed marked differences in the antennal morphology between males and females
- Females have smaller and simpler antennae compared to males, which support the hypothesis that males use their antennae to locate females at night
- Females produce a specific blend of at least two compounds that attract males
- These compounds act synergistically, because males are only caught when both compounds are present in a specific ratio
- The compound identities and effective ratios and dispenser design has been provisionally patented
- This pheromone can now be used to gain a better understanding of the distribution of the pest and possibly even finding the indigenous host of *C. tristis*
- The pheromone blend and dispenser will be commercially available in the near future
- Future work will aim to optimize the numbers of males that can be caught by investigating the active ratio in more detail and possibly including other pheromone compounds that were also detected in our samples

Acknowledgements

We thank Dr. Brett Hurley and Mr. Hardus Hatting for their help in collecting field samples and Dr Yvette Naude, Department of Chemistry, University of Pretoria for assistance in analyzing samples. Members of the Tree Protection Co-operative Programme (TPCP), the THRIP initiative of the Department of Trade and Industry and the DST/NRF Centre of Excellence in Tree Health Biotechnology (CTHB) are acknowledged for financial support.

FIGURE G.3: Poster presented at Forestry science symposium 2014

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