Studying Volatile Emissions of Fruit Flies as Chemical Lures

Sally Noushini

Bachelor of Chemistry

Master of Organic Chemistry

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

Department of Molecular Sciences

Faculty of Science and Engineering

Macquarie University

Sydney, Australia

November 2020

Declaration of Originality

I declare that the work presented in this thesis written in the format of thesis by publication has not been submitted, either in the whole or in part, for a higher degree to any other university or institution, and to the best of my knowledge is my own and original work, except as acknowledged in the text.

Sally Noushini
(ID:)

November 2020

Table of Contents

Abstract

Fruit fly species are amongst the most damaging horticultural pests globally and have a devastating impact on food production. Fruit flies typically release volatile compounds, usually interpreted as sex pheromones, as an integral element of their sexual biology. Understanding the composition and function of released volatiles is an important aspect of understanding fruit fly sexual biology and can also provide valuable knowledge for the development of attractants that can be used for fruit fly monitoring and control.

This PhD thesis provides the chemical profiles of rectal gland contents and emissions of four pest species, *Bactrocera musae*, *B. frauenfeldi*, *B. bryoniae* and *B. kraussi*, by using gas chromatography-mass spectrometry; and evaluates the detection and function of natural blends of both sexes by gas chromatography-electroantennogram detection and/or gas chromatography-electropalpogram detection and Y-tube olfactometry. Across both sexes of all species, four chemical classes were present including carboxylic acid, esters, spiroacetals and acetamides. Analysis showed that esters and spiroacetals were the two electrophysiologically active classes of compounds in these four species. Interestingly, Y-tube olfactometer assays for *B. kraussi* showed that the natural blend of female rectal glands attracted males, while for *B. frauenfeldi* the females were attracted to male rectal glands. For *B. bryoniae*, both males and females were attracted to the conspecific natural rectal gland blends. Males of this species were also attracted to the conspecific male rectal glands, while this was not observed for *B. kraussi* and *B. frauenfeldi*. No females were attracted to volatiles from conspecific female rectal glands across all species.

This PhD thesis also examined the most common techniques for sampling rectal glands and headspace volatile compounds of fruit flies, using *B. tryoni* as a model organism as it has been studied previously for its chemistry. Extraction of the rectal glands with the different polarity solvents *n*-hexane, dichloromethane and ethanol was conducted, with both crushed and non-crushed glands examined. Headspace collection of the gland volatiles was also investigated using static and dynamic methods. For the static methods, three different types of solid phase microextraction fibres (polydimethylsiloxane, polydimethylsiloxane/divinylbenzene and polyacrylate) were compared. For the dynamic methods, the performance of two common sorbent polymers, Tenax-GR and PoraPak Q, and the effect of sampling duration on performance of each sorbent, were also examined. Both

male and female *B. tryoni* were found to emit a wide range of volatile compounds, including acetamides, spiroacetals and esters. Six new compounds that have not been reported in previous studies, ethyl propanoate, ethyl isobutyrate, ethyl 2-methylbutanoate, propyl isobutyrate, ethyl 2-methylpentanoate and diethyl succinate, were identified in the male rectal glands and headspace samples, along with six previously reported amides. Three new compounds, propyl laurate, methyl myristate and *N*-(2-methylbutyl)acetamide, were also identified in female rectal glands and headspace samples, as well as nineteen compounds previously reported including fatty acid esters, spiroacetals and amides. For the rectal gland extractions, dichloromethane was found to extract greater amounts of amides, while there was no significant difference between the three solvents in extraction of spiroacetals. No significant differences were observed for esters between the *n*-hexane and dichloromethane extracts. Ethanol was found to be an unsuitable solvent as it did not contain as many of the short chain esters. Extraction of the crushed and non-crushed rectal gland samples afforded the same range of compounds, however, the crushed glands provided higher concentrations of each compound. For static headspace samples, polydimethylsiloxane performed better in collecting spiroacetals while type of fiber did not affect the amounts of esters and amides. Polyacrylate had relatively low affinity for spiroacetals, ethyl isobutyrate and ethyl-2-methylbutanoate. The results from the dynamic methods showed that Tenax was more suitable for amides and esters except ethyl isobutyrate. Sampling duration was a critical factor for the dynamic headspace methods. These studies show that when interpreting volatile profiles of fruit flies, and when comparing species, it is important to consider the biases that can be introduced by sampling methods.

Overall, this PhD study provides insight into the development of new lures for fruit fly control and also provides guidelines for method selection for fruit fly pheromone studies.

Acknowledgement

I am really grateful to my supervisors Joanne Jamie, Phil Taylor and Ian Jamie for their constant support, guidance and encouragement. They have been invaluable sources of knowledge, and their constructive advice and feedback throughout my research, manuscripts and thesis writing enhanced the outcome of this thesis. I feel very privileged to have them as supervisors.

I greatly appreciate Soo and Jeanneth for their immeasurable support and for always being available to help with everything relating to my experiments. I cannot imagine how much harder these three years would have been without them. I especially want to thank them for their collaborative efforts in helping with Y-tube observations and providing their valuable feedback on the manuscripts.

I would like to thank the rest of the fruit fly team at Macquarie University, especially Donald, Benjamin, Tahereh and Maryam for their friendship and making the workplace comfortable and pleasant. I thank Vivian for assisting me with the EAG research, and I am in debt to Danielle for her preliminary work on identification of volatile compounds from *Bactrocera kraussi*, *B. frauenfeldi* and *B. musae*, which was the foundation for my project. I am also thankful to all the other people at Macquarie University who have helped me in various ways over the years. I would also like to express my appreciation to the Queensland Department of Agriculture and Fisheries (QDAF), especially Sybilla for supplying fruit flies for this study.

This study would not have been possible without the financial support of the Australian Research Council Industrial Transformation Training Centre (ITTC) for Fruit Fly Biosecurity Innovation (Project IC50100026), funded by the Australian Government.

Last but not least, I would like to express my profound thanks to my wonderful family for their never-ending support, love and encouragement. Thank you for understanding that my research took over my life for the past three years. A very special thanks goes to Ali for his patience and for always being happy to listen to me talk about my research. Thank you love for helping me survive all the stressful times during my study and for not letting me give up. It would not have been possible to have completed this thesis without you.

List of Abbreviations

AcOH	Acetic acid
ANOVA	Analysis of variance
APVMA	Australia Pesticide and Veterinary Medicines Authority
bp	Boiling point
CAR	Carboxen
$CDCl_3$	Deuterated chloroform
C_6D_6	Deuterated benzene
cm	Centimetre
δ	NMR chemical shifts in parts per million
d	Doublet
dd	Doublet of doublets
ddt	Doublet of doublet of triplets
DCM	Dichloromethane
DVB	Divinylbenzene
EAG	Electroantennography
EI	Electron impact
Et	Ethyl
EtOH	Ethanol
eV	Electron volt
FID	Flame ionization detector
FHS	Female headspace
FRG	Female rectal gland
g	Gram
G	Generation
GC	Gas chromatography
GC-EAD	Gas chromatography-electroantennogram detection
GC-EPD	Gas chromatography-electropalpogram detection
GC-MS	Gas chromatography-mass spectrometry
$HClO_4$	Perchloric acid
$Hg(OAc)_2$	Mercury(II) acetate
HPLC	High-performance liquid chromatography
HS	Headspace

Hz	Hertz
ID	Inner diameter
ITTC	Industrial Transformation Training Centre
J	Coupling constant
KI	Kovats index
KOH	Potassium hydroxide
L	Litre
µL	Microlitre
m	Multiplet
mm	Millimetre
µm	Micrometre
m/z	Mass to charge
MAT	Male annihilation technique
MHS	Male headspace
MRG	Male rectal gland
MHz	Megahertz
min	Minute
mg	Milligram
mL	Millilitre
MS	Mass spectrometry
MW	Molecular weight
NaOH	Sodium hydroxide
$NaBH_4$	Sodium borohydride
ND	Not detected
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
ns	Not significant
NSW	New south wales
P	P-value
PA	Polyacrylate
PBS	Phosphate-buffered saline
PDMS	Polydimethylsiloxane
PDMS/DVB	Polydimethylsiloxane/divinylbenzene
ppm	Parts per million

Pr	Propyl
q	Quartet
QDAF	Queensland Department of Agriculture and Fisheries
RG	Rectal gland
RH	Relative humidity
RI	Retention index
rt	Room temperature
RT	Retention time
s	Singlet
sep	Septet
SE	Standard error
SIT	Sterile insect technique
SPME	Solid phase microextraction
Et_3N	Triethylamine
t	Triplet
THF	Tetrahydrofuran

Chapter One

Introduction

Introduction

This PhD study investigated chemical mediation of sexual communication in four economically important pest fruit fly species; the banana fruit fly *Bactrocera musae* (Tryon), the mango fruit fly *Bactrocera frauenfeldi* (Schiner), *Bactrocera bryoniae* (Tryon) and *Bactrocera kraussi* (Hardy). Of the four species, the composition of chemical profile of male *B. kraussi* has been studied before, but the research into the other species as well as female *B. kraussi* is entirely novel. Using the most economically destructive fruit fly in Australia, *B. tryoni* (Froggatt), as a model this study also compared sampling methods used to collect fruit fly volatiles, identifying numerous previously unreported volatiles in this species.

1. 1. The fruit fly life cycle

There are more than 4000 species of true fruit flies (Diptera: Tephritidae), with many regarded as significant horticultural pests.[1,2] In 2014, total damage caused by fruit flies was estimated to be more than $2 billion USD annually.[3] In Australia alone, losses caused by fruit flies was valued at $159 million AUD per annum.[4] Adding to this economic burden, the presence of fruit flies in fruit-producing areas also impedes trade, with many countries imposing strict quarantine restrictions before importation of produce is allowed. Of the tephritid fruit flies, members of the genus *Bactrocera* are among the most damaging. Many are serious quarantine pests and significant contributors to economic losses in production of fruits and vegetables, and are regarded as a major concern for international trade of fresh fruits and vegetables.[5–9] Thus, efficient and effective detection and control measures are essential.

The tephritid fruit fly life cycle consists of four different stages: egg, larva, pupa and adult (Figure 1). Adult female fruit flies cause direct damage by laying eggs under the skin of fruits and vegetables. Bacteria can infect the site of oviposition which results in the partial or complete degradation of the crop and hence render the crop unmarketable. The eggs hatch into larvae that feed on the internal tissue of their host, damaging the crop and making it inedible or resulting in premature fruit drop.[7,10] After several days, when the larvae have grown sufficiently, they jump out of the host to pupate in the soil. [11–14] The adult flies emerge

from the pupal cases in the soil and then disperse from the soil surface. Fruit fly life cycles differ according to species and environmental conditions. Maturation from eggs to adult flies that are able to lay eggs usually takes 3-5 weeks in favourable conditions for many tropical species.[6] The short life cycle of fruit flies can enable rapid population increases.

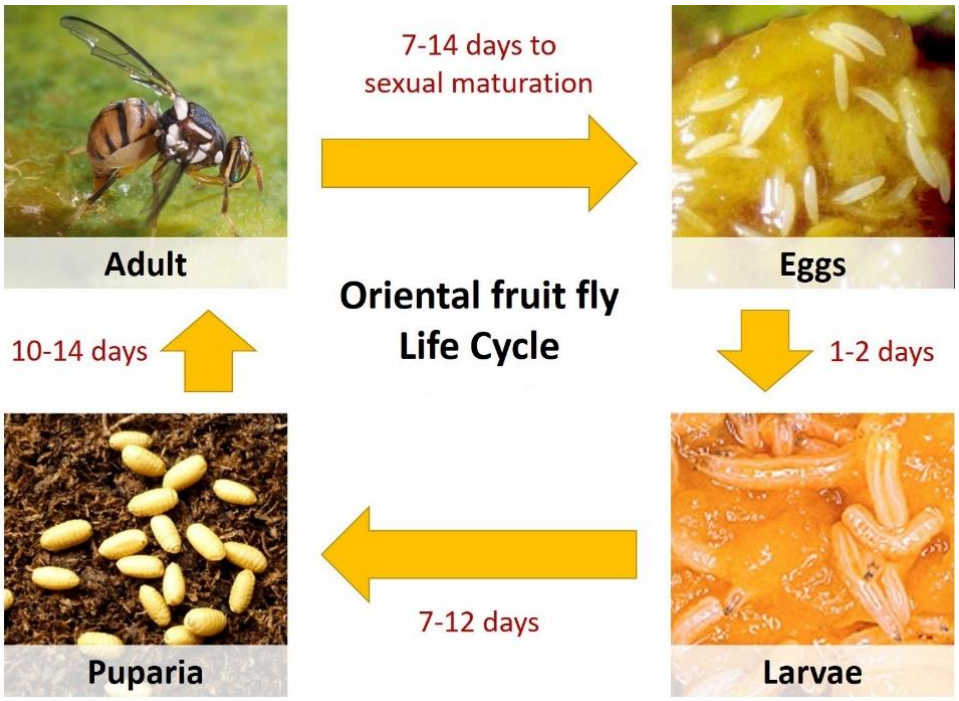


Figure 1. Schematic diagram of tephritid fruit fly life cycle; *Bactrocera dorsalis* (Oriental fruit fly) as a model.[15,16]

In Australia, an estimated 46 exotic and endemic species of fruit fly are considered to present significant economic threats to agriculture,[6,17] with the Queensland fruit fly (*Bactrocera tryoni*) as the most destructive pest fruit fly species. *Bactrocera tryoni* infests around 250 different crops, including many economically important horticulture crops such as apple, peach, kiwi fruit, orange and tomatoes.[6,7,18,19] *Bactrocera tryoni* is native to northern and eastern states of Australia, having spread southward from Queensland through New South Wales, and is now established in much of Victoria as well as some Pacific Islands.[7,20,21] Climate change and inadequate control may have contributed to the expansion of *B. tryoni* into the southern states of Australia.[7,18,22] In addition to *B. tryoni*, other economically important pest fruit fly species in Australia include *B. musae* (Tryon) (the banana fruit fly), *B. frauenfeldi* (Schiner) (the mango fruit fly), *B. bryoniae* (Tryon) and *B. kraussi* (Hardy) (Figure 2). These species are polyphagous pests that are known to infest a range of both wild and commercial host plants. *Bactrocera musae* has been recorded on 16 hosts from nine plant families including Musaceae, Caricaceae and Myrtaceae, and its major hosts are

banana, papaya and guava.[2,23] *Bactrocera frauenfeldi* has been found on 109 hosts from 37 plant families including Anacardiaceae, Caricaceae, Moraceae, Musaceae, Myrtaceae, Oxalidaceae, Passifloraceae, Rutaceae, Sapotaceae and Solanaceae. Its major commercial hosts include mango, banana, citrus, guava, papaya and star apple.[2,23–25] *Bactrocera bryoniae* has been recorded on 9 hosts from 5 families including Cucurbitaceae, Loganiaceae, Musaceae, Passifloraceae and Solanaceae, and chilli is the main commercial host for this species.[2,26,27] *Bactrocera kraussi* has been recorded on 106 hosts from 31 families including Anacardiaceae, Musaceae, Myrtaceae, Oxalidaceae, Passifloraceae, Rosaceae, Rubiaceae, Rutaceae, Sapindaceae, Sapotaceae and Solanaceae. Its major commercial hosts include mango, banana, guava, feijoa, carambola, peach, citrus and tamarind.[2,23]

Bactrocera musae is distributed throughout mainland Papua New Guinea. In Australia, it is very common along the eastern coast of Queensland as far south as Townsville.[2,28] *Bactrocera frauenfeldi* is endemic to Papua New Guinea[29] and has become established on several Pacific Islands, including the Solomon Islands, the Federated States of Micronesia, the Republic of Kiribati, Marshall Islands, Palau, Nauru, West Papua in Indonesia[30–32] and in Australia.[2,23,28,33,34] *Bactrocera bryoniae* is well established throughout Indonesia (Papua), Papua New Guinea (every province except Bougainville and Manus), and parts of Australia (Queensland, Northern Western Australia, Northern Territory, east coast south to Sydney, New South Wales, and the Torres Strait Islands).[2,35,36] *Bactrocera kraussi* is found in the Torres Strait Islands and Northeast Queensland, reaching as far south as Townsville.[2] In Australia, *B. musae*, *B. frauenfeldi*, *B. bryoniae* and *B. kraussi* are all currently restricted to specific regions[20] but with climate change and availability of hosts they have potential to become established in other regions.[22]

Figure 2. Up left: male *B. musae*[26], up right: female *B. bryoniae*[26], bottom left: female *B. frauenfeldi*[26], bottom right: female *B. kraussi* (photo by author).

1. 2. Fruit fly control methods

A number of control methods have been deployed to manage fruit fly populations. The most successful and widely employed method is use of insecticides, mainly dimethoate and fenthion which have been used over the past 50 years.[19] These organophosphate insecticides only require contact rather than ingestion to be effective. Insecticides are most often applied to the entire tree by cover spraying, with the intention to kill adult fruit flies prior to oviposition (laying eggs) into the fruit.[19] Organophosphates are cholinesterase inhibitors,[19] and are hazardous to humans as they can inhibit human acetyl cholinesterase. Exposure to these insecticides can cause severe hypotension, and affect the function of the central nervous system[7,19] leading to several serious neurotoxic effects and death.[37,38] Moreover, organophosphate insecticides are hazardous to birds and aquatic life and are also non-discriminatory to insects, killing natural enemies and pollinators as well as pests.[39,40] Dimethoate and fenthion, along with other organophosphates, are being phased out or are now used only under restrictive regulations.[19,41,42] The Australia Pesticide and Veterinary Medicines Authority (APVMA) conducted reviews into dimethoate and fenthion, determining that many common historical use patterns of dimethoate and fenthion would exceed the acceptable daily intake and/or associated acute reference dose. As a result, the use of dimethoate has been suspended on many crops in Australia, whilst fenthion use has ceased.[41,42]

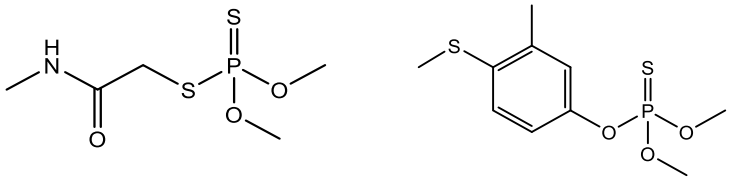


Figure 3. Structures of dimethoate (*O,O*-dimethyl *S*-[2-(methylamino)-2-oxoethyl] phosphorodithioate) (left) and fenthion (*O,O*-dimethyl *O*-[3-methyl-4-(methylsulfanyl)phenyl] phosphorothioate) (right).

With restrictions on the use of organophosphate insecticides, other fruit fly control methods are and will become more heavily relied on to protect crops. More environmentally benign approaches that have been deployed include the sterile insect technique (SIT) and 'lure and kill'. SIT involves producing and releasing sterile males of the target pest species to compete with wild males in mating with wild females, hence preventing the females from reproducing and reducing the fruit fly population over generations.[43,44] The 'lure and kill' technique has an emphasis on food-based or semiochemical-based lures in combination with an insecticide in a trap. Food-based lures attract both male and female fruit flies,[45,46] while semiochemical-based lures such as cuelure, zingerone, raspberry ketone and methyl eugenol (Figure 3)[7,47,48] attract males for the 'male annihilation technique' (MAT). Semiochemical-based male attractants are generally more powerful attractants than the food-based lures.[47]

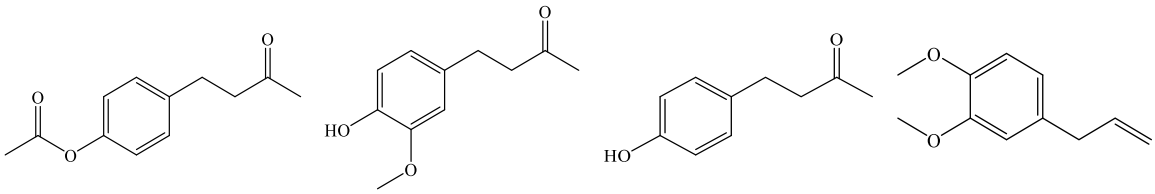


Figure 4. Chemical structures of the male lures cuelure, zingerone, raspberry ketone and methyl eugenol.

While available chemical lures are attractive to many *Bactrocera* and closely related *Dacus* and *Zeugodacus* species, approximately 50% of the identified species do not respond to any known lure or have only a weak response.[7,47,49,50] Female fruit flies cause direct damage to crops, but available female lures have very limited efficacy. There remains significant interest in developing new lures for females and for males of species that do not respond to known lures.

1. 3. Fruit fly pheromones

Semiochemicals are produced by organisms and are involved in biological communication.[51,52] Semiochemicals are subdivided into allelochemicals and pheromones. Allelochemicals are emitted by one species in order to modify the behaviour of another species. Allelochemicals are further classified into allomones (benefit only the originator but not the receiver), kairomones (benefit only the receiver but not the originator) and synomones (benefit both originator and receiver). Pheromones are like allelochemicals, except they are emitted by members of a species to modify the behaviour of other members of the same species. Pheromones are subdivided into several categories based on the nature of the interaction, including sex pheromones (released by members of one sex to attract the opposite sex), aggregation pheromones (attract both males and females to a small area), and alarm pheromones (alert individuals to danger).[51–53]

The rectal gland of tephritid fruit flies (Figure 4) is well known as a sex pheromone secreting organ.[54–59] Emission of volatile sex pheromones into the atmosphere during calling and courtship has been considered as mechanism for short and long range attraction of the opposite sex.[60–63] The volatile emissions also play an important role in attracting the same sex to mating aggregations.[64,65] During periods of sexual activity, which is usually restricted to a specific time of day, mature males release pheromones to attract females.[8,65] The males start sexual behaviour by releasing sex pheromones from the rectal gland through the anus. They rub the rectal gland content onto the wings using the hind legs, and disperse the contents by rapid wing fanning that produces unique audible pulses of buzzing that is generally known as ‘calling’.[58,66,67]

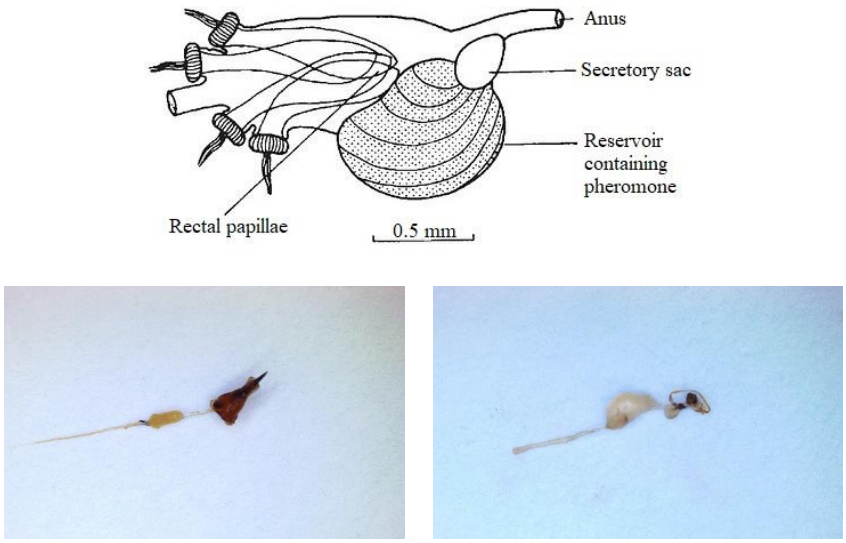


Figure 5. Up: schematic diagram of the anatomy of a rectum of a sexually mature *Bactrocera* species showing the rectal gland (reservoir containing pheromone) [59]. Bottom left: female *B. bryoniae* rectal gland (photo by author). Bottom right: male *B. bryoniae* rectal gland (photo by author).

Of more than 450 species in the genus *Bactrocera*, the sex and aggregation pheromones of only a few pest species have been investigated for potential applications as attractants.[68] One of the first investigations was conducted by B. S. Fletcher, who showed that male *B. tryoni* (Froggatt) produce and release sex pheromones.[59] This was reported as a blend containing six aliphatic amides (Figure 5).[69] Male fruit flies have been assumed to be the main pheromone producer for most tephritid fruit flies[70–75] and most studies have focused on chemical profiles of males.[69,76,77] However, there are at least three studies that show females also produce sex pheromones. For example, 1,7-dioxaspiro[5,5]undecane has been described as a female-produced pheromone of *B. oleae*,[78] although later studies also reported this compound in rectal glands of young males.[79] While 1,7-dioxaspiro[5,5]undecane has been used extensively for monitoring and mass trapping of *B. oleae*,[80] sex specific olfactory cues of *B. oleae* are driven by synergistic actions of a number of compounds that are not yet fully understood.[8,81] Similarly, in the melon fly, *Z. cucurbitae* (Coquillett), females are attracted by male rectal gland secretions containing three aliphatic amides, two pyrazines and an aromatic acid,[82] while males are attracted by female headspace constituents containing 2,8-dialkyl-1,7-dioxaspiro[5,5]undecanes and *N*-(3-methylbutyl)acetamide.[83] Similarly, males of the oriental fruit fly, *B. dorsalis* (Hendel), produce two phenols and an aliphatic cyclic alcohol in rectal glands that show pheromonal activity towards females,[84,85] while females emit several spiroacetals that attract males.[83]

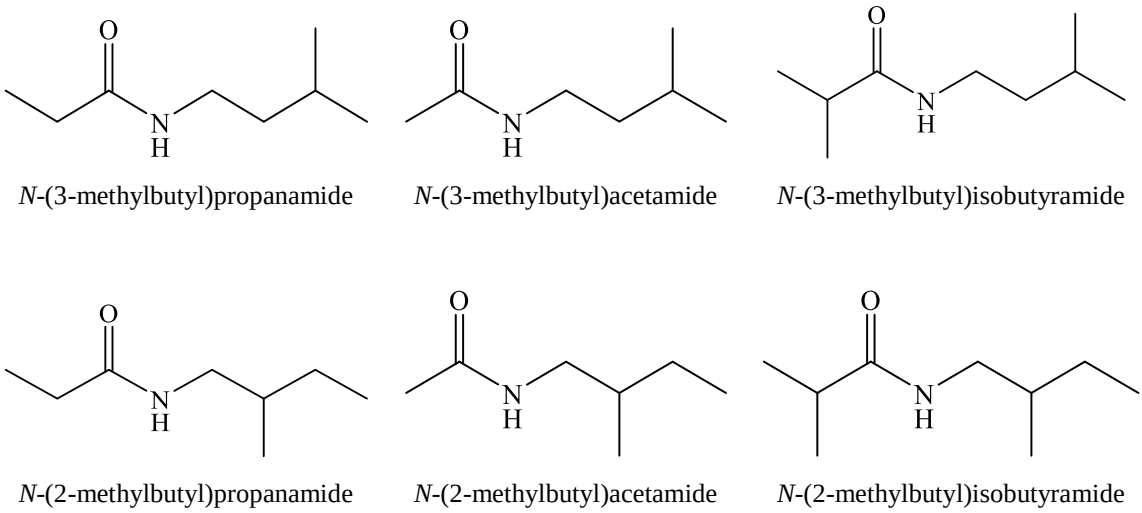


Figure 6. Structures of amides reported in the pheromone profile of male *B. tryoni*.

Given that chemical-mediated sexual communication is a key component in the reproductive biology of tephritid fruit flies,[8,86] and the potential application of volatiles as attractants, understanding of the chemical communication of economically important species is of particularly interest. To better understand this, the PhD study aimed to identify and characterise rectal gland composition and volatiles released from males and females of four important *Bactrocera* species, *B. musae*, *B. frauenfeldi*, *B. bryoniae* and *B. kraussi*. This was done using gas chromatography-mass spectrometry to identify the volatile emissions; gas chromatography-electroantennogram detection and gas chromatography-electropalpogram detection to identify electrophysiologically active components present in the emissions of each sex; and y-maze olfactometers to evaluate the attractiveness of the volatile compounds to the opposite and same sex.[87–89]

1. 4. Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) permits a screening of compounds in complex chemical mixtures such as fruit fly pheromones, and is a common practice for the identification of fruit fly volatile compounds.[89–93] GC separates complex mixtures into individual components, allowing sequential analysis of the components by MS. Individual components can be tentatively identified by matching the fragmentation pattern with a mass spectral library. If the compound is not available in a spectral library, detailed analysis of the fragmentation pattern provides an alternative approach to identification. Finally, comparison

of mass spectra and retention times to authentic samples provides definitive identification of each compound.

1. 5. Electrophysiological assays

Electroantennogram and electropalpogram detection (EAD and EPD, respectively) measure the difference in electrical potential between the tip and base of the antenna/maxillary palps on exposure to volatiles. When a chemical stimulus is introduced to the antenna/palps, if there are receptors that detect the stimulus, the depolarisation of receptor neurons is measured as the electrical response (amplitude) in millivolts. Coupling GC to an EAD/EPD permits a rapid screening of compounds in complex mixtures; the electrophysiological response of the antenna/palp to different compounds (peaks) indicates which components in a blend are detectable by the insect sensory system (Figure 6). The electrophysiological responses do not reliably indicate whether a compound will influence the behaviour of an insect or what the behaviour might be. Therefore, behavioural assays are needed to evaluate behavioural responses, such as attraction.

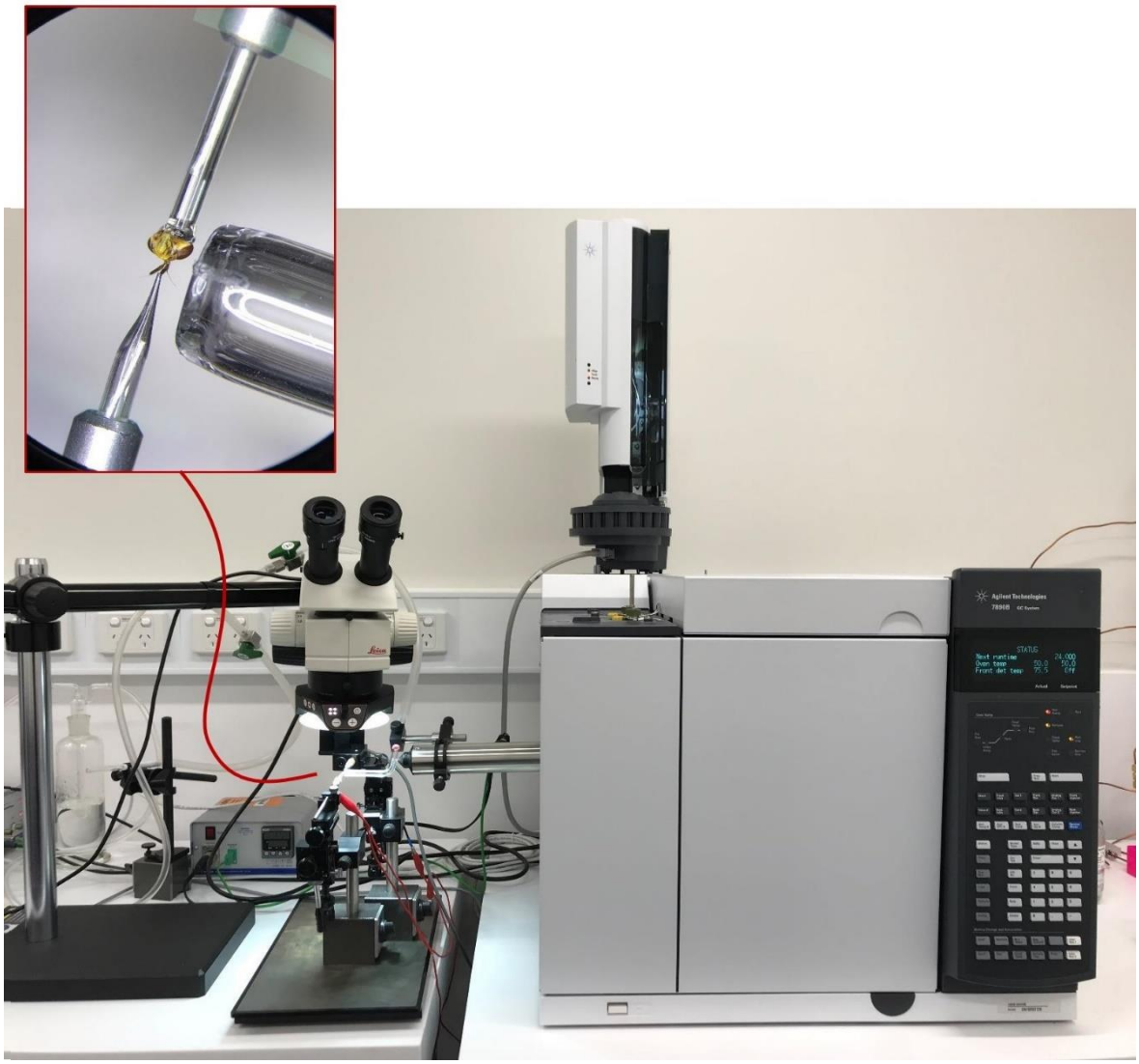


Figure 7. GC-EAD apparatus used in this study (photo by author).

1. 6. Behavioural assays

Olfactometer experiments, in which insects are given the choice between two or more odour sources to test behavioural preferences, are commonly used in chemical ecology studies. Y-Tube olfactometers, in particular, have been widely used in the studies of sex and aggregation pheromones of fruit flies.[87–89] They comprise of a Y-shaped tube with one central arm in which the release chamber is located, and two upwind side arms, each of which is connected to a control or stimulus source (Figure 7). Once the fly reaches the junction of the Y, they then make a choice between the two arms. Compared to other behavioural assays, Y-tube olfactometry provides clearer responses and enables much higher throughput because it is possible to run multiple trials in parallel. This is particularly

important when studying volatiles associated with mating, for which many fruit flies are commonly only responsive for ~30 minutes each day.

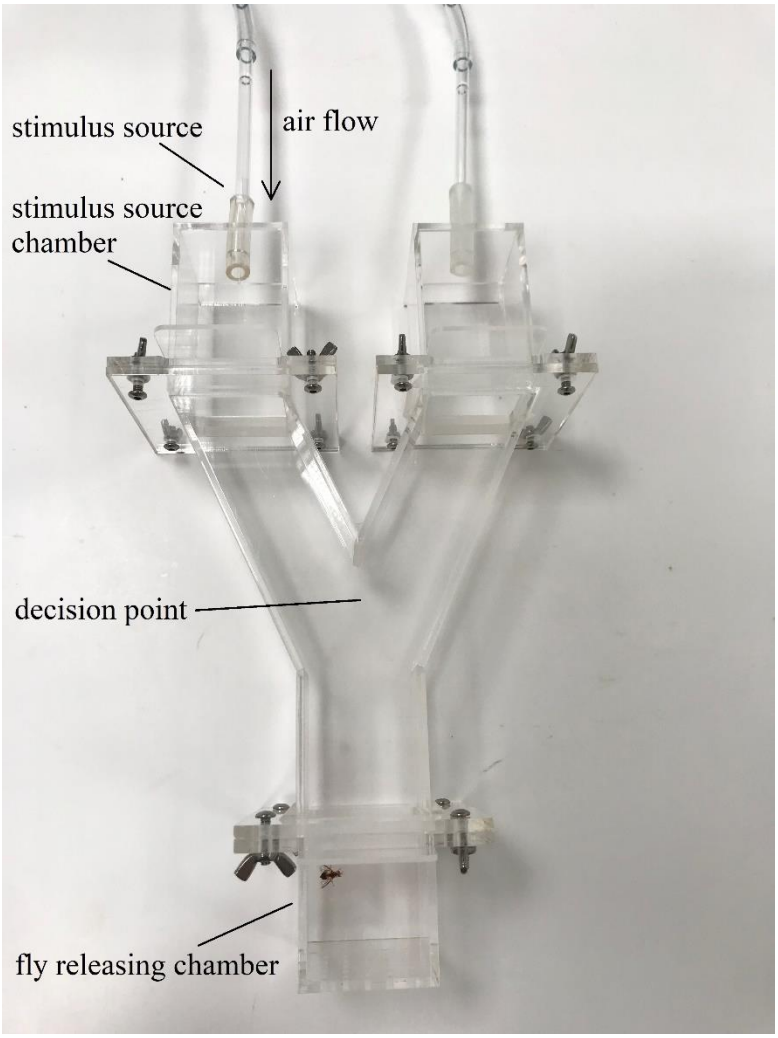


Figure 8. Y-Tube apparatus used in this study (photo by author).

1. 7. Semiochemical sampling techniques

Diverse sampling methods have been used for the collection of insect volatile semiochemicals. Because semiochemicals are commonly produced and released at low concentrations, efficient sampling methods are needed for collection and subsequent identification and quantification of composition.[55,94–97] Each sampling method may have different sensitivities to different compounds, and may be insensitive to some compounds. This has consequences both for understanding of each species' semiochemical profile and for comparisons across species. The most common method deployed for sampling of

tephritid fruit fly rectal gland volatiles involves immersion of rectal glands in organic solvents. Selection of the extraction solvent depends on the specific nature of compounds being targeted and the extraction yield can be affected by the polarity of the solvent. Common solvents that have been used for fruit fly rectal gland extraction differ in polarity and include *n*-pentane, *n*-hexane, acetone, dichloromethane and ethanol.[56,70,71,87,89] Some studies have extracted compounds from intact rectal glands by immersing them in the solvent, but in other studies the glands have been crushed.[61,71,90,98] Instead of focusing on compounds stored in the rectal glands, other studies have focused on the volatiles released by live fruit flies. In most studies, the isolation technique entails trapping volatiles onto an adsorbent material using either dynamic or static sampling techniques.[99] Dynamic sampling techniques involve purging and trapping volatiles onto an adsorbent material such as Porapak (ethylvinylbenzene-divinylbenzene copolymer), activated charcoal or Tenax (porous polymer based on 2,6-diphenyl-*p*-phenylene oxide) by passing a constant air flow. Tenax[71,100,101] and Porapak[74,102] have been broadly used in sampling of fruit fly semiochemicals. Static sampling techniques involve use of adsorbent materials under static air. The most commonly deployed static sampling method uses SPME (solid phase microextraction) adsorbent fibres, such as this with polydimethylsiloxane (PDMS), carboxen (CAR), divinylbenzene (DVB), polyacrylate (PA) or mixed-phase coatings, depending on the polarity of compounds of interest. The PDMS fibre is extensively used for extraction of non-polar semiochemicals.[79,103–105] Polyacrylate (PA) has been used for less volatile semiochemicals due to its high affinity to more polar compounds.[106,107] Polydimethylsiloxane/divinylbenzene (PDMS/DVB), a mixed-phase coating that covers a broader spectrum due to PDMS and DVB's different polarities, has also been used for collection of semiochemicals.[63,90,98,106,108,109] Because adsorbent materials differ in affinity for particular groups of semiochemicals, an inappropriate selection of adsorbent can result in substantial under-sampling, or failure to detect, even compounds that are quite abundant. Thus, chemical and physical characteristics need to be carefully considered in employing both sampling tools and materials. This PhD study compared the effects of common sampling methods and materials on the detection and quantification of fruit fly semiochemicals and provides guidance for choosing the most suitable method.

1. 8. Objectives of study

Current restrictive regulations on the use of insecticides for fruit fly control has raised the need to develop alternative fruit fly monitoring and control methods. The central role of chemical-mediated sexual communication in the reproduction of fruit flies, and the potential application of volatiles as attractants, as well as the fact that little is known about fruit fly pheromones and sexual selection in the genus *Bactrocera*, highlight the need to characterise the pheromone profile of more *Bactrocera* species. Thus, the core objectives of this study were to:

- examine and identify the rectal gland compositions and volatiles released from males and females of important pest species; *Bactrocera musae*, *B. frauenfeldi*, *B. bryoniae* and *B. kraussi* using GC-MS;
- compare the effects of common sampling methods and materials on the detection and quantification of fruit fly semiochemicals using *B. tryoni*, Australia's most economically damaging fruit fly species as a model.

1. 9. References

1. Díaz-Fleischer, F. & Aluja, M. Behavior of Tephritid Flies: A Historical Perspective. in *Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior* (eds. Aluja, M. & Norrbom, A. L.) 39–69 (CRC Press, 2000).
2. Schutze, M. *et al. The Australian Handbook for the Identification of Fruit Flies. Version 3.1*. (Plant Health Australia, 2018). doi:10.1016/j.jasms.2007.01.008
3. Malavasi, A. *Trapping and the Detection, Control, and Regulation of Tephritid Fruit Flies*. (Springer, 2014). doi:10.1007/978-94-017-9193-9
4. PHA. Fruit flies | Plant Health Australia. (2019). Available at: https://www.planthealthaustralia.com.au/national-programs/fruit-fly/. (Accessed: 16th December 2019)
5. Vijaysegaran, S. Fruit fly research and development in tropical Asia. in *Management of Fruit Flies in the Pacific* (ed. Allwood, A., Drew, R, E.) 21–29 (Australian Centre for International Agricultural Research Proceedings, 1997).
6. Hickey, P. *et al. National fruit fly strategy implementation action plan*. (Plant Health Australia, 2010).
7. Clarke, A. R., Powell, K. S., Weldon, C. W. & Taylor, P. W. The ecology of *Bactrocera tryoni* (Diptera: Tephritidae): what do we know to assist pest management? *Ann. Appl. Biol.* **158**, 26–54 (2011).
8. Benelli, G. *et al.* Sexual communication and related behaviours in Tephritidae: current knowledge and potential applications for integrated pest management. *J. Pest Sci.* **87**, 385–405 (2014).
9. Dominiak, B. C. & Mapson, R. Revised distribution of *Bactrocera tryoni* in eastern Australia and effect on possible incursions of Mediterranean fruit fly: development of Australia's eastern trading block. *J. Econ. Entomol.* **110**, 2459–2465 (2017).
10. Ekesi, S. & Mohamed, S. A. Mass rearing and quality control parameters for tephritid fruit flies of economic importance in Africa. in *Wide Spectra of Quality Control* (ed. Akyar, I.) (InTech, 2011). doi:10.5772/21330
11. Exley, E. M. Comparative morphological studies of the larvae of some Queensland Dacinae (Trypetidae, Diptera). *Queensl. J. Agric. Sci.* **12**, 119–150 (1955).
12. Anderson, D. T. The larval development of *Dacus tryoni* (Frogg.) (Diptera: Trypetidae) I. Larval Instars, imaginal discs, and haemocytes. *Aust. J. Zool.* **11**, 202–218 (1963).

13. Anderson, D. T. The larval development of *Dacus tryoni* (Frogg.) (Diptera: Trypetidae) II. Development of imaginal rudiments other than the principal discs. *Aust. J. Zool.* **12**, 1–8 (1964).
14. Elson-Harris, M. M. Morphology of the immature stages of Dacus tryoni (Froggatt) (Diptera: Tephritidae). *Austral Entomol.* **27**, 91–98 (1988).
15. Daniel, C. & Grunder, J. Integrated management of European cherry fruit fly *Rhagoletis cerasi* (L.): situation in Switzerland and Europe. *Insects* **3**, 956–988 (2012).
16. *Bactrocera dorsalis* - Oriental fruit fly. Available at: https://wiki.nus.edu.sg/display/TAX/Bactrocera+dorsalis+-+Oriental+fruit+fly.
17. Plant Health Australia. *The National Plant Biosecurity*. (2018).
18. Sutherst, R. W., Collyer, B. S. & Yonow, T. The vulnerability of Australian horticulture to the Queensland fruit fly, *Bactrocera (Dacus) tryoni*, under climate change. *Aust. J. Agric. Res.* **51**, 467–480 (2000).
19. Dominiak, B. C. & Ekman, J. H. The rise and demise of control options for fruit fly in Australia. *Crop Prot.* **51**, 57–67 (2013).
20. Royer, J. E. & Hancock, D. L. New distribution and lure records of Dacinae (Diptera: Tephritidae) from Queensland, Australia, and description of a new species of *Dacus* Fabricius. *Aust. J. Entomol.* **51**, 239–247 (2012).
21. Drew, R. A. I., Hooper, G. H. S. & Bateman, M. A. *Economic Fruit Flies of the South Pacific Region*. (Queensland Department of Primary Industries, 1978).
22. Sultana, S., Baumgartner, J. B., Dominiak, B. C., Royer, J. E. & Beaumont, L. J. Impacts of climate change on high priority fruit fly species in Australia. *bioRxiv* (2019). doi:10.1101/567321
23. Hancock, D. L., Hamacek, E. L., Lloyd, A. C. & Elson-Harris, M. M. *The distribution and host plants of fruit flies (Diptera: Tephritidae) in Australia*. (Queensland Department of Primary Industries, 2000).
24. Leblanc, L., William, J. & Allwood, A., J. Host fruit of mango fly (Bactrocera frauenfeldi (Schiner)) (Diptera: Tephritidae) in the Federated States of Micronesia. *Micronesica* **37**, 21–31 (2004).
25. Metcalf, R. L. & Metcalf, E. R. *Plant kairomones in insect ecology and control*. (Chapman and Hall, 1992).
26. Leblanc, L. *et al. Fruit flies in Papua New Guinea*. *Plant Protection Service* (2001).
27. Hamacek, E. Host records of fruit flies in the South Pacific. in *Management of Fruit Flies in the Pacific* (eds. Allwood, A. J. & Drew, R. A. I.) 102 (Australian Centre

for International Agricultural Research, 1997).

28. Osborne, R. *et al.* Australian distribution of 17 species of fruit flies (Diptera: Tephritidae) caught in cue lure traps in February 1994. *Aust. J. Entomol.* **36**, 45–50 (1997).
29. Leblanc, L. & Allwood, A. J. Mango fruit fly (*Bactrocera frauenfeldi*): why so many in federated states of Micronesia. in *Management of Fruit Flies in the Pacific* (eds. Allwood, A. J. & Drew, R. A. I.) 125–130 (Australian Centre for International Agricultural Research, 1997).
30. Hardy, D. E. & Adachi, M. Diptera: Tephritidae. in *Insects of Micronesia* 1–28 (B.P. Bishop Museum Bulletin, 1956).
31. Allwood, A. J. & Leblanc, L. Losses caused by fruit flies (Diptera: Tephritidae) in seven Pacific Island countries. in *Management of Fruit Flies in the Pacific* 208–2011 (Australian Centre for International Agricultural Research, 1997).
32. Mararuai, A. N. Market access of Papua New Guinea bananas (Musa spp.) with particular respect to banana fly (Bactrocera musae (Tryon)) (Diptera: Tephritidae). (PhD Thesis, School of Natural Resource Sciences, Queensland University of Technology, 2010).
33. Drew, R. A. I., Hancock, D. L. & Romig, M. C. New species and records of fruit flies (Diptera: Tephritidae: Dacinae) from north Queensland. *Australian Entomologist* **26**, 1–12 (1999).
34. Royer, J. E., Wright, C. L. & Hancock, D. L. *Bactrocera frauenfeldi* (Diptera: Tephritidae), an invasive fruit fly in Australia that may have reached the extent of its spread due to environmental variables. *Austral Entomol.* **55**, 100–111 (2016).
35. Leblanc, L., Tora, E., Drew, R. A. I. & Allwood, A. J. Host plant records for fruit flies (Diptera: Tephritidae: Dacini) in the Pacific Islands. *Proc. Hawaiian Entomol. Soc.* **44**, 11–53 (2013).
36. Drew, R. A. I. & Romig, M. C. *Tropical Fruit Flies of South-East Asia*. *Tropical Fruit Flies of South-East Asia* (CAB International, 2013).
37. Davies, J., Roberts, D., Eyer, P., Buckley, N. & Eddleston, M. Hypotension in severe dimethoate self-poisoning. *Clin. Toxicol.* **46**, 880–884 (2008).
38. Senanayake, N. & Karalliedde, L. Neurotoxic effects of organohosphorus insecticides. *N. Engl. J. Med.* **316**, 761–763 (1987).
39. Vittozzi, L. & De Angelis, G. A critical review of comparative acute toxicity data on freshwater fish. *Aquatic Toxicology* **19**, 167–204 (1991).
40. DeWeese, L. R., McEwen, L. C., Settimi, L. A. & Deblinger, R. D. Effects on birds

of fenthion aerial application for mosquito control. *J. Econ. Entomol.* **76**, 906–911 (1983).

41. Australian Pesticides and Veterinary Medicines Authority. *Dimethoate residues and dietary risk assessment report*. (Australian Pesticides and Veterinary Medicines Authority, 2016).
42. Australian Pesticides and Veterinary Medicines Authority. *Fenthion residues and dietary risk assessment report*. (Australian Pesticides and Veterinary Medicines Authority, 2012).
43. Klassen, W. Area-wide integrated pest management and SIT. in *Sterile Insect Technique Principles and Practice in Area-Wide Integrated Pest Management* (eds. Dyck, V. A., Hendrichs, J. & Robinson, A. S.) 39–68 (Springer, 2005).
44. Klassen, W. & Curtis, C. F. History of the sterile insect technique. in *Sterile Insect Technique Principles and Practice in Area-Wide Integrated Pest Management* (eds. Dyck, V. A., Hendrichs, J. & Robinson, A. S.) 3–38 (Springer, 2005).
45. Epsky, N. D., Kendra, P. E. & Schnell, E. Q. History and development of food-based attractants. in *Trapping and the Detection, Control, and Regulation of Tephritid Fruit Flies* 75–118 (Springer Netherlands, 2014). doi:10.1007/978-94-017-9193-9_3
46. Dominiak, B. C. & Nicol, H. I. Field performance of Lynfield and McPhail traps for monitoring male and female sterile *Bactrocera tryoni* (Froggatt) and wild *Dacus newmani* (Perkins). *Pest Manag. Sci.* **66**, 741–744 (2010).
47. Royer, J. E. Responses of fruit flies (Tephritidae: Dacinae) to novel male attractants in north Queensland, Australia, and improved lures for some pest species. *Austral Entomol.* **54**, 411–426 (2015).
48. El-Sayed, A. M., Suckling, D. M., Byers, J. A., Jang, E. B. & Wearing, C. H. Potential of "lure and kill" in long-term pest management and eradication of invasive species. *J. Econ. Entomol.* **102**, 815–835 (2009).
49. Fay, H. A. C. A highly effective and selective male lure for *Bactrocera jarvisi* (Tryon) (Diptera: Tephritidae). *Aust. J. Entomol.* **51**, 189–197 (2012).
50. Tan, K. H., Nishida, R., Jang, E. B. & Shelly, T. E. Pheromones, male lures, and trapping of tephritid fruit flies. in *Trapping and the Detection, Control, and Regulation of Tephritid Fruit Flies* 15–74 (Springer Netherlands, 2014). doi:10.1007/978-94-017-9193-9_2
51. Karuppuchamy, P. & Venugopal, S. Integrated pest management. in *Ecofriendly Pest Management for Food Security* 651–684 (Elsevier Inc., 2016).

doi:10.1016/B978-0-12-803265-7.00021-X

52. Kalaisekar, A., Padmaja, P. G., Bhagwat, V. R. & Patil, J. V. Pest management strategies and technologies. in *Insect Pests of Millets* 143–183 (Elsevier, 2017). doi:10.1016/b978-0-12-804243-4.00005-7

53. Vargas, R. I., Shelly, T. E., Leblanc, L. & Piñero, J. C. Recent Advances in Methyl Eugenol and Cue-Lure Technologies for Fruit Fly Detection, Monitoring, and Control in Hawaii. in 575–595 (2010). doi:10.1016/S0083-6729(10)83023-7

54. Perkins, M. V., Fletcher, M. T., Kitching, W., Drew, R. A. I. & Moore, C. J. Chemical studies of rectal gland secretions of some species of *Bactrocera dorsalis* complex of fruit flies (diptera: Tephritidae). *J. Chem. Ecol.* **16**, 2475–2487 (1990).

55. Piccardi, P. Insect sex-communication and prospects for pheromones in pest management. *Bolletino di Zool.* **47**, 397–408 (1980).

56. Tokushima, I., Orankanok, W., Tan, K. H., Ono, H. & Nishida, R. Accumulation of phenylpropanoid and sesquiterpenoid volatiles in male rectal pheromonal glands of the guava fruit fly, *Bactrocera correcta*. *J. Chem. Ecol.* **36**, 1327–1334 (2010).

57. Wee, S. L. & Tan, K. H. Female sexual response to male rectal volatile constituents in the fruit fly, *Bactrocera carambolae* (Diptera: Tephritidae). *Appl. Entomol. Zool.* **40**, 365–372 (2005).

58. Fletcher, B. S. The structure and function of the sex pheromone glands of the male Queensland fruit fly, *Dacus tryoni*. *J. Insect Physiol.* **15**, 1309–1322 (1969).

59. Fletcher, B. S. Storage and release of sex pheromone by the Queensland fruit fly, *Dacus tryoni* (Diptera:Trypetidae). *Nature* **219**, 631–632 (1968).

60. Nation, J. L. Courtship behavior and evidence for a sex attractant in the male Caribbean fruit fly, *Anastrepha suspensa*. *Ann. Entomol. Soc. Am.* **65**, 1364–1367 (1972).

61. Perkins, M. V. Characterisation and synthesis of Bactrocera fruit fly pheromones. (PhD Thesis, Department of Chemistry.The University of Queensland, Australia, 1990). doi:https://doi.org/10.14264/uql.2015.222

62. Sivinski, J. *et al.* Topics in the evolution of sexual behavior in the Tephritidae. in *Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior* (eds. Aluja, M. & Norrbom, A. L.) 751–792 (CRC Press, 2000). doi:10.1201/9781420074468

63. Cruz-López, L., Malo, E. A. & Rojas, J. C. Sex pheromone of *Anastrepha striata*. *J. Chem. Ecol.* **41**, 458–464 (2015).

64. Sivinski, J. M. & Calkins, C. Use of pheromones in tropical crops: pheromones and parapheromones in the control of tephritids. *Florida Entomol.* **69**, 157–168 (1986).

65. Hendrichs, J., Robinson, A. S., Cayol, J. P. & Enkerlin, W. Medfly areawide sterile insect technique programmes for prevention, suppression or eradication: the importance of mating behavior studies. *Fla Entomol.* **85**, 1–14 (2002).
66. Tychsen, P. Mating behaviour of the Queensland fruit fly, *Dacus tryoni* (Diptera: Tephritidae), in field cages. *J. Aust. Entomol. Soc* **16**, 459–465 (1977).
67. Mankin, R. W., Lemon, M., Harmer, A. M. T., Evans, C. S. & Taylor, P. W. Time-pattern and frequency analyses of sounds produced by irradiated and untreated male *Bactrocera tryoni* (Diptera: Tephritidae) during mating behavior. *Ann. Entomol. Soc. Am.* **101**, 664–674 (2008).
68. Doorenweerd, C., Leblanc, L., Norrbom, A. L., Jose, M. S. & Rubinoff, D. A global checklist of the 932 fruit fly species in the tribe Dacini (Diptera, Tephritidae). *Zookeys* **730**, 19–56 (2018).
69. Bellas, T. E. & Fletcher, B. S. Identification of the major components in the secretion from the rectal pheromone glands of the Queensland fruit flies *Dacus tryoni* and *Dacus neohumeralis* (Diptera: Tephritidae). *J. Chem. Ecol.* **5**, 795–803 (1979).
70. Kitching, W. *et al.* Spiroacetals in rectal gland secretions of Australasian fruit fly species. *J. Chem. Soc. Chem. Commun.* **11**, 853 (1986).
71. Kitching, W. *et al.* Chemistry of fruit flies. Composition of the rectal gland secretion of (male) *Dacus cucumis* (cucumber fly) and *Dacus halfordiae*. Characterization of (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. *J. Org. Chem.* **54**, 3893–3902 (1989).
72. Fletcher, M. T. & Kitching, W. Chemistry of fruit flies. *Chem. Rev.* **95**, 789–828 (1995).
73. Hayes, P., Fletcher, M. T., Moore, C. J. & Kitching, W. Synthesis and absolute stereochemistry of a constitutionally new spiroacetal from an insect. *J. Org. Chem.* **66**, 2530–2533 (2001).
74. Heath, R. R., Landolt, P. J., Robacker, D. C., Dueben, B. D. & Epsky, N. D. Sexual pheromones of tephritid flies: clues to unravel phylogeny and behavior. in *Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior* (eds. Aluja, M. & Norrbom, A. L.) 793–809 (CRC Press, 2000).
75. El-Sayed, A. M. The Pherobase: Database of pheromones and semiochemicals. (2019). Available at: http://www.pherobase.com/. (Accessed: 17th March 2019)
76. Perkins, M. V., Kitching, W., Drew, R. A. I., Moore, C. J. & Konig, W. A. Chemistry of fruit flies: composition of the male rectal gland secretions of some

species of South-East Asian Dacinae. Re-examination of *Dacus cucurbitae* (melon fly). *J. Chem. Soc. Perkin Trans. 1* 1111–1117 (1990). doi:10.1039/P19900001111

77. Krohn, S. *et al.* Chemistry of fruit flies: Nature of glandular secretion and volatile emission of *Bactrocera (bactrocera) cacuminatus* (Héring). *J. Chem. Ecol.* **17**, 485–495 (1991).
78. Baker, R. & Herbert, R. H. Isolation and synthesis of 1,7-dioxaspiro[5.5]undecane and 1,7-dioxaspiro[5.5]undecan-3-and -4-ols from the olive fly (*Dacus oleae*). *J. Chem. Soc. Perkin Trans. 1* 1123 (1987). doi:10.1039/p19870001123
79. Levi-Zada, A. *et al.* Analyzing diurnal and age-related pheromone emission of the olive fruit fly, *Bactrocera oleae* by sequential SPME-GCMS analysis. *J. Chem. Ecol.* **38**, 1036–1041 (2012).
80. Haniotakis, G. E., Mazomenos, B. E. & Tumlinson, J. H. A sex attractant of the olive fruit fly, *Dacus oleae* and its biological activity under laboratory and field conditions. *Entomol. Exp. Appl.* **21**, 81–87 (1977).
81. Benelli, G., Bonsignori, G., Stefanini, C., Raspi, A. & Canale, A. The production of female sex pheromone in *Bactrocera oleae* (Rossi) young males does not influence their mating chances. *Entomol. Sci.* **16**, 47–53 (2013).
82. Baker, R., Herbert, R. H. & Lomer, R. A. Chemical components of the rectal gland secretions of male *Dacus cucurbitae*, the melon fly. *Experientia* **38**, 232–233 (1982).
83. Baker, R. & Bacon, A. J. The identification of spiroacetals in the volatile secretions of two species of fruit fly (*Dacus dorsalis, Dacus curcurbitae*). *Experientia* **41**, 1484–1485 (1985).
84. Nishida, R. *et al.* Accumulation of phenylpropanoids in the rectal glands of males of the Oriental fruit fly, *Dacus dorsalis*. *Experientia* **44**, 534–536 (1988).
85. Nishida, R., Tan, K. H. & Fukami, H. *Cis*-3,4-dimethoxycinnamyl alcohol from the rectal glands of male oriental fruit fly, *Dacus dorsalis*. *Chem. Express* **3**, 207–210 (1988).
86. Witzgall, P., Kirsch, P. & Cork, A. Sex pheromones and their impact on pest management. *J. Chem. Ecol.* **36**, 80–100 (2010).
87. Canale, A. *et al.* Behavioural and electrophysiological responses to overlooked female pheromone components in the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae). *Chemoecology* **25**, 147–157 (2015).
88. Biasazin, T. D. *et al.* Detection of volatile constituents from food lures by tephritid fruit flies. *Insects* **9**, (2018).

89. Zhang, X. *et al.* Chemical compounds from female and male rectal pheromone glands of the guava fruit fly, *Bactrocera correcta*. *Insects* **10**, (2019).
90. Booth, Y. K. *et al.* A diverse suite of spiroacetals, including a novel branched representative, is released by female Bactrocera tryoni (Queensland fruit fly). *Chem. Commun.* 3975–3977 (2006). doi:10.1039/B611953K
91. El-Sayed, A. M. *et al.* Chemical composition of the rectal gland and volatiles released by female Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae). *Environ. Entomol.* (2019). doi:10.1093/ee/nvz061
92. Fletcher, M. T. *et al.* Chemistry of fruit-flies. Spiroacetal-rich secretions in several *Bactrocera* species from the South-West Pacific region. *J. Chem. Soc. Perkin Trans. 1* 2827–2831 (1992). doi:10.1039/P19920002827
93. Heath, R. R. *et al.* Analysis, synthesis, formulation, and field testing of three major components of male mediterranean fruit fly pheromone. *J. Chem. Ecol.* **17**, 1925–1940 (1991).
94. Nojima, S., Classen, A., Groot, A. T. & Schal, C. Qualitative and quantitative analysis of chemicals emitted from the pheromone gland of individual *Heliothis subflexa* females. *PLoS One* **13**, (2018).
95. Golub, M. A. & Weatherston, I. Techniques for extracting and collecting sex pheromones from live insects and from artificial sources. in *Techniques in Pheromone Research* (eds. Hummel, H. E. & Miller, T. A.) 223–285 (Springer, 1984). doi:10.1007/978-1-4612-5220-7_10
96. Al-Khshema, H., Agarwal, M. & Ren, Y. Optimization and validation for determination of volatile organic compounds from Mediterranean fruit fly (Medfly) *Ceratitis capitata* (Diptera: Tephritidae) by using HS-SPME-GC-FID/MS. *J. Biol. Sci.* **17**, 347–352 (2017).
97. Baker, T. C., Gaston, L. K., Pope, M. M., Kuenen, L. P. S. & Vetter, R. S. A high-efficiency collection device for quantifying sex pheromone volatilized from female glands and synthetic sources. *J. Chem. Ecol.* **7**, 961–968 (1981).
98. Schwartz, B. D., Booth, Y. K., Fletcher, M. T., Kitching, W. & Voss, J. J. De. Spiroacetal biosynthesis in fruit flies is complex : distinguishable origins of the same major spiroacetal released by different *Bactrocera* spp. 1526–1528 (2010). doi:10.1039/b917977a
99. Barbosa-Cornelio, R., Cantor, F., Coy-Barrera, E. & Rodríguez, D. Tools in the investigation of volatile semiochemicals on insects: From sampling to statistical analysis. *Insects* **10**, 241 (2019).

100. Nation, J. L. Biology of pheromone release by male Caribbean fruit flies, *Anastrepha suspensa* (Diptera: Tephritidae). *J. Chem. Ecol.* **16**, 553–572 (1990).
101. Pérez, J., Park, S. J. & Taylor, P. W. Domestication modifies the volatile emissions produced by male Queensland fruit flies during sexual advertisement. *Sci. Rep.* **8**, 1–10 (2018).
102. Ohinata, K. *et al.* Oriental fruit fly and melon fly: Biological and chemical studies of smoke produced by males. *J. Environ. Sci. Heal. Part A Environ. Sci. Eng.* **17**, 197–216 (1982).
103. Booth, Y. K., Kitching, W. & De Voss, J. J. Biosynthesis of insect spiroacetals. *Nat. Prod. Rep.* **26**, 490–525 (2009).
104. Ohmura, W. *et al.* Chemical composition of the defensive secretion of the longhorned beetle, *Chloridolum loochooanum*. *J. Chem. Ecol.* **35**, 250–255 (2009).
105. Robacker, D. C., Aluja, M., Bartelt, R. J. & Patt, J. Identification of chemicals emitted by calling males of the sapote fruit fly, *Anastrepha serpentina*. *J. Chem. Ecol.* **35**, 601–609 (2009).
106. Cerkowniak, M., Boguś, M. I., Włóka, E., Stepnowski, P. & Gołębiowski, M. Application of headspace solid-phase microextraction followed by gas chromatography coupled with mass spectrometry to determine esters of carboxylic acids and other volatile compounds in *Dermestes maculatus* and *Dermestes ater* lipids. *Biomed. Chromatogr.* **32**, (2018).
107. Levi-Zada, A. *et al.* Circadian release of male-specific components of the greater date moth, *Aphomia (Arenipses) Sabella*, Using Sequential SPME/GC/MS Analysis. *J. Chem. Ecol.* **40**, 236–243 (2014).
108. Schwartz, B. D. *et al.* Spiroacetal biosynthesis in insects from Diptera to Hymenoptera: The giant ichneumon wasp *Megarhyssa nortoni nortoni* Cresson. *J. Am. Chem. Soc.* **130**, 14853–14860 (2008).
109. Gilley, D. C., DeGrandi-Hoffman, G. & Hooper, J. E. Volatile compounds emitted by live European honey bee (*Apis mellifera* L.) queens. *J. Insect Physiol.* **52**, 520–527 (2006).

Chapter Two

Rectal gland chemistry, volatile emissions, and antennal responses of male and female banana fruit fly, *Bactrocera musae*

Published in Insects

Author Contributions:
Sample collection in 2017 was performed by S. Noushini. Sample in 2014 was collected by J. Perez. GC-MS and GC-EAD experiments was carried out by S. Noushini. Author analysed all data and drafted the manuscript. All authors read the manuscript and provided feedback.

At the begging of this chapter there is a short summery of phylogenetic situation of *Bactrocera musae* and species which are the known close relatives to this species.

At the end of this chapter, after the manuscript figure 4 has been provided summarising structure of all identified compounds in male and female *Bactrocera musae*.

Preamble

Bactrocera musae (Tryon) is a polyphagous pest species belongs to order Diptera and family Tephritidae. Similar species to *B. musae* are *B. bancroftii* (Tryon), *B. endiandrae* (Perkins and May*), B. opiliae* (Drew and Hardy) and *B. dorsalis* (Hendel).[1] *Bactrocera musae* differs from *B. bancroftii* in not having an apical band on the scutellum and having a wider costal band and narrower anal streak. They differ from *B. endiandrae* in having a wider costal band, narrower anal streak, longer less tapered vittae and less of a distinct T or wraparound T on the abdomen. Difference between *B. musae* and *B. opiliae* is that *B. musae* has a wider costal band that overlaps R_{2+3}, a slightly wider anal streak, and usually less patterning on the abdomen (*B. opilae* has a distinct T). This species differs from *B. dorsalis* in having a wider costal band that overlaps at R_{2+3}, a slightly wider anal streak, slightly tapering and shorter vittae that usually end at the intra-alar setae, and usually less patterning on the abdomen.[1]

All molecular markers separate *B. musae* from *B. endiandrae* and *B. bancroftii*; however, RPA2 does not separate *B. musae* from *B. opiliae* and *B. dorsalis.*[1,2]

Among these species only pheromone profile of *B. dorsalis* has been studied. Both males and females of this species have been reported to be attracted to the volatiles from opposite sex.[3–5] Baker and Bacon reported *N*-3-methylbutyl acetamide as the major component of the aeration extract of female *B. dorsalis*.[6] Three spiroacetals were also reported from female volatiles. The two major components were (*E*,*E*)-2,8-dimethyl-l,7-dioxaspiro[5.5]undecane and (*E*,*E*)-8-ethyl-2-methyl-l,7-dioxaspiro[5.5]undecane and the minor spiroacetal component was reported as (*E*,*E*)-8-methyl-2-propyl-l,7-dioxaspiro[5.5]undecane. Moreover, a series of fatty acid esters including ethyl laurate, ethyl myristate, ethyl myristoleate and ethyl palmitate were reported for female.[6] Later Shen et al reported that a new female based-compound, 4-allyl-2,6-dimethoxyphenol to be robustly attractive to conspecific males.[3] For males of *B. dorsalis* long-chain fatty acids including lauric acid, myristic acid, palmitic acid, palmitoleic acid, oleic acid, elaidic acid and ethyl laurate were reported as the major components.[7] Other minor compounds that have been reported in males include (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, 3-hydroxy-2,8-dimethyl-l,7-dioxaspiro[5.5]undecane, *N*-(3-methylbutyl)acetamide,

trimethyl pyrazine, 3,5-dihydroxy-2-methyl-4H-pyran-4-one, 2-hydroxy-3-methylbutanoic acid.[7]

References

1. Schutze, M. *et al. The Australian Handbook for the Identification of Fruit Flies. Version 3.1*. (Plant Health Australia, 2018). doi:10.1016/j.jasms.2007.01.008
2. Drew, R. A. I., Ma, J., Smith, S. & Hughes, J. M. The taxonomy and phylogenetic relationships of species in the Bactrocera musae complex of fruit flies (Diptera: Tephritidae: Dacinae) in Papua new guinea. *Reffles Bull. Zool.* **59**, 145–162 (2011).
3. Shen, J. *et al.* Allyl-2,6-dimethoxyphenol, a female-biased compound, is robustly attractive to conspecific males of *Bactrocera dorsalis* at close range. *Entomol. Exp. Appl.* **167**, 811–819 (2019).
4. Ohinata, K. *et al.* Oriental fruit fly and melon fly: Biological and chemical studies of smoke produced by males. *J. Environ. Sci. Heal. Part A Environ. Sci. Eng.* **17**, 197–216 (1982).
5. Nishida, R. *et al.* Accumulation of phenylpropanoids in the rectal glands of males of the Oriental fruit fly, *Dacus dorsalis*. *Experientia* **44**, 534–536 (1988).
6. Baker, R. & Bacon, A. J. The identification of spiroacetals in the volatile secretions of two species of fruit fly (*Dacus dorsalis, Dacus curcurbitae*). *Experientia* **41**, 1484–1485 (1985).
7. Perkins, M. V., Fletcher, M. T., Kitching, W., Drew, R. A. I. & Moore, C. J. Chemical studies of rectal gland secretions of some species of *Bactrocera dorsalis* complex of fruit flies (diptera: Tephritidae). *J. Chem. Ecol.* **16**, 2475–2487 (1990).

Noushini, S., Perez, J., Park, S. J., Holgate, D., Jamie, I., Jamie, J., & Taylor, P. (2020). Rectal gland chemistry, volatile emissions, and antennal responses of male and female banana fruit fly, *Bactrocera musae*. *Insects*, *11*(1), [32].

DOI: https://doi.org/10.3390/insects11010032

insects

MDPI

Article

Rectal Gland Chemistry, Volatile Emissions, and Antennal Responses of Male and Female Banana Fruit Fly, *Bactrocera musae*

Saeedeh Noushini [1,2,*], Jeanneth Perez [2,3], Soo Jean Park [2,3], Danielle Holgate [1], Ian Jamie [1,2], Joanne Jamie [1] and Phillip Taylor [2,3]

1 Department of Molecular Sciences, Macquarie University, Sydney NSW 2109, Australia; danielle.holgate@hdr.mq.edu.au (D.H.); ian.jamie@mq.edu.au (I.J.); joanne.jamie@mq.edu.au (J.J.)
2 Australian Research Council Industrial Transformation Training Centre for Fruit Fly Biosecurity Innovation, Macquarie University, Sydney NSW 2109, Australia; jeanneth.perez@mq.edu.au (J.P.); soojean.park@mq.edu.au (S.J.P.); Phil.Taylor@mq.edu.au (P.T.)
3 Applied BioSciences, Macquarie University, Sydney NSW 2109, Australia
* Correspondence: saeedeh.noushini1@hdr.mq.edu.au or sally.noushini@mq.edu.au

Received: 4 December 2019; Accepted: 29 December 2019; Published: 31 December 2019

Abstract: The banana fruit fly, *Bactrocera musae* (Tryon) (Diptera: Tephritidae), is an economically important pest endemic to Australia and mainland Papua New Guinea. The chemistry of its rectal glands, and the volatiles emitted during periods of sexual activity, has not been previously reported. Using gas chromatography–mass spectrometry (GC-MS), we find that male rectal glands contain ethyl butanoate, *N*-(3-methylbutyl) acetamide, ethyl laurate and ethyl myristate, with ethyl butanoate as the major compound in both rectal gland and headspace volatile emissions. Female rectal glands contain four major compounds, ethyl laurate, ethyl myristate, ethyl palmitate and (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, as well as 11 minor compounds. For both male and female *B. musae*, all compounds found in the headspace were also present in the rectal gland extracts, suggesting that the rectal gland is the main source of the headspace volatiles. Gas chromatography–electroantennography (GC-EAD) of rectal gland extracts confirms that male antennae respond to male-produced ethyl laurate and female-produced (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, while female antennae respond to male-produced ethyl butanoate but no female-produced compounds. This is an important step in understanding the volatiles involved in the chemical communication of *B. musae*, their functional significance, and potential application.

Keywords: *B. musae*; headspace; electroantennography; insect volatile; GC-EAD

1. Introduction

In tephritid fruit flies (Diptera: Tephritidae), courtship and mating are typically mediated by pheromones [1,2]. Sex pheromones are usually secreted and stored in the rectal glands and emitted during periods of mating activity [3–6]. The volatile compounds released by fruit flies are known to attract the opposite sex in many species [7–10], as well as members of the same sex to form mating aggregations [11,12]. Although males are commonly thought of as the major sex pheromone producers [13,14], there are also examples of female fruit flies that produce and release sex pheromones. For example, in *Bactrocera oleae* (Rossi), sex pheromones are produced mainly by the female [15,16], while for *Zeugodacus cucurbitae* (Coquillett) and *Bactrocera dorsalis* (Hendel), both male and female volatile emissions attract the opposite sex [17–20].

Bactrocera musae (Tryon) is a polyphagous pest species endemic to Australia and mainland Papua New Guinea [21]. In Australia, *B. musae* is very common along the eastern coast of Queensland as far south as Townsville [22], where bananas (*Musa* spp.) are an important commercial crop [23]. Unlike many other fruit fly species that oviposit in ripe fruits, *B. musae* lays eggs in unripe bananas [24,25]. Therefore, harvesting bananas at a green stage to avoid fruit fly infestation is not a solution in these regions [24]. Although banana is its major host, papaya (*Carica papaya* L.) and guava (*Psidium guajava* L.) are also occasional hosts of *B. musae* [23]. Sex pheromones of many of the most economically important *Bactrocera* species have been documented [2,15,17,18,26–30]. However, the chemical profiles of rectal gland and volatile emissions of *B. musae* are unknown, despite its moderate to high pest status [31]. This study presents the rectal gland chemical profiles of both male and female *B. musae*, as well as headspace samples, using gas chromatography–mass spectrometry (GC-MS), and evaluates the antennal electrophysiological response of male and female *B. musae* to the volatiles produced by the opposite sex using gas chromatography–electroantennogram detection (GC-EAD).

2. Materials and Methods

2.1. Banana Fruit Fly Rearing

Laboratory-reared populations of *B. musae* were obtained from the Queensland Government Department of Agriculture and Fisheries (QDAF) in September 2014 (Mareeba) and November 2017 (Cairns).

2014 collections: Flies were kept in mixed-sex cages at QDAF, Mareeba, in a controlled environment room at 26 ± 1 °C, 70 ± 5% relative humidity (RH) and with a natural light cycle so the flies experienced a natural dusk. The adult flies were fed with sugar and yeast hydrolysate provided separately, and water through a soaked sponge. Flies used for rectal gland extractions were 13–18 days old.

2017 collections: Flies were kept at Macquarie University, Sydney, in a controlled environment room at 25 ± 0.5 °C, 65 ± 5% RH and 11.5:0.5:11.5:0.5 light/dusk/dark/dawn photoperiod. The adult flies were fed with sugar and yeast hydrolysate (MP Biomedicals LLC, Santa Ana, CA, USA) provided separately, and water through a soaked sponge. Flies that had been received as pupae were reared through one generation using a standard carrot diet [32], following the method described by Pérez et al. [33]. Flies were sorted by sex within three days after emergence and transferred to 12.5 L clear plastic cages (180 flies per cage). No mating was observed before separating the flies. Flies used for all experiments were 13–18 days old.

2.2. Rectal Gland Extractions

n-Hexane extracts of rectal glands of flies collected in both 2014 and 2017 were obtained using sexually mature male and female *B. musae* (13–18 days old) following literature procedures [34]. Flies were chilled on dry ice and then dissected. The abdomen was gently squeezed with tweezers such that the gland protruded slightly, and the gland was gently pulled out with fine forceps and carefully placed in a tear-drop vial (1.1 mL) in dry ice. For each sex and for each year of collection, 20 glands were combined in the vials. The vials were taken out of the dry ice, and 200 µL of *n*-hexane (HPLC grade, Sigma-Aldrich, St. Louis, MO, USA) was added to each vial. Samples were left to stand at room temperature for 10 min, then the extracts were transferred to a new labelled vial and stored at −20 °C until analysed. Three replicates per sex were collected in 2014, and six replicates were collected in 2017.

2.3. Headspace Collections

Collections of volatile emissions from male and female *B. musae* obtained from QDAF in 2017 were performed at Macquarie University, Sydney, in a controlled environment room at 25 ± 0.5 °C and 65 ± 5% RH. Like many other *Bactrocera*, *B. musae* mate at dusk [35]. Thirty sexually mature males and 30 sexually mature females (13–18 days old) were separately placed into a cylindrical glass chamber (150 mm long and 40 mm inner diameter) 30 min before dusk. A charcoal-filtered air stream at a flow

rate of 0.5 L/min (air pulling system) was drawn over the flies for one hour, from the beginning of dusk. Released volatiles were adsorbed onto traps of 50 mg of Tenax-GR adsorbent (Scientific Instrument Services, Inc, Ringoes, NJ, USA, Tenax-GR Mesh 60/80) packed into 6 × 50 mm glass tubes and fitted with glass wool plugs. Volatiles were eluted with 1 mL of *n*-hexane (HPLC grade, Sigma-Aldrich). An air control sample comprising an empty glass chamber was run and analysed along with each volatile collection. Samples were stored at −20 °C until analysis. Six replicates of 30 flies per sex were obtained. Prior to each headspace collection, Tenax traps were conditioned at 200 °C for three hours under a nitrogen stream (75 mL/min). Glass chambers were washed with 5% Extran aqueous solution, rinsed with hot tap water, and heated at 200 °C for 18 h. Activated charcoal filters were thermally conditioned by heating them at 200 °C for 18 h prior to each headspace collection [36].

2.4. GC-MS Analysis

Mass spectra were recorded on a Shimadzu GCMS-QP2010 instrument. The GC was equipped with a non-polar capillary column with 5% diphenyl/95% dimethyl polysiloxane as the stationary phase (SH-Rtx-5MS, 30 m × 0.25 mm ID × 0.25 µm film thickness, Shimadzu, Japan) and helium (99.999%) (ultra-high purity, BOC, Australia) as a carrier gas with a constant flow of 1 mL/min. A 1 µL sample was injected in the splitless mode. The injector temperature was set at 270 °C. The temperature program was 50 °C (4 min) to 250 °C (6 min) at a rate of 10 °C/min. The interphase and ion source temperatures were set at 290 °C and 200 °C, respectively. Mass spectra were recorded in electron impact mode (70 eV), scanning from 40 to 620 *m*/*z*. A peak was considered of interest if it was not present in the air control samples. The identification of compounds was confirmed by comparing their mass spectra and retention times to those of commercial standards or synthesised samples. Commercial samples were purchased from Sigma-Aldrich (Castle Hill, Australia), Alfa-Aesar (Heysham, Lancashire, United Kingdom) and Nu-Chek-Prep, INC (Minneapolis, MN, USA). These compounds included ethyl butanoate, ethyl caprate, methyl laurate, ethyl laurate, ethyl tridecanaote, methyl myristate, ethyl myristate, ethyl myristoleate, methyl palmitate, ethyl palmitate and ethyl oleate. (*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (racemate), *N*-(3-methylbutyl)acetamide, propyl laurate, ethyl palmitoleate and ethyl elaidate were not commercially available and were synthesised following literature procedures (see Supplementary Materials for synthesis details). (*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane was tentatively identified based on the literature mass spectral fragmentation pattern [37].

2.5. GC-EAD Experiments

Gas chromatography–electroantennographic detection (GC-EAD) was conducted using a gas chromatography flame ionisation detector (FID, Agilent 7890B) coupled to an electroantennogram (Ockenfels Syntech GmbH, Kirchzarten, Germany). The GC was equipped with a non-polar capillary column with (5%-phenyl)-methylpolysiloxane as the stationary phase (Agilent HP-5, 30 m × 0.32 mm ID × 0.25 µm film thickness). The carrier gas was hydrogen (99.999% pure) supplied by a generator (MGG-2500-220 Parker Balston, NY, USA) with a constant flow of 2.5 mL/min. The initial temperature was set at 50 °C (4 min) then increased to 250 °C (6 min) at a rate of 10 °C/min. The injector and detector temperatures were set at 270 °C and 290 °C, respectively. The effluent of the column was mixed with 30 mL/min make-up nitrogen gas and split in a ratio of 1 (FID) to 1.5 (EAD) through a heated transfer line (Syntech, TC-02, Ockenfels Syntech GmbH, Kirchzarten, Germany) and kept at 200 °C.

A female or male B. musae head was carefully severed and a silver glass capillary electrode filled with phosphate-buffered saline (PBS) was inserted into the back of the head. The tip of the antenna was inserted into the tip of the recording glass capillary electrode. The mounted heads were placed under a charcoal-filtered and humidified air flow (400 mL/min) controlled by a flow controller (Syntech Stimulus Controller CS-55, Ockenfels Syntech GmbH, Kirchzarten, Germany) and were subjected to each stimulus. Electrophysiological responses were captured and processed by a data acquisition controller (IDAC-4, Ockenfels Syntech GmbH, Kirchzarten, Germany). Before the injection of the

sample into the airstream, the antenna was stimulated with 1-hexanol to check its sensitivity; then, 1 µL of the rectal gland extract was injected. EAD signals were analysed using GC-EAD 2014 software version 1.2.5. Nine successful GC-EAD recordings were obtained for each sex. The electrophysiological responses of male and female antennae to the conspecific opposite sex rectal glands extract were recorded. A response was considered genuine if it was present in at least six out of the nine replicates collected. The identity of the compounds eliciting an electrophysiological response was confirmed by comparing retention times with those of GC-MS chromatograms.

3. Results

3.1. Analysis of Volatile Compounds

GC-MS analyses confirmed the presence of 17 compounds in rectal gland extracts and headspace collections of sexually mature male and female *B. musae* (Table 1). This included 14 esters, one amide and two spiroacetals. For both males and females, a more complex blend was detected in the rectal glands, with fewer compounds detected for the headspace collections for both sexes (Figures 1 and 2). No additional compounds were detected in the headspace samples. The most abundant chemicals in female rectal glands, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**), (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**), ethyl caprate (**5**), methyl laurate (**6**), ethyl laurate (**7**), methyl myristate (**10**), ethyl myristate (**11**), ethyl myristoleate (**12**), ethyl palmitate (**14**) and ethyl palmitoleate (**15**) were also detected in the headspace collections. Similarly, ethyl butanoate (**1**) and *N*-(3-methylbutyl)acetamide (**2**) were found in both the male rectal glands and headspace samples. Overall, there was a large difference in the chemical profiles of males and females. Males predominantly produced and released a short carbon chain ester, ethyl butanoate (**1**). Females predominantly produced and released longer carbon chain esters, ethyl laurate (**7**) and ethyl myristate (**11**).

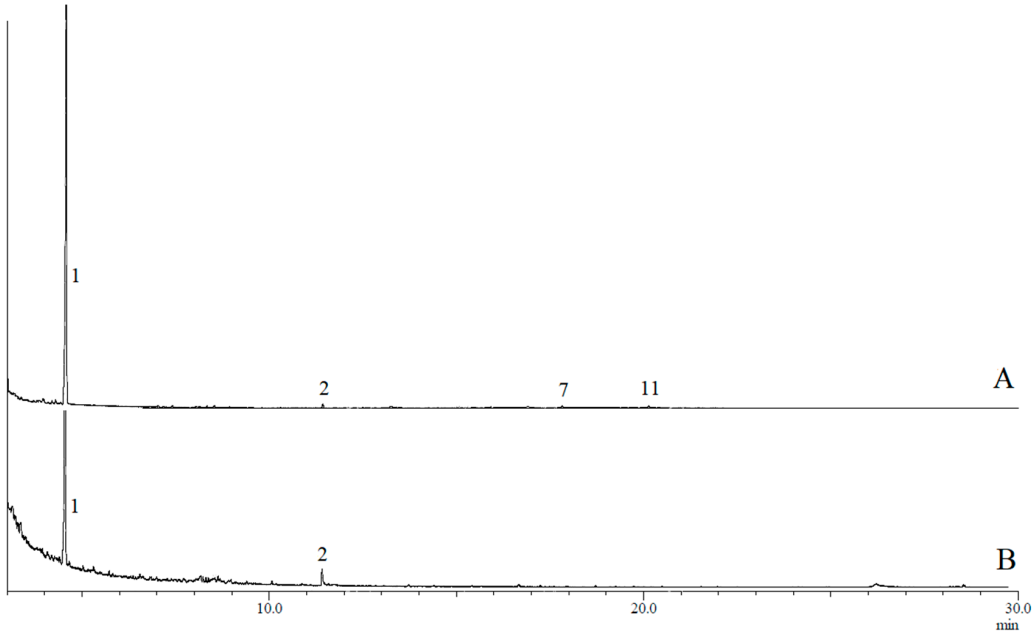


Figure 1. Typical gas chromatogram of (**A**) rectal gland extract and (**B**) headspace collections of *B. musae* males. Numbered peaks indicate detected compounds: ethyl butanoate (**1**), *N*-(3-methylbutyl)acetamide (**2**), ethyl laurate (**7**) and ethyl myristate (**11**).

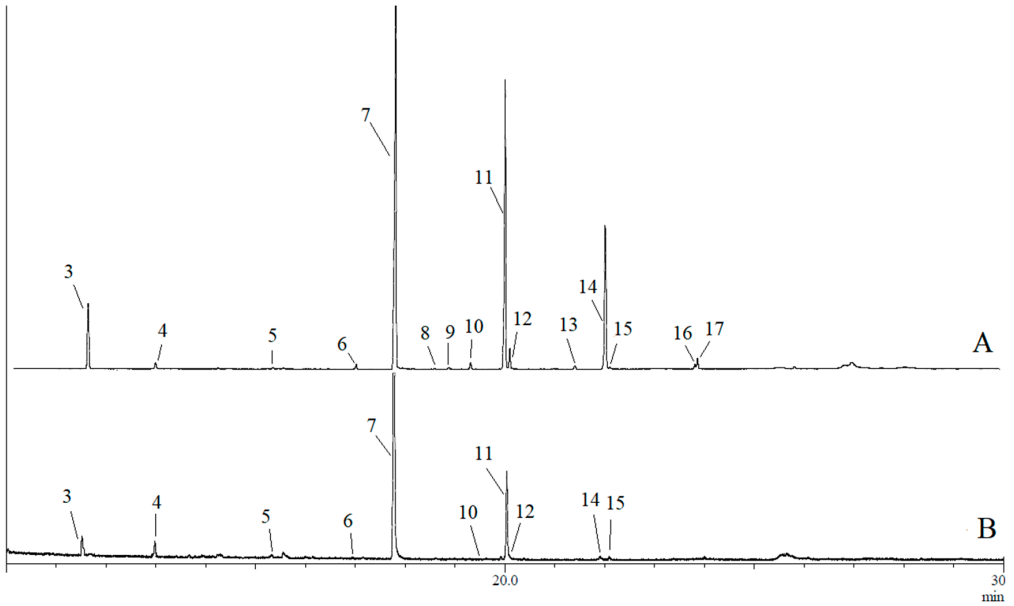


Figure 2. Typical gas chromatogram of (**A**) rectal gland extract and (**B**) headspace collections of *B. musae* females. Numbered peaks indicate detected compounds: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**), (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**), ethyl caprate (**5**), methyl laurate (**6**), ethyl laurate (**7**), ethyl tridecanaote (**8**), propyl laurate (**9**), methyl myristate (**10**), ethyl myristate (**11**), ethyl myristoleate (**12**), methyl palmitate (**13**), ethyl palmitate (**14**), ethyl palmitoleate (**15**), ethyl oleate (**16**) and ethyl elaidate (**17**).

Table 1. Relative amount of compounds identified in chemical profiles for *B. musae*. KI = Kováts retention index, ND = not detected.

KI	Compound	Female			Male		
		Rectal Glands		Headspace	Rectal Glands		Headspace
		2014	2017	2017	2014	2017	2017
828	Ethyl butanoate (**1**)	ND	ND	ND	99.0	99.2	98.3
1162	*N*-(3-Methylbutyl)acetamide (**2**)	ND	ND	ND	<1	<1	1.7
1179	(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**3**)	5.0	5.7	2.1	ND	ND	ND
1266	(*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**)	<1	<1	2.0	ND	ND	ND
1437	Ethyl caprate (**5**)	<1	<1	<1	ND	ND	ND
1570	Methyl laurate (**6**)	<1	<1	<1	ND	ND	ND
1637	Ethyl laurate (**7**)	40.0	47.3	72.5	<1	ND	ND
1705	Ethyl tridecanaote (**8**)	<1	<1	ND	ND	ND	ND
1731	Propyl laurate (**9**)	<1	<1	ND	ND	ND	ND
1771	Methyl myristate (**10**)	<1	<1	<1	ND	ND	ND
1837	Ethyl myristate (**11**)	21.8	25.2	19.2	<1	ND	ND
1845	Ethyl myristoleate (**12**)	1.9	1.7	<1	ND	ND	ND
1974	Methyl palmitate (**13**)	<1	<1	ND	ND	ND	ND
2037	Ethyl palmitate (**14**)	21.7	16.4	1.6	ND	ND	ND
2047	Ethyl palmitoleate (**15**)	<1	<1	<1	ND	ND	ND
2233	Ethyl oleate (**16**)	<1	<1	ND	ND	ND	ND
2239	Ethyl elaidate (**17**)	<1	<1	ND	ND	ND	ND

3.2. Assignment of Compounds

Amide: Compound **2** was found to have an odd molecular ion at *m*/*z* 129, indicating the presence of a single nitrogen atom in the molecule, a fragment ion at *m*/*z* 114 due to loss of a methyl group, and an *m*/*z* 60 fragment ion consistent with β-cleavage of acetamides [38]. It also showed a fragment ion at *m*/*z* 86, consistent with the loss of a C_3H_7 or $COCH_3$ moiety. The NIST library showed high similarity for compound **2** with *N*-(3-methylbutyl)acetamide, and this identity was confirmed by comparing the retention time and mass spectral fragmentation pattern of the compound in the rectal gland extracts and headspace collections with the synthesized amide.

Methyl esters: Compounds **6**, **10** and **13** all produced a base peak at *m*/*z* 74, which is characteristic of the McLafferty rearrangement of methyl esters [39]. Compounds **6**, **10** and **13** exhibited molecular ions at *m*/*z* 214, *m*/*z* 242 and *m*/*z* 270, respectively, and they all showed loss of *m*/*z* 31 consistent with cleavage of a methoxy group, suggesting they were methyl esters of saturated C_{12}, C_{14} and C_{16} chains, respectively. A NIST library search showed high similarity with methyl laurate, methyl myristate and methyl palmitate for compounds **6**, **10** and **13**, respectively. The identities of the compounds were confirmed by comparing retention times and mass spectral fragmentation patterns of the compounds in the rectal gland extracts and headspace collections with the authentic commercial methyl esters.

Ethyl esters: Compounds **5**, **7**, **8**, **11** and **14** exhibited molecular ions at *m*/*z* 200, *m*/*z* 228, *m*/*z* 242, *m*/*z* 256 and *m*/*z* 284, respectively, along with the characteristic McLafferty fragmentation product of ethyl esters at *m*/*z* 88 as a base peak [39]. They also all showed similar fragmentation patterns, including fragment ions from the cleavage of an ethyl group, ethoxy group, and propyl group from the molecular ion. These data indicated compounds **5**, **7**, **8**, **11** and **14** were ethyl esters of saturated C_{10}, C_{12}, C_{14}, C_{15} and C_{16} chains, respectively. Compounds **12**, **15**, **16** and **17** had molecular ions at *m*/*z* 254, *m*/*z* 282 and *m*/*z* 310, respectively. They all showed a loss of ethanol from the molecular ion, and the McLafferty fragment ion at *m*/*z* 88 was present but less abundant than for the saturated esters **5**, **7**, **8**, **11** and **14**. A NIST library search suggested compounds **5**, **7**, **8**, **11** and **14** were the saturated esters ethyl caprate, ethyl laurate, ethyl tridecanaote, ethyl myristate and ethyl palmitate and **12**, **15**, **16** and **17** were the unsaturated esters ethyl myristoleate, ethyl palmitoleate, ethyl oleate and ethyl elaidate, respectively. The identities of the esters were confirmed by comparing retention times and mass spectral fragmentation patterns of the compounds in the rectal gland extracts and headspace collections with the authentic commercial or synthesized samples.

Propyl ester: Compound **9** showed a molecular ion at *m*/*z* 242 and a McLafferty fragment ion at *m*/*z* 102, suggesting a propyl/isopropyl ester with a C_{12} saturated chain [39]. It also exhibited a fragment ion at *m*/*z* 183 for loss of a propoxy (C_3H_7O) group. Given that propyl and isopropyl esters are structural isomers and will therefore produce very similar mass spectra, both propyl laurate and isopropyl laurate were synthesized. Compound **9** was found to have very similar mass spectral fragmentation patterns to the propyl isomer (but not isopropyl laurate). The identification of compound **9** was further confirmed as propyl laurate based on a comparison of retention time by the co-injection of the rectal gland extracts and synthesized propyl laurate. Isopropyl laurate showed a slightly different retention time when compared to compound **9**.

Spiroacetals: Compounds **3** and **4** had molecular ions of *m*/*z* 184 and 198, respectively, and similar mass spectral fragmentation patterns, indicating a difference of CH_2 only. This was further supported by both compounds exhibiting a fragment ion at *m*/*z* 169, indicating a loss of CH_3 for compound **3** and CH_2CH_3 for compound **4**. Both compounds also showed characteristic fragmentation patterns of methyl substituted dioxaspiro[5.5]undecane spiroacetals [40], including a strong doublet at *m*/*z* 112 and *m*/*z* 115, due to the retro-cleavage of one of the six-membered rings leading to a methyl methylene heterocycle ($CH_3(C_5H_7O)=CH_2$) at *m*/*z* 112 and *m*/*z* 115 ($CH_3(C_5H_7O)=OH$) due to the alternate ring cleavage accompanied by intramolecular hydrogen transfer. The presence of another set of ions at *m*/*z* 126 and *m*/*z* 129 for compound **4** indicated it also had an ethyl substituted ring. Compound **3** was consistent with a dimethylated dioxaspiro[5.5]undecane, while compound **4** was consistent with a methyl and ethyl substituted dioxaspiro[5.5]undecane [40]. Compound **3** was

confirmed as (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane by comparing the retention time and mass spectral fragmentation pattern of the compound in the rectal gland extracts and headspace collections with the authentic synthesized (racemic) sample. Compound **4** was tentatively identified as (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane based on the literature mass spectral fragmentation pattern [8,37,41].

For *B. musae* males, four compounds were identified in rectal gland extracts obtained from the flies collected in 2014, including ethyl butanoate (**1**), *N*-(3-methylbutyl)acetamide (**2**), ethyl laurate (**7**) and ethyl myristate (**11**), while only compounds **1** and **2** were detected for the 2017 collections. Of the four compounds, only ethyl butanoate (**1**) and *N*-(3-methylbutyl)acetamide (**2**) were found in the headspace samples. In contrast, for *B. musae* females, 15 compounds were identified in rectal gland extracts obtained from flies collected in both 2014 and 2017 (Table 1). Identified compounds included 13 saturated/unsaturated fatty acid esters, including ethyl caprate (**5**), methyl laurate (**6**), ethyl laurate (**7**), ethyl tridecanaote (**8**), propyl laurate (**9**), methyl myristate (**10**), ethyl myristate (**11**), ethyl myristoleate (**12**), methyl palmitate (**13**), ethyl palmitate (**14**), ethyl palmitoleate (**15**), ethyl oleate (**16**) and ethyl elaidate (**17**), as well as two spiroacetals (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**) and (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**). Of these, ten compounds were also found in headspace collections. The main compound present in female gland extracts and headspace samples was ethyl laurate (**7**), although it was found in higher proportions in the headspace samples (~73% vs. 47%).

3.3. GC-EAD Experiment

The electroantennogram response of female and male *B. musae* to the rectal gland extract of the conspecific opposite sex is shown in Figure 3. Ethyl butanoate (**1**), the most abundant compound emitted by male *B. musae*, elicited antennal responses from *B. musae* females. Two compounds, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**) and ethyl laurate (**7**), elicited antennal responses from conspecific males.

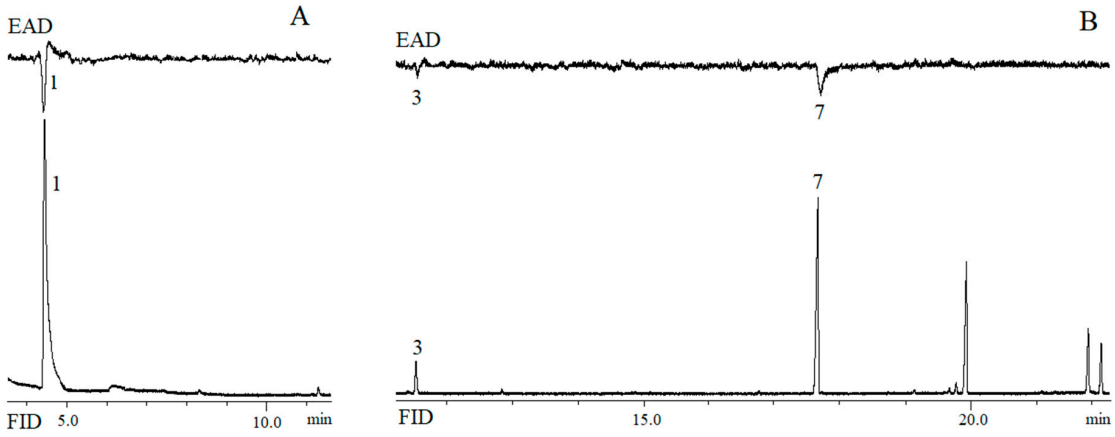


Figure 3. Simultaneous response of flame ionisation detector (FID) and electroantennographic detection (EAD) using *B. musae* (**A**) female antenna with rectal gland extract from conspecific males and (**B**) male antenna to rectal gland extract from conspecific females. Numbered peaks indicate EAD-active compounds: ethyl butanoate (**1**), (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**) and ethyl laurate (**7**).

4. Discussion

The present study describes for the first time the chemical profiles of rectal gland extracts and volatiles released during dusk, the period of sexual activity in *B. musae*, as well as the EAD-active compounds for both sexes. Females are found to release a more complex blend than males, and

the major compound(s) present in the chemical profile of each sex elicited antennal responses in the opposite sex.

Female rectal gland volatile profiles from colonies collected in 2014 and 2017 were similar in composition and relative amounts. There were some differences in the male rectal gland profiles between the two collections. Male rectal glands from the flies obtained in 2014 contained trace amounts of ethyl laurate and ethyl myristate (<0.5%), which were not observed in flies collected in 2017. Qualitative and/or quantitative changes in the volatile composition may appear as a result of age, nutritional and mating status [30,42–44], or due to the domestication process [33]. The differences found between the two collections cannot easily be explained as larvae and adults used for both collections were fed the same diet (carrot diet for larvae and sugar and hydrolysate yeast for adults), and the adults used for the extractions were in the same age range (13–18 days old). Therefore, it is unlikely that these factors can cause this slight difference. The difference may have arisen because the samples collected in 2014 were from mixed sex cages of flies whereas the samples collected in 2017 were from single sex cages.

Ethyl butanoate (**1**), the major component found in male *B. musae* rectal gland and headspace extracts, has not been previously found in volatile secretions/emissions of other tephritid fruit fly species. It is commonly found in fruits, such as mangos, and is known to elicit EAD responses in female *B. dorsalis* [45]. *N*-(3-Methylbutyl)acetamide (**2**) has been reported in other fruit fly pheromone profiles, and elicits female attraction in *Z. cucurbitae*, *B. dorsalis* and *Bactrocera carambolae* Drew & Hancock [3,6,18]. Ethyl laurate (**7**) and ethyl myristate (**11**) were only minor compounds in males, but represent more than 90% of abundance in female *B. musae*.

Saturated/unsaturated fatty acid esters are commonly found in rectal glands of female *Bactrocera* [46]. All saturated/unsaturated fatty acid esters from female *B. musae* have been previously reported in rectal gland extracts of female *Bactrocera tryoni* (Froggatt) [46–48]. Ethyl myristate (**11**) and ethyl palmitate (**14**) are also found in rectal gland extracts of male *Bactrocera jarvisi* (Tryon) [47]. These two compounds, as well as methyl laurate (**6**), have also been reported as minor components in the rectal gland extracts of female *B. oleae* [49]. Ethyl laurate (**7**), the most abundant compound found in *B. musae* females, has been previously reported in rectal gland extracts of female *B. oleae* [49] and male *B. jarvisi* [47]. The relative abundance of this compound is higher in headspace than rectal glands due to its lower chain length (C_{12}) and hence its higher volatility in headspace compared to the C_{14}, C_{16} or C_{18} esters. Males of *B. dorsalis* exhibit EAD responses to this compound, which was found in the cuticle extraction [50]. Surprisingly, Ethyl laurate (**7**) only elicited EAD responses from *B. musae* males.

Both spiroacetals, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**) and (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**), have been reported as volatile emissions of fruit flies. (*E*,*E*)- 2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**3**) has been found in rectal glands of many fruit flies including *B. dorsalis*, *B. nigrotibialis* (Perkins), *B. albistrigata* (Meijere), *B. jarvisi*, *B. kirki* (Froggatt), *B. kraussi* (Hardy), *Z. cucumis* and *B. tryoni* [34,46–48,51,52]. (*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**) has been previously reported as part of male emissions of male *B. nigrotibialis*, *B. halfordiae* (Tryon), *B. dorsalis*, *B. kirki*, *B. latifrons* (Hendel) and *B. occipitalis* (Bezzi) as well as female *B. tryoni* [2,3,46,53,54]. For *B. nigrotibialis* and *Z. cucumis*, the spiroacetals were found to be solely the 2*S*,6*R*,8*S* enantiomers [34,55]. It is likely that the spiroacetals (**3**) and (**4**) found in *B. musae* also exist as single enantiomers, but this was not investigated in our study. The finding that (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**) elicited an antennal response in males suggests a likely biological role of this compound, together with ethyl butanoate (**1**) and ethyl laurate (**7**).

5. Conclusions

The investigation of rectal gland and airborne volatiles of *B. musae* at dusk, the period of mating activity in this species, revealed that males and females produce and release distinctly different volatile compounds. Females were found to release a more complex blend of volatile compounds than males. Males predominantly produce a short carbon chain ester—ethyl butanoate—while females predominantly produce longer carbon chain esters—ethyl laurate, ethyl myristate, ethyl palmitate.

For both males and females, all compounds found in the headspace collections were also present in the rectal gland extracts. Furthermore, GC-EAD results showed that the major compound present in the chemical profile of each sex elicited an antennal response in the opposite sex, suggesting a possible biological role of these compounds in the mating system of *B. musae*. Knowing the volatiles that are released during mating activity and those that elicit antennal responses is an important step toward understanding the chemical communication system of the banana fruit fly *B. musae*. However, further behavioural studies are required in order to investigate the functions of the volatiles we identified in male and female rectal glands to conspecific females or males (e.g., attraction, species and sex identification, indicators of mate quality). Such insight into the sexual communication of this species could reveal new applications for the control of this pest.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/11/1/32/s1: synthesis of *N*-(3-methylbutyl)acetamide (**2**), 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**), propyl laurate (**9**), isopropyl laurate, ethyl palmitoleate (**15**) and ethyl elaidate (**17**).

Author Contributions: Conceptualization, S.N., P.T., I.J. and J.J.; investigation, S.N., J.P., S.J.P. and D.H.; data analysis, S.N.; writing—Original draft preparation, S.N.; writing—Review and editing, all authors. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded through the Australian Research Council Industrial Transformation Training Centre (ITTC) for Fruit Fly Biosecurity Innovation (Project IC50100026), funded by the Australian Government.

Acknowledgments: We are grateful to the Queensland Department of Agriculture and Fishers (QDAF, Cairns and Mareeba), especially Sybilla Oczkowicz, Peter Leach and Stefano DeFaveri, for suppling fruit flies and pupae and providing space for the 2014 sample collection.

Conflicts of Interest: The authors declare no conflict of interest.

References

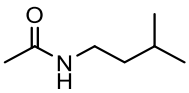
1. Witzgall, P.; Kirsch, P.; Cork, A. Sex pheromones and their impact on pest management. *J. Chem. Ecol.* **2010**, *36*, 80–100. [CrossRef] [PubMed]
2. Benelli, G.; Daane, K.M.; Canale, A.; Niu, C.-Y.; Messing, R.H.; Vargas, R.I. Sexual communication and related behaviours in Tephritidae: Current knowledge and potential applications for integrated pest management. *J. Pest Sci.* **2014**, *87*, 385–405. [CrossRef]
3. Perkins, M.V.; Fletcher, M.T.; Kitching, W.; Drew, R.A.I.; Moore, C.J. Chemical studies of rectal gland secretions of some species of *Bactrocera dorsalis* complex of fruit flies (diptera: Tephritidae). *J. Chem. Ecol.* **1990**, *16*, 2475–2487. [CrossRef] [PubMed]
4. Piccardi, P. Insect sex-communication and prospects for pheromones in pest management. *Bolletino di Zool.* **1980**, *47*, 397–408. [CrossRef]
5. Tokushima, I.; Orankanok, W.; Tan, K.H.; Ono, H.; Nishida, R. Accumulation of phenylpropanoid and sesquiterpenoid volatiles in male rectal pheromonal glands of the guava fruit fly, *Bactrocera correcta*. *J. Chem. Ecol.* **2010**, *36*, 1327–1334. [CrossRef] [PubMed]
6. Wee, S.L.; Tan, K.H. Female sexual response to male rectal volatile constituents in the fruit fly, *Bactrocera carambolae* (Diptera: Tephritidae). *Appl. Entomol. Zool.* **2005**, *40*, 365–372. [CrossRef]
7. Nation, J.L. Courtship behavior and evidence for a sex attractant in the male Caribbean fruit fly, *Anastrepha suspensa*. *Ann. Entomol. Soc. Am.* **1972**, *65*, 1364–1367. [CrossRef]
8. Perkins, M.V. Characterisation and Synthesis of Bactrocera Fruit Fly Pheromones. Ph.D. Thesis, Department of Chemistry, The University of Queensland, Brisbane, Australia, 1990.
9. Sivinski, J.; Aluja, M.; Dodson, G.N.; Freidberg, A.; Headrick, D.H.; Kaneshiro, K.Y.; Landolt, P.J. Topics in the evolution of sexual behavior in the Tephritidae. In *Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior*; Aluja, M., Norrbom, A.L., Eds.; CRC Press: Boca Raton, FL, USA, 2000; pp. 751–792. ISBN 9781420074468.
10. Cruz-López, L.; Malo, E.A.; Rojas, J.C. Sex pheromone of *Anastrepha striata*. *J. Chem. Ecol.* **2015**, *41*, 458–464. [CrossRef]
11. Sivinski, J.M.; Calkins, C. Use of pheromones in tropical crops: Pheromones and parapheromones in the control of tephritids. *Fla. Entomol.* **1986**, *69*, 157–168. [CrossRef]

12. Hendrichs, J.; Robinson, A.S.; Cayol, J.P.; Enkerlin, W. Medfly areawide sterile insect technique programmes for prevention, suppression or eradication: The importance of mating behavior studies. *Fla. Entomol.* **2002**, *85*, 1–14. [CrossRef]
13. El-Sayed, A.M. The Pherobase: Database of Pheromones and Semiochemicals. Available online: http://www.pherobase.com/ (accessed on 17 March 2019).
14. Heath, R.R.; Landolt, P.J.; Robacker, D.C.; Dueben, B.D.; Epsky, N.D. Sexual pheromones of tephritid flies: Clues to unravel phylogeny and behavior. In *Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior*; Aluja, M., Norrbom, A.L., Eds.; CRC Press: Boca Raton, FL, USA, 2000; pp. 793–809.
15. Haniotakis, G.E. Sexual attraction in the olive fruit fly, *Dacus oleae* (Gmelin). *Environ. Entomol.* **1974**, *3*, 82–86. [CrossRef]
16. Mazomenos, B.E.; Haniotakis, G.E. Male olive fruit fly attraction to synthetic sex pheromone components in laboratory and field tests. *J. Chem. Ecol.* **1985**, *11*, 397–405. [CrossRef]
17. Baker, R.; Herbert, R.H.; Lomer, R.A. Chemical components of the rectal gland secretions of male *Dacus cucurbitae*, the melon fly. *Experientia* **1982**, *38*, 232–233. [CrossRef]
18. Baker, R.; Bacon, A.J. The identification of spiroacetals in the volatile secretions of two species of fruit fly (*Dacus dorsalis*, *Dacus curcurbitae*). *Experientia* **1985**, *41*, 1484–1485. [CrossRef]
19. Nishida, R.; Tan, K.H.; Serit, M.; Lajis, N.H.; Sukari, A.M.; Takahashi, S.; Fukami, H. Accumulation of phenylpropanoids in the rectal glands of males of the Oriental fruit fly, *Dacus dorsalis*. *Experientia* **1988**, *44*, 534–536. [CrossRef]
20. Nishida, R.; Tan, K.H.; Fukami, H. *Cis*-3,4-dimethoxycinnamyl alcohol from the rectal glands of male oriental fruit fly, *Dacus dorsalis*. *Chem. Express* **1988**, *3*, 207–210.
21. Drew, R.A.I.; Hooper, G.H.S.; Bateman, M.A. *Economic fruit flies of the South Pacific Region*, 2nd ed.; Queensland Department of Primary Industries: Brisbane, Australia, 1982.
22. Hancock, D.L.; Hamacek, E.L.; Lloyd, A.C.; Elson-Harris, M.M. *The Distribution and Host Plants of Fruit Flies (Diptera: Tephritidae) in Australia*; Queensland Department of Primary Industries: Brisbane, Australia, 2000.
23. Schutze, M.; McMahon, J.; Krosch, M.; Strutt, F.; Royer, J.; Bottrill, M.; Woods, N.; Cameron, S.; Woods, B.; Blacket, M. *The Australian Handbook for the Identification of Fruit Flies*; Version 3.1; Plant Health Australia: Canberra, Australia, 2018; ISBN 9780648245605.
24. Hamacek, E. Host records of fruit flies in the South Pacific. In *Management of Fruit Flies in the Pacific*; Allwood, A.J., Drew, R.A.I., Eds.; Australian Centre for International Agricultural Research: Canberra, Australia, 1997; p. 102.
25. Leblanc, L.; Tora, E.; Drew, R.A.I.; Allwood, A.J. Host plant records for fruit flies (Diptera: Tephritidae: Dacini) in the Pacific Islands. *Proc. Hawaiian Entomol. Soc.* **2013**, *44*, 11–53.
26. Baker, R.; Herbert, R.H. Isolation and synthesis of 1,7-dioxaspiro[5.5]undecane and 1,7-dioxaspiro[5.5]undecan-3-and -4-ols from the olive fly (Dacus oleae). *J. Chem. Soc. Perkin Trans. 1* **1987**, 1123. [CrossRef]
27. Baker, R.; Herbert, R.; Howse, P.E.; Jones, O.T.; Francke, W.; Reith, W. Identification and synthesis of the major sex pheromone of the olive fly (*Dacus oleae*). *J. Chem. Soc. Chem. Commun.* **1980**, 52–53. [CrossRef]
28. Fletcher, B.S. Storage and release of sex pheromone by the Queensland fruit fly, *Dacus tryoni* (Diptera:Trypetidae). *Nature* **1968**, *219*, 631–632. [CrossRef]
29. Bellas, T.E.; Fletcher, B.S. Identification of the major components in the secretion from the rectal pheromone glands of the Queensland fruit flies *Dacus tryoni* and *Dacus neohumeralis* (Diptera: Tephritidae). *J. Chem. Ecol.* **1979**, *5*, 795–803. [CrossRef]
30. Nation, J.L. Biology of pheromone release by male Caribbean fruit flies, *Anastrepha suspensa* (Diptera: Tephritidae). *J. Chem. Ecol.* **1990**, *16*, 553–572. [CrossRef] [PubMed]
31. White, I.M.; Elson-Harris, M.M. *Fruit Flies of Economic Significance: Their Identification and Bionomics*; CAB International: Wallingford, UK, 1992; ISBN 0851987907.
32. Steiner, L.F.; Mitchell, S. Tephritid fruit flies. In *Insect Colonization and Mass Production*; Smith, C.N., Ed.; Academic Press: Cambridge, MA, USA, 1966; pp. 555–583. ISBN 978-0-12-395601-9.
33. Pérez, J.; Park, S.J.; Taylor, P.W. Domestication modifies the volatile emissions produced by male Queensland fruit flies during sexual advertisement. *Sci. Rep.* **2018**, *8*, 16503. [CrossRef] [PubMed]

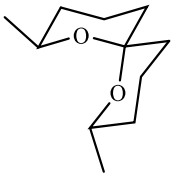
34. Kitching, W.; Lewis, J.A.; Perkins, M.V.; Drew, R.; Moore, C.J.; Schurig, V.; Koenig, W.A.; Francke, W. Chemistry of fruit flies. Composition of the rectal gland secretion of (male) *Dacus cucumis* (cucumber fly) and *Dacus halfordiae*. Characterization of (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. *J. Org. Chem.* **1989**, *54*, 3893–3902. [CrossRef]
35. Aluja, M.; Norrbom, A.L. *Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior*; Aluja, M., Norrbom, A.L., Eds.; CRC Press: Boca Raton, FL, USA, 2000; ISBN 0849312752.
36. El-Sayed, A.M.; Byers, J.A.; Manning, L.M.; Jürgens, A.; Mitchell, V.J.; Suckling, D.M. Floral scent of Canada thistle and its potential as a generic insect attractant. *J. Econ. Entomol.* **2008**, *101*, 720–727. [CrossRef] [PubMed]
37. Booth, Y.K.; Hayes, P.Y.; Moore, C.J.; Lambert, L.K.; Kitching, W.; De Voss, J.J. Synthesis and absolute configuration of a constitutionally-new [5.6] spiroacetal from *B. tryoni* (Queensland fruit fly). *Org. Biomol. Chem.* **2007**, *5*, 1111–1117. [CrossRef]
38. Gilpin, J.A. Mass spectrometric analysis of aliphatic amides. *Anal. Chem.* **1959**, *31*, 935–939. [CrossRef]
39. Sharkey, A.G.; Shultz, J.L.; Friedel, R.A. Mass spectra of esters. Formation of rearrangement ions. *Anal. Chem.* **1959**, *31*, 87–94. [CrossRef]
40. Francke, W.; Kitching, W. Spiroacetals in insects. *Curr. Org. Chem.* **2001**, *5*, 233–251. [CrossRef]
41. Schwartz, B.D.; Booth, Y.K.; Fletcher, M.T.; Kitching, W.; Voss, J.J. De Spiroacetal biosynthesis in fruit flies is complex: Distinguishable origins of the same major spiroacetal released by different *Bactrocera* spp. *Chem. Commun.* **2010**, *46*, 1526–1528. [CrossRef]
42. López-Guillén, G.; Cruz-López, L.; Malo, E.A.; González-Hernández, H.; Cazares, C.L.; López-Collado, J.; Toledo, J.; Rojas, J.C. Factors influencing the release of volatiles in *Anastrepha obliqua* males (Diptera: Tephritidae). *Environ. Entomol.* **2008**, *37*, 876–882. [CrossRef] [PubMed]
43. Bosa, C.F.; Cruz-López, L.; Zepeda-Cisneros, C.S.; Valle-Mora, J.; Guillén-Navarro, K.; Liedo, P. Sexual behavior and male volatile compounds in wild and mass-reared strains of the Mexican fruit fly *Anastrepha ludens* (Diptera: Tephritidae) held under different colony management regimes. *Insect Sci.* **2016**, *23*, 105–116. [CrossRef] [PubMed]
44. Vaníčková, L.; Do Nascimento, R.R.; Hoskovec, M.; Ježková, Z.; Břízová, R.; Tomčala, A.; Kalinová, B. Are the wild and laboratory insect populations different in semiochemical emission? the case of the medfly sex pheromone. *J. Agric. Food Chem.* **2012**, *60*, 7168–7176. [CrossRef] [PubMed]
45. Kamala Jayanthi, P.D.; Woodcock, C.M.; Caulfield, J.; Birkett, M.A.; Bruce, T.J.A. Isolation and identification of host cues from mango, *Mangifera indica*, that attract gravid female Oriental fruit fly, *Bactrocera dorsalis*. *J. Chem. Ecol.* **2012**, *38*, 361–369. [CrossRef] [PubMed]
46. El-Sayed, A.M.; Venkatesham, U.; Unelius, C.R.; Sporle, A.; Pérez, J.; Taylor, P.W.; Suckling, D.M. Chemical composition of the rectal gland and volatiles released by female Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae). *Environ. Entomol.* **2019**, *48*, 807–814. [CrossRef] [PubMed]
47. Fletcher, M.T.; Kitching, W. Chemistry of fruit flies. *Chem. Rev.* **1995**, *95*, 789–828. [CrossRef]
48. Booth, Y.K.; Schwartz, B.D.; Fletcher, M.T.; Lambert, L.K.; Kitching, W.; De Voss, J.J. A diverse suite of spiroacetals, including a novel branched representative, is released by female Bactrocera tryoni (Queensland fruit fly). *Chem. Commun.* **2006**, 3975–3977. [CrossRef]
49. Mazomenos, B.E.; Haniotakis, G.E. A multicomponent female sex pheromone of *Dacus oleae* Gmelin: Isolation and bioassay. *J. Chem. Ecol.* **1981**, *7*, 437–444. [CrossRef]
50. Shen, J.; Hu, L.; Zhou, X.; Dai, J.; Chen, B.; Li, S. Allyl-2,6-dimethoxyphenol, a female-biased compound, is robustly attractive to conspecific males of *Bactrocera dorsalis* at close range. *Entomol. Exp. Appl.* **2019**, *167*, 811–819. [CrossRef]
51. Fletcher, M.T.; Jacobs, M.F.; Kitching, W.; Krohn, S.; Drew, R.A.I.; Haniotakis, G.E.; Francke, W. Absolute stereochemistry of the 1,7-dioxaspiro[5.5]undecanols in fruit-fly species, including the olive-fly. *J. Chem. Soc. Chem. Commun.* **1992**, 1457–1459. [CrossRef]
52. Baker, R.; Herbert, R.H.; Grant, G.G. Isolation and identification of the sex pheromone of the Mediterranean fruit fly, *Ceratitis capitata* (Wied). *J. Chem. Soc. Chem. Commun.* **1985**, 824–825. [CrossRef]
53. Symonds, M.R.E.; Moussalli, A.; Elgar, M.A. The evolution of sex pheromones in an ecologically diverse genus of flies. *Biol. J. Linn. Soc.* **2009**, *97*, 594–603. [CrossRef]

54. Perkins, M.V.; Kitching, W.; Drew, R.A.I.; Moore, C.J.; Konig, W.A. Chemistry of fruit flies: Composition of the male rectal gland secretions of some species of South-East Asian Dacinae. Re-examination of *Dacus cucurbitae* (melon fly). *J. Chem. Soc. Perkin Trans. 1* **1990**, 1111–1117. [CrossRef]
55. Perkins, M.V.; Kitching, W.; König, W.A.; Drew, R.A.I. An (*S*)-(+)-lactic acid route to (2*S*,6*R*,8*S*)-2,8 dimethyl-1,7-dioxaspiro[5,5]undecane and (2*S*,6*R*,8*S*)-2-ethyl-8-methyl-1,7-dioxaspiro[5,5]undecane and demonstration of their presence in the rectal glandular se. *J. Chem. Soc. Perkin Trans. 1* **1990**, 2501–2506. [CrossRef]

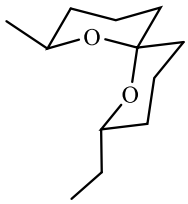
Figure 4. Structure of components identified in *Bactrocera musae* chemical profiles.



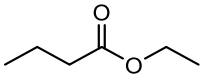
N-(3-methylbutyl)acetamide



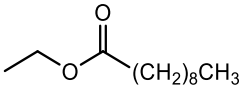
(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane



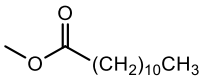
(*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane



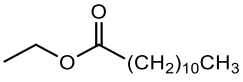
Ethyl butanoate



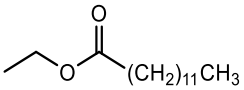
Ethyl caprate



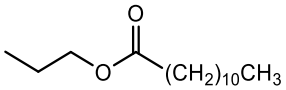
Methyl laurate



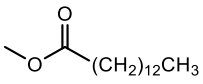
Ethyl laurate



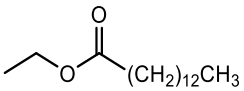
Ethyl tridecanoate



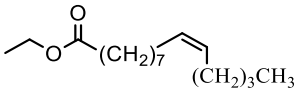
Propyl laurate



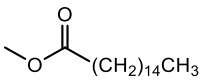
Methyl myristate



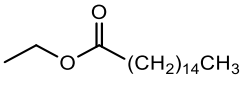
Ethyl myristate



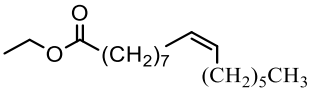
Ethyl myristoleate



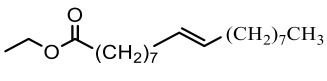
Methyl palmitate



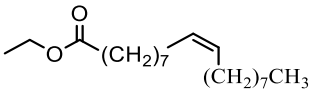
Ethyl palmitate



Ethyl palmitoleate



Ethyl elaidate

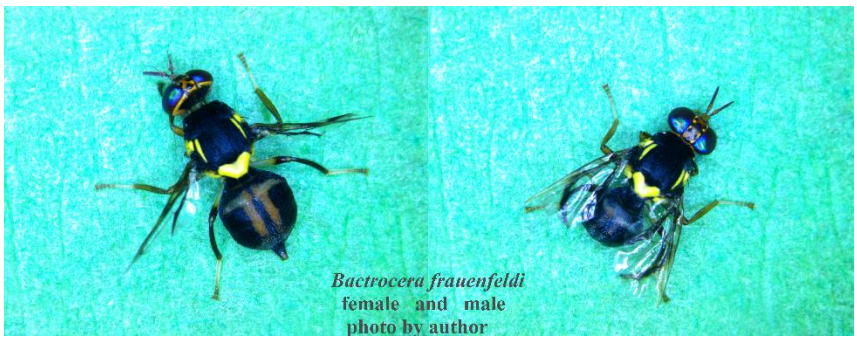


Ethyl oleate

Chapter Three

Attraction and electrophysiological response to identified rectal gland volatiles in *Bactrocera frauenfeldi* (Schiner)

Published in Molecules



Author contributions:

All experiment was conducted by S. Noushini with assistant from J. Perez and S. J. Park for Y-tube observations. S. J. Park and V. Mendez helped with EAG experiment. All data analysis was carried out by S. Noushini. The manuscript was drafted by S. Noushini. All authors read the manuscript and provided critical feedback.

At the begging of this chapter there is a short summery of phylogenetic situation of *Bactrocera frauenfeldi* and species which are known as close relatives to this species.

At the end of this chapter, after the manuscript figure 5 has been provided summarising structure of all identified compounds in male and female *Bactrocera frauenfeldi*.

Preamble

Bactrocera frauenfeldi (Schiner) is an economically important fruit fly pest species belongs to order Diptera and family Tephritidae.[1,2] This species is very similar to *B. albistrigata* (Meijere) but has dark postpronotal lobes (sometimes red-brown postpronotal lobes).[2] Current molecular markers do not adequately separate species in *B. frauenfeldi* complex. The molecular diagnostic locus that best separate these two confused species is EIF3L. Otherwise they are similar in other loci includind RPA2, COI, POP4 and DDOSTS2.[2] Other similar species to *B. frauenfeldi* are *B. trilineola* Drew, *B. kirki* (Froggatt) and *B. psidii* (Froggatt). *Bactrocera frauenfeldi* differs from *B. trilineola* by having lateral vittae present and facial spots instead of a facial mask.[2] They differ from *B. kirki* by having dark postpronotal lobes, lateral vittae present and a distinct band on the wing.[2] They differ from *B. psidii* by having dark postpronotal lobes, a medial line and lateral bands on terga III-V, legs with dark patterning and a distinct band on the wing.[2]

Among these species only putative pheromonal blend of *B. albistrigata* and *B. kirki* is known.[3–5] Composition of the male rectal gland secretions of *B. albistrigata* has been reported to be rich in methyl 4-hydroxybenzoate.[3] The (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecanes and (*E*,*Z*)-2,8-dimethyl-1,7- dioxaspiro[5.5]undecanes were also present.[3] In the glandular extract of male *B. kirki*, (*E*,*E*)-2,8-dimethyl-l,7-dioxaspiro[5.5lundecane was shown to be the major component.[4] (*E*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and (*E*,*E*)-2-ethyl-8-methyl-l,7-dioxaspiro[5.5lundecane were also reported as minor components of male rectal gland extract.[4]

References:

1. Royer, J. E., Wright, C. L. & Hancock, D. L. *Bactrocera frauenfeldi* (Diptera: Tephritidae), an invasive fruit fly in Australia that may have reached the extent of its spread due to environmental variables. *Austral Entomol.* **55**, 100–111 (2016).
2. Schutze, M. *et al. The Australian Handbook for the Identification of Fruit Flies. Version 3.1*. (Plant Health Australia, 2018). doi:10.1016/j.jasms.2007.01.008
3. Perkins, M. V., Kitching, W., Drew, R. A. I., Moore, C. J. & Konig, W. A. Chemistry of fruit flies: composition of the male rectal gland secretions of some species of South-East Asian Dacinae. Re-examination of *Dacus cucurbitae* (melon

fly). *J. Chem. Soc. Perkin Trans. 1* 1111–1117 (1990). doi:10.1039/P19900001111

4. Fletcher, M. T. *et al.* Chemistry of fruit-flies. Spiroacetal-rich secretions in several *Bactrocera* species from the South-West Pacific region. *J. Chem. Soc. Perkin Trans. 1* 2827–2831 (1992). doi:10.1039/P19920002827
5. Symonds, M. R. E., Moussalli, A. & Elgar, M. A. The evolution of sex pheromones in an ecologically diverse genus of flies. *Biol. J. Linn. Soc.* **97**, 594–603 (2009).

Noushini, S., Perez, J., Jean Park, S., Holgate, D., Alvarez, V. M., Jamie, I., Jamie, J., & Taylor, P. (2020). Attraction and electrophysiological response to identified rectal gland volatiles in *Bactrocera frauenfeldi* (Schiner). *Molecules*, *25*(6), 1275.

DOI: https://doi.org/10.3390/molecules25061275

molecules

MDPI

Article

Attraction and Electrophysiological Response to Identified Rectal Gland Volatiles in *Bactrocera frauenfeldi* (Schiner)

Saeedeh Noushini [1,2,*], Jeanneth Perez [2,3], Soo Jean Park [2,3], Danielle Holgate [1], Vivian Mendez Alvarez [2,3], Ian Jamie [1,2], Joanne Jamie [1,2] and Phillip Taylor [2,3]

1 Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia; danielle.holgate@hdr.mq.edu.au (D.H.); ian.jamie@mq.edu.au (I.J.); joanne.jamie@mq.edu.au (J.J.)
2 Australian Research Council Industrial Transformation Training Centre for Fruit Fly Biosecurity Innovation, Macquarie University, Sydney, NSW 2109, Australia; jeanneth.perez@mq.edu.au (J.P.); soojean.park@mq.edu.au (S.J.P.); vivian.mendez@mq.edu.au (V.M.A.); Phil.Taylor@mq.edu.au (P.T.)
3 Applied BioSciences, Macquarie University, Sydney, NSW 2109, Australia
* Correspondence: sally.noushini@mq.edu.au

Received: 18 February 2020; Accepted: 7 March 2020; Published: 11 March 2020

Abstract: *Bactrocera frauenfeldi* (Schiner) (Diptera: Tephritidae) is a polyphagous fruit fly pest species that is endemic to Papua New Guinea and has become established in several Pacific Islands and Australia. Despite its economic importance for many crops and the key role of chemical-mediated sexual communication in the reproductive biology of tephritid fruit flies, as well as the potential application of pheromones as attractants, there have been no studies investigating the identity or activity of rectal gland secretions or emission profiles of this species. The present study (1) identifies the chemical profile of volatile compounds produced in rectal glands and released by *B. frauenfeldi*, (2) investigates which of the volatile compounds elicit an electroantennographic or electropalpographic response, and (3) investigates the potential function of glandular emissions as mate-attracting sex pheromones. Rectal gland extracts and headspace collections from sexually mature males and females of *B. frauenfeldi* were analysed by gas chromatography-mass spectrometry. Male rectal glands contained (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro [5.5]undecane as a major component and (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane as a moderate component. Minor components included palmitoleic acid, palmitic acid, and ethyl oleate. In contrast, female rectal glands contained (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and ethyl laurate as major components, ethyl myristate and ethyl palmitoleate as moderate components, and 18 minor compounds including amides, esters, and spiroacetals. Although fewer compounds were detected from the headspace collections of both males and females than from the gland extractions, most of the abundant chemicals in the rectal gland extracts were also detected in the headspace collections. Gas chromatography coupled electroantennographic detection found responses to (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane from the antennae of both male and female *B. frauenfeldi*. Responses to (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane were elicited from the antennae of females but not males. The two spiroacetals also elicited electropalpographic responses from both male and female *B. frauenfeldi*. Ethyl caprate and methyl laurate, found in female rectal glands, elicited responses in female antennae and palps, respectively. Y-maze bioassays showed that females were attracted to the volatiles from male rectal glands but males were not. Neither males nor females were attracted to the volatiles from female rectal glands. Our findings suggest (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane as components of a sex-attracting pheromone in *B. frauenfeldi*.

Keywords: *B. frauenfeldi*; mango fruit fly; insect volatiles; GC-EAD; olfaction

1. Introduction

Many tephritid fruit flies, belonging to the genus *Bactrocera*, are significant horticultural pests, causing direct damage to fruit and impeding trade [1–4]. The mango fruit fly, *Bactrocera frauenfeldi* (Schiner) (Diptera: Tephritidae), is an economically important tephritid pest, with hosts including guava (*Psidium guajava*), mango (*Mangifera indica*), beach almond (*Terminalia catappa*), and Alexandrian laurel (*Calophyllum inophyllum*) [5–7]. Endemic to Papua New Guinea [8], *B. frauenfeldi* has become established on several Pacific Islands, including the Solomon Islands, the Federated States of Micronesia, the Republic of Kiribati, Marshall Islands, Palau, Nauru, West Papua in Indonesia [9–11], and in Australia [6,12–15]. Surveillance, monitoring, and control of tephritid fruit flies involves the use of lures [16]. Food-based lures have been used as attractants in traps for detecting and monitoring tephritid flies [17]. However they are not as powerful as male lures, such as cuelure, zingerone, and methyl eugenol [18]. *Bactrocera frauenfeldi* adult males are highly attracted to cuelure and raspberry ketone, and are weakly attracted to zingerone [15,18]. Raspberry ketone and zingerone are naturally occurring compounds found in many plants [19,20]. Raspberry ketone is also known as a fungal metabolite [21]. While cuelure has not been found in nature, it hydrolyses to raspberry ketone [16]. Ingested raspberry ketone, zingerone, and cuelure accumulate in the rectal gland of some male s of some fruit fly species in their original form, while zingerone and cuelure are also to some extent transformed [22–24]. However, similar to other fruit flies, there is a lack of a specific attractant for females of *B. frauenfeldi*.

Volatile compounds produced in the rectal glands of tephritid fruit flies and emitted during calling and courtship have been described as the key elements for long and short-range attraction of the opposite sex [25–28]. The volatile emissions also play an important role in attracting the same sex to mating aggregations [29,30]. Rectal gland secretions of some fruit fly species have been studied for potential applications as attractants, including *B. tryoni* (Froggatt) [31], *Zeugodacus cucumis* (French) [32–35], *B. dorsalis* (Hendel) [36], *Z. cucurbitae* (Coquillet) [37], *B. oleae* (Rossi) [38], and *B. correcta* (Bezzi) [39]. In most tephritid fruit flies, males are thought to produce sex pheromones to attract females [40]. However, there are several notable exceptions. For example, 1,7-dioxaspiro[5,5]undecane has been described as a female-produced pheromone of *B. oleae* [38], although later studies reported on this compound in rectal glands of young males of *B. oleae* [41]. While 1,7-dioxaspiro[5.5]undecane has been used extensively for the monitoring and mass trapping of *B. oleae* [42], the sex specific olfactory cues of *B. oleae* are driven by synergistic actions of a number of compounds that are not yet fully understood [4,43]. In *Z. cucurbitae*, females are attracted by male rectal gland secretions containing three aliphatic amides, two pyrazines, and an aromatic acid [44], while males are attracted by headspace constituents containing 2,8-dialkyl-1,7-dioxaspiro[5.5]undecanes and *N*-(3-methylbutyl)acetamide [45]. Similarly, males of *B. dorsalis* produce two phenols and an aliphatic cyclic alcohol in their rectal glands that show pheromonal activity towards females [46,47], while females emit several spiroacetals that attract males [45].

The chemical profiles of the *B. frauenfeldi* volatile compounds are unknown. Given the central role of chemical-mediated sexual communication in the reproductive biology of tephritid fruit flies [4,48], and the potential application of volatiles as attractants, the present study analysed the rectal gland extracts and headspace collections from both males and females of *B. frauenfeldi* using gas chromatography-mass spectrometry (GC-MS). In order to identify electrophysiologically active components present in the emissions of each sex, we used gas chromatography-electroantennogram detection (GC-EAD) and gas chromatography-electropalpogram detection (GC-EPD) to test the responses of male and female antennae and maxillary palps to the emissions of male and female rectal glands. The rectal gland contents were tested for attraction of the opposite and same sex using y-maze olfactometers [49–51].

2. Methods and Materials

2.1. Insects

Pupae of *B. frauenfeldi* (9 generations from wild) were obtained from the Queensland Department of Agriculture and Fisheries (Cairns, Australia). Approximately 500 pupae were allowed to emerge in a 47.5 × 47.5 × 47.5 cm fine mesh cage (Megaview Bugdorm 4S4545, Taiwan) in a controlled environment room (25 ± 0.5 °C, 65 ± 5% relative humidity) and with light:dusk:dark:dawn 11.5:0.5:11.5:0.5 h photoperiod (these conditions were maintained for all rearing and experiments). The adult flies were fed sugar and yeast hydrolysate (MP Biomedicals LLC) and were provided water through a soaked sponge. The flies were reared through one generation at Macquarie University, Sydney, using a standard carrot larval diet [52], following methods described by Pérez et al. [53]. The flies were separated by sex within three days after emergence and transferred to 12.5 L clear plastic cages (180 flies per cage). No mating was observed before separating the flies. The flies used for all the experiments were 13–18 days old.

2.2. Gland Extraction

The flies were killed by chilling them on dry ice. The rectal glands were extracted under a stereomicroscope by gently pressing the abdomen and pulling the gland out with fine forceps. The glands were carefully placed in a 1.1 mL tear-drop vial in dry ice. Once 10 glands were collected, the vials were removed from the dry ice and 100 µL of *n*-hexane (HPLC grade, Sigma-Aldrich, St. Louis, MO, USA) was added. Vials containing *n*-hexane and glands were left to stand at room temperature for 10 min, and then the extracts were transferred to a new vial, labelled, and stored at −20 °C until analysed [54]. Ten replicates of 10 glands were collected for each sex.

2.3. Headspace Collections

Based on our preliminary observations, *B. frauenfeldi* appeared to mate at any time during the day, with the mating peak at noon. Ten males or females were separately placed into a cylindrical glass chamber (150 mm long and 40 mm ID) 30 min before the mating peak. A charcoal-filtered air stream at a flow rate of 0.5 L/min (air pulling system) was drawn over the flies for 1 h. The released volatiles were adsorbed onto traps of 50 mg of Tenax-GR Mesh 60/80 adsorbent (Scientific Instrument Services, Inc, Palmer, MA, USA) packed into 6 × 50 mm glass tubes and fitted with glass wool plugs. The volatiles were eluted with 1 mL of *n*-hexane. The samples were stored at −20 °C until analysed. Seven replicates were collected for each sex. To distinguish any possible contaminants, an air control sample comprising an empty glass chamber was run and analysed along with each volatile collection.

Before each headspace collection, the glass chambers were washed with 5% Extran aqueous solution, rinsed with hot tap water, and heated at 200 °C for 18 h. The tenax traps were thermally conditioned at 200 °C for three hours under a 75 mL/min nitrogen stream. The activated charcoal filters were conditioned by heating them at 200 °C for 18 h prior to each headspace collection [55].

2.4. GC-MS Analysis

Mass spectra were recorded on a Shimadzu GCMS-TQ8040 instrument (Kyoto, Japan), using a capillary column with 5% diphenyl/95% dimethyl polysiloxane as the stationary phase (SH-Rtx-5MS, 30 m × 0.25 mm ID × 0.25 µm film thickness, Shimadzu, Japan) and helium (99.999%) (ultra-high purity, BOC, Sydney, Australia) as a carrier gas with a constant flow of 1 mL/min. A 1 µL sample was injected in the splitless mode. The injector temperature was set at 270 °C. The temperature program was 40 °C (1 min) to 250 °C (3 min) at a rate of 10 °C/min. The interphase and ion source temperatures were set at 290 °C and 200 °C, respectively. Mass spectra were recorded in electron impact mode (70 eV), scanning from 40 to 500 *m*/*z*. A peak was considered of interest if it was not present in the air control samples. Compounds including esters, amides, and spiroacetals were identified through comparison with retention times and fragmentation patterns of authentic samples,

with the exception of compound **4** which was tentatively identified based on literature mass spectral fragmentation patterns [56,57]. Ethyl caprate (**5**), methyl laurate (**6**), ethyl laurate (**7**), ethyl tridecanaote (**8**), propyl laurate (**9**), methyl myristate (**10**), myristic acid (**11**), ethyl myristoleate (**12**), ethyl myristate (**13**), methyl palmitoleate (**14**), methyl palmitate (**15**), palmitoleic acid (**16**), palmitic acid (**17**), ethyl palmitate (**19**), methyl elaidate (**20**), and ethyl oleate (**21**) were purchased from Sigma-Aldrich (Castle Hill, Australia), Alfa-Aesar (United Kingdom), Nu-Chek-Prep, and INC (Minneapolis, USA). Ethyl palmitoleate (**18**), ethyl elaidate (**22**), *N*-(2-methylbutyl)acetamide (**1**), *N*-(3-methylbutyl)acetamide (**2**), and (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5,5]undecane (**3**) were synthesised (see Supplementary Materials for synthesis details).

2.5. Electrophysiological Assays

Gas chromatography-electroantennographic detection (GC-EAD) and gas chromatography-electropalpogram detection (GC-EPD) were carried out to identify the antennal- or palpal-active components from male and female rectal gland extracts. The system consisted of a gas chromatography flame ionization detector (Agilent 7890B, CA, USA) coupled to an electroantennogram (Syntech, Hilversum, The Netherlands). The GC was equipped with a polar capillary column with (35%-phenyl)-methylpolysiloxane as the stationary phase (Agilent HP-5, 30 m × 0.32 mm ID × 0.25 µm film thickness). The carrier gas was hydrogen (99.999% pure) supplied by a generator (MGG-2500-220 Parker Balston, NY, USA) with a constant flow of 2.5 mL/min. The initial temperature was set at 50 °C (1 min) then increased to 250 °C (3 min) at a rate of 10 °C/min. The injector and detector temperatures were set at 270 °C and 290 °C, respectively. The effluent of the column was mixed with 30 mL/min make-up nitrogen gas and split in a ratio of 1 (FID) to 1.5 (EAD) through a heated transfer line (Syntech, TC-02, Syntech, Hilversum, The Netherlands) and kept at 200 °C.

A female or male *B. frauenfeldi* head was carefully severed and a borosilicate glass capillary electrode filled with electrically conductive gel (Spectra 360) was attached onto the back of the head. The tip of the antenna or maxillary palp was inserted into the tip of the recording glass capillary electrode filled with phosphate-buffered saline (PBS). The mounted heads were under charcoal filtered and humidified air flow (400 mL/min) controlled by a flow controller (Syntech Stimulus Controller CS-55, Syntech, Hilversum, The Netherlands) and were subjected to each stimulus. The electrophysiological responses were captured and processed by a data acquisition controller (IDAC-4, Syntech, Hilversum, The Netherlands). Before the injection of the sample into the airstream, the antenna/palp was stimulated with 1-hexanol to check its sensitivity. EAD/EPD signals were analysed using GC-EAD 2014 software version 1.2.5. Nine successful GC-EAD/EPD recordings were obtained for each sex. The electrophysiological responses of male and female antennae and palps to the conspecific opposite and same sex rectal gland extracts were recorded. A response was considered genuine if it was present in at least six out of the nine replicates collected. The identity of the compounds eliciting an electrophysiological response was confirmed by comparing the retention times with that of the GC-MS chromatograms using the same column and method as for the GC(FID)-EAD experiments.

2.6. Y-maze Bioassays

The response of sexually mature (13–18 days old) *B. frauenfeldi* males and females toward rectal gland contents of the same and opposite sex was evaluated using Y-maze olfactometers. The system consisted of a clear Plexiglas Y shape tube with one central arm (6.5 cm × 4.5 cm × 5 cm) in which the release chamber (5 cm × 5 cm × 5 cm) was located, and two upwind lateral arms (12.5 cm × 4.5 cm × 5 cm), each of which was connected to a rectangular chamber (7.5 cm × 5 cm × 5 cm) (see Supplementary Materials, Figure S1). The Y-maze olfactometer was positioned horizontally on a white table and a humidified and charcoal-filtered air stream was passed through the Y-maze at a flow rate of 140 ± 5 mL/min. The stimulus cartridge was prepared by crushing 15 rectal glands (male or female) on a 1 cm^2 filter paper (Advantec, Tokyo, Japan) inserted in a glass Pasteur pipette (145 mm long). The control cartridge was prepared using 1 cm^2 filter paper inserted in the same type of

glass Pasteur pipette. One cartridge of each type was fitted to one of the Y-maze upwind arms using a Tygon tube (Tygon®formula E-3603, Sigma-Aldrich, St. Louis, MO, USA). An individual fly was placed in the release chamber to acclimatise for 30 min before each experiment. The experiment was carried out at noon in a controlled environment room, under the same conditions the flies were kept. Each trial lasted 30 min. Once the two cartridges (stimulus and control) were connected to the upwind arms, the system was allowed to equilibrate for two minutes and then the barriers of the two upwind arms and the release chamber were removed. A choice was recorded when the fly reached one of the two upwind arms and stayed there for at least one minute. Flies that did not make any choice, i.e. remained in the release chamber, did not reach one of the two upwind arms, or did not stay there for one minute, were excluded. For each treatment, 45–50 replicates from responsive flies were carried out over multiple days with no more than 15 replicates on any day. The position (left or right) of the stimulus and the control was reversed for every trial to minimise the positional effects. The flies used in this experiment were obtained from multiple batches (two per week) and each fly was tested only once. Freshly dissected rectal glands were used each day. Before each replicate, the Y-maze olfactometer was washed with 5% Extran aqueous solution, rinsed with hot tap water, and air-dried. To compare the number of flies choosing the stimulus over the control, a binomial test with the probability level of $p < 0.05$ was used.

3. Results

The GC-MS results showed that male and female *B. frauenfeldi* have different rectal gland and volatile emission compositions. Females produced a more complex blend than did males (Figure 1). A total of twenty-two compounds were identified including amides (**1** and **2**), spiroacetals (**3** and **4**), esters (**5**–**10**, **12**–**15**, **18**–**21**, and **22**), and fatty acids (**11**, **16,** and **17**). The identities of the twenty-one compounds were confirmed by comparison with GC retention times and mass fragmentation patterns of authentic samples.

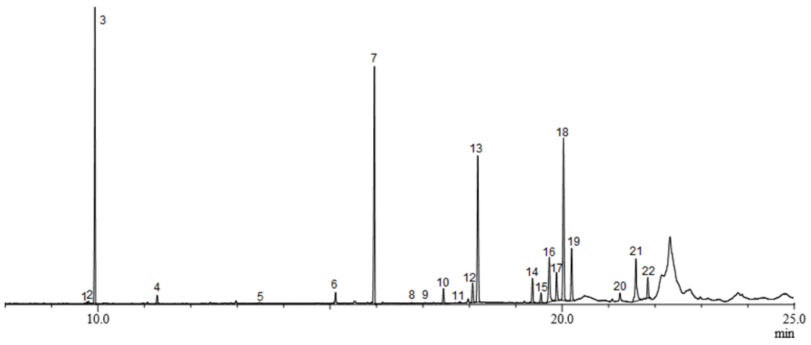


Figure 1. Gas chromatogram of the rectal gland extract of *B. frauenfeldi* females. The numbered peaks indicate detected compounds: *N*-(2-methylbutyl)acetamide (**1**), *N*-(3-methylbutyl)acetamide (**2**), (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5,5]undecane (**3**), (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**), ethyl caprate (**5**), methyl laurate (**6**), ethyl laurate (**7**), ethyl tridecanaote (**8**), propyl laurate (**9**), methyl myristate (**10**), myristic acid (**11**), ethyl myristoleate (**12**), ethyl myristate (**13**), methyl palmitoleate (**14**), methyl palmitate (**15**), palmitoleic acid (**16**), palmitic acid (**17**), ethyl palmitoleate (**18**), ethyl palmitate (**19**), methyl elaidate (**20**), ethyl oleate (**21**), and ethyl elaidate (**22**).

Of the twenty-two compounds that were identified in female *B. frauenfeldi*, nine compounds were detected in rectal gland extracts and headspace samples including *N*-(3-methylbutyl)acetamide (**2**), (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**), (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**), methyl laurate (**6**), ethyl laurate (**7**), methyl myristate (**10**), ethyl myristoleate (**12**), ethyl myristate (**13**), and ethyl palmitoleate (**18**) (Table 1). The main compounds present in female gland extracts and headspace samples were (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**) and ethyl laureate (**7**), although they were found in higher proportions in the headspace samples (Table 1).

Table 1. Percentage of compounds identified in chemical profiles for *B. frauenfeldi*. RT = retention time, KI = Kovats index, ND = not detected.

Compound	Females		Males		RT	KI	Diagnostic Ions *m*/*z* (%)
	Headspace (%)	Rectal Gland (%)	Headspace (%)	Rectal Gland (%)			
N-(2-Methyl-butyl)acetamide (**1**)	ND	<1	ND	ND	9.7	1133	129 (M^+, 5.2), 100 (62.2), 73 (β-cleavage/H rearrangement, 76.4), 72 (M – C_4H_9, 100), 60 ($CH_3C(OH)NH^+$, 54.8)
N-(3-Methylbutyl)acetamide (**2**)	<1	<1	ND	ND	9.8	1137	129 (M^+, 6.6), 114 (18.2), 86 (28.4), 73 (β-cleavage/H rearrangement, 100), 72 (M – C_4H_9, 74.4), 60 ($CH_3C(OH)NH^+$, 32.6)
(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**3**)	53.9	20.1	24.6	16.6	9.9	1147	184 (M^+, 9.7), 169 (2.1), 140 (17.8), 125 (9.7), 115 ($CH_3(C_5H_7O)=OH^+$, 98.1), 112 ($CH_3(C_5H_7O)=CH_2$, 100), 97 (75.4), 69 (33.3), 55 (31.2)
(*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**)	5.7	<1	75.4	70.3	11.3	1237	198 (M^+, 10.7), 169 (14.1), 140 (17.5), 129 ($CH_3CH_2(C_5H_7O)=OH^+$, 52), 126 ($CH_3CH_2(C_5H_7O)=CH_2$, 40.1), 115 $CH_3(C_5H_7O)=OH^+$, 94.2), 112 ($CH_3(C_5H_7O)=CH_2$, 100), 97 (66.5), 69 (43.5), 55 (49.1)
Ethyl caprate (**5**)	ND	<1	ND	ND	13.5	1396	200 (M^+, 1.7), 171 (4.2), 157 (19.5), 155 (M – OC_2H_5, 15.9), 115 (9.7), 101 (44.7), 88 (100), 73 ($COOC_2H_5$, 23.6), 70 (27.6)
Methyl laurate (**6**)	2.1	<1	ND	ND	15.1	1524	214 (M^+, 3.7), 183 (M – OCH_3, 7.8), 171 (14.6), 143 (18.2), 87 (60), 74 (100), 59 ($COOCH_3$, 8.4), 55 (22.8)
Ethyl laurate (**7**)	30.3	18.9	ND	ND	15.9	1595	228 (M^+, 4.3), 199 (4.7), 183 (M – OC_2H_5, 11.6), 157 (18.2), 101 (52.9), 88 (100), 73 ($COOC_2H_5$, 20.9), 70 (25.8), 61 (13.6), 55 (21.3)
Ethyl tridecanaote (**8**)	ND	<1	ND	ND	16.8	1667	242 (M^+, 4.5), 213 (11.9), 199 (15.6), 197 (M – OC_2H_5, 2.3), 157 (31.7), 101 (60.9), 88 (100), 73 ($COOC_2H_5$, 5.8), 57 (25.9), 55 (24.4)
Propyl laurate (**9**)	ND	<1	ND	ND	17.1	1691	242 (M^+, 1.6), 201 (40.4), 199 (1.1), 183 (M – OC_3H_7, 36.5), 115 (26.7), 102 (29.7), 87 ($COOC_3H_7$, 11.2), 61 (100), 60 (34), 55 (30.4)
Methyl myristate (**10**)	<1	1.4	ND	ND	17.4	1727	242 (M^+, 6.6), 211 (M – OCH_3, 6.3), 199 (16.2), 143 (25.6), 87 (64.4), 74 (100), 59 ($COOCH_3$, 7.8), 55 (23.4)
Myristic acid (**11**)	ND	<1	ND	ND	17.8	1759	228 (M^+, 19.8), 185 (44.6), 171 (26.6), 143 (25.2), 129 (67.6), 115 (24.5), 97 (22.1), 87 (33.1), 85 (21.2), 83 (25.7), 73 (100), 69 (39.3), 60 (CH_3COOH, 90.6), 57 (68), 55 (64)
Ethyl myristoleate (**12**)	2.6	1.9	ND	ND	18.1	1785	254 (M^+, 4.1), 209 (M – OC_2H_5, 13.9), 208 (M – C_2H_5OH, 14.9), 166 (28.8), 124 (23.7), 88 (46.3), 73 ($COOC_2H_5$, 16.6), 69 (52.1), 55 (100)

Table 1. *Cont.*

Compound	Females		Males		RT	KI	Diagnostic Ions *m*/*z* (%)
	Headspace (%)	Rectal Gland (%)	Headspace (%)	Rectal Gland (%)			
Ethyl myristate (**13**)	1.9	14.6	ND	ND	18.2	1795	256 (M^+, 7.1), 213 (13.8), 211 (M – OC_2H_5, 8.16), 157 (21.9), 101 (53.8), 88 (100), 73 ($COOC_2H_5$, 17.8), 70 (22.1), 55 (20.1)
Methyl palmitoleate (**14**)	ND	2.5	ND	ND	19.3	1909	268 (M^+, 5.1), 237 (M – OCH_3, 14.2), 236 (M – CH_3OH, 18.5), 194 (17.9), 152 (24.1), 96 (51.3), 74 (52.3), 59 ($COOCH_3$, 17.1), 55 (100)
Methyl palmitate (**15**)	ND	<1	ND	ND	19.5	1928	270 (M^+, 12.5), 227 (14.8), 143 (23.6), 87 (68.2), 74 (100), 69 (12.5), 59 ($COOCH_3$, 7.2), 55 (24.8)
Palmitoleic acid (**16**)	ND	5.5	ND	4.4	19.7	1825	254 (M^+, 2.2), 236 (13.6), 152 (9.2), 111 (23.8), 98 (33.8), 97 (50.3), 96 (35.2), 83 (56.4), 73 (15.3), 69 (73.7), 60 (CH_3COOH, 10), 57 (24.8), 55 (100)
Palmitic acid (**17**)	ND	3.1	ND	3.9	19.9	1962	256 (M^+, 38.1), 227 (9.9), 213 (M – COOH, 31.3), 185 (26.9), 157 (31.4), 129 (61.8), 115 (26.5), 97 (33.2), 87 (36.7), 85 (37), 83 (39), 73 (100), 69 (45.9), 60 (CH_3COOH, 84.8), 57 (88.9), 55 (75.4)
Ethyl palmitoleate (**18**)	2.5	16.1	ND	ND	20.0	1977	282 (M^+, 2.9), 237 (M – OC_2H_5, 19.1), 236 (M – C_2H_5OH, 21.3), 194 (23.2), 152 (28.6), 88 (57.3), 73 ($COOC_2H_5$, 16.8), 69 (68.7), 55 (100)
Ethyl palmitate (**19**)	ND	5.3	ND	ND	20.2	1995	284 (M^+, 11.2), 255 (4.1), 241 (13.2), 239 (M – OC_2H_5, 7.5), 157 (21.3), 101 (57.5), 88 (100), 73 ($COOC_2H_5$, 16.1)
Methyl elaidate (**20**)	ND	<1	ND	ND	21.2	2102	296 (M^+, 5.3), 265 (M – OCH_3, 17.8), 264 (26.7), 222 (16.9), 152 (13.6), 97 (62.2), 74 (47.5), 69 (66.2), 55 (100)
Ethyl oleate (**21**)	ND	6.4	ND	4.8	21.6	2144	310 (M^+, 1.2), 265 (M – OC_2H_5, 8.8), 264 (M – C_2H_5OH, 16.9), 222 (5.4), 123 (13.6), 110 (22.8), 97 (59.7), 88 (54.1), 83 (62.9), 73 ($COOC_2H_5$, 15.1), 69 (72.1), 55 (100)
Ethyl elaidate (**22**)	ND	1.9	ND	ND	21.8	2172	310 (M^+, 7.9), 265 (M – OC_2H_5, 24.5), 264 (M – C_2H_5OH, 31.7), 222 (22.1), 180 (20.4), 110 (31.4), 97 (65.5), 88 (57.9), 83 (63.4), 73 ($COOC_2H_5$, 15.2), 69 (68.4), 55 (100)

The GC-MS analysis of male rectal gland extracts showed a blend of six compounds (Figure 2), being (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**) as the main compound and (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**) as the second major compound, representing 70% and 17% of the blend, respectively. Male headspace samples showed only (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane and (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, in a similar ratio to that detected in the male rectal gland samples.

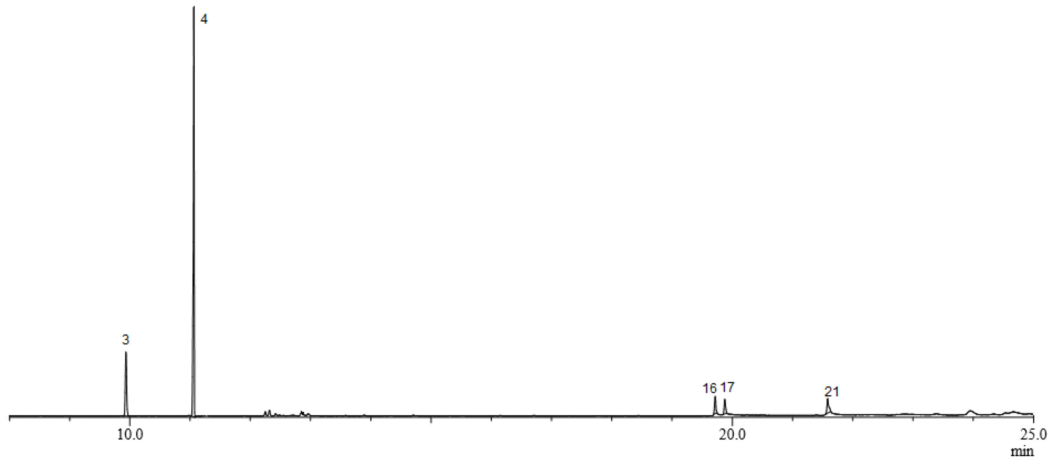


Figure 2. Typical Gas chromatogram of rectal gland extract of *B. frauenfeldi* males. The numbered peaks indicate detected compounds: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**), (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**), palmitoleic acid (**16**), palmitic acid (**17**), and ethyl oleate (**21**).

3.1. Electrophysiological Responses

The analysis by GC-EAD of the male rectal gland samples showed that female and male antennae responded consistently to the main spiroacetal, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**). The female antennae also responded to (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**) from the male rectal gland, while the male antennae did not. The female and male palps responded to (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**) and (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**) (Figure 3A).

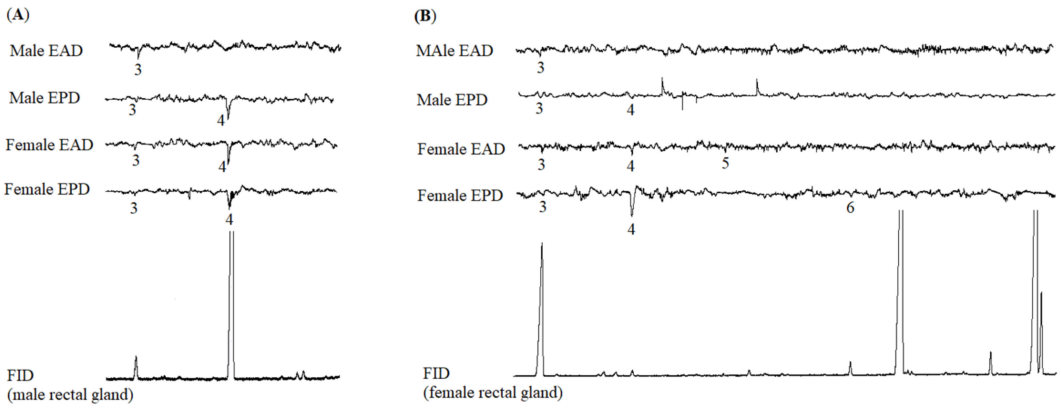


Figure 3. Flame ionization detector (FID) response and electrophysiological responses of antennae (EAD) and maxillary palps (EPD) using *Bactrocera frauenfeldi* males and females to (**A**) rectal gland extracts from conspecific males and (**B**) rectal gland extracts from conspecific females. The numbered peaks indicate EAD- and EPD-active compounds: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**), (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**), ethyl caprate (**5**), and methyl laurate (**6**).

In contrast, GC-EAD analysis of female rectal gland samples revealed that (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**) elicited an antennal response in both males and females. The female antennae responded to two more compounds from the female rectal gland including (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**) and ethyl caprate (**5**). GC-EPD analysis of the female rectal gland samples showed that the female and male maxillary palps responded to (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**) and (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**4**). Methyl laurate (**6**) was only detected by the female palps (Figure 3B).

3.2. Y-maze Bioassays

Sexually mature *B. frauenfeldi* females significantly preferred the upwind arm containing male rectal glands over the control ($p = 0.01$). In contrast, *B. frauenfeldi* males did not show a significant preference for female rectal glands over the control ($p = 0.1$). Neither females nor males exhibited significant preferences when the rectal gland content of the same sex was presented ($p = 0.06$ and $p = 0.1$, respectively) (Figure 4).

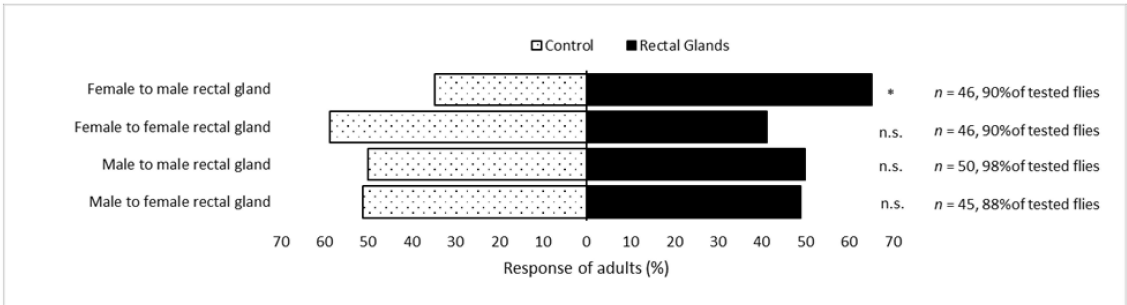


Figure 4. Response of sexually mature virgin *Bactrocera frauenfeldi* males and females to rectal gland volatiles of the same and opposite sex, vs. control (clean filter paper) in Y-maze bioassays. * significantly different at 0.01 level, ns not significantly different, n total number of responded flies.

4. Discussion

We report here the first identification, electrophysiological detection, and behavioural evaluation of rectal gland volatiles produced by *B. frauenfeldi* males and females. Our data show that females of this species produced and emitted a greater diversity of compounds than males. The compounds included aliphatic amides, spiroacetals, and saturated and unsaturated acids and esters. The two amides, *N*-(2-methylbutyl)acetamide (**1**) and *N*-(3-methylbutyl)acetamide (**2**), found in this study have been previously reported in rectal glands of other species, including *B. tryoni* [31,58], *Z. cucumis* (French) [34], *B. dorsalis* (Hendel), and *Z. cucurbitae* (Coquillett) [45]. *N*-(3-Methylbutyl)acetamide is one of the major components in the rectal glands of *B. tryoni* males. The two spiroacetals that were found in *B. frauenfeldi* have also been reported in other *Bactrocera* species, and closely related *Zeugodacus*. For instance, *EE*, *EZ*, and *ZZ* isomers of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane have been reported in the rectal glands of *Z. cucumis* [33]. (*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**3**) have been also found in the rectal glands of *B. dorsalis*, *B. nigrotibialis* (Perkins), *B. albistrigata* (Meijere), *B. jarvisi* (Tryon), *B. kirki* (Froggatt), *B. kraussi* (Hardy), *B. musae* (Tryon), and *B. tryoni* [33,34,45,58–61]. This compound was also found in both sexes of *B. frauenfeldi*, although it was less abundant in males. The most abundant compound in males was (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane. The spiroacetal (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane has been previously reported in the rectal glands of male *B. nigrotibialis* (Perkins), *B. halfordiae* (Tryon), *B. dorsalis* (Hendel), *B. kirki* (Froggatt), *B. latifrons* (Hendel), and *B. occipitalis* (Bezzi) and in female *B. tryoni* (Froggatt) and *B. musae* [4,58,61–63]. In addition to the compounds found in males, the female rectal gland and headspace extracts included saturated and unsaturated acids and esters. The compounds, ethyl caprate (**5**), methyl laurate (**6**),

ethyl laurate (**7**), ethyl tridecanaote (**8**), propyl laurate (**9**), methyl myristate (**10**), myristic acid (**11**), ethyl myristoleate (**12**), ethyl myristate (**13**), methyl palmitoleate (**14**), methyl palmitate (**15**), ethyl palmitoleate (**18**), ethyl palmitate (**19**), methyl elaidate (**20**), and ethyl elaidate (**22**) were female-specific. Nine of the saturated and unsaturated esters in *B. frauenfeldi* have also been reported in *B. oleae*, with ethyl myristate and ethyl palmitate as the major compounds in both species [49]. *B. musae* rectal glands were also reported to contain twelve of the esters found in female *B. frauenfeldi* with ethyl laurate, ethyl myristate, and ethyl palmitate as the most abundant components [61]. Ethyl caprate, which was EAG active for females of *B. frauenfeldi*, has been found to attract both males and females of *B. oleae* [49]. The other electrophysiologically active ester in this study, methyl laurate, has not been reported as an attractant in any other species, but the similar saturated methyl ester, methyl palmitate, has been reported to attract both male and female *B. oleae* [49]. Many of the saturated/unsaturated acids and esters that we found in *B. frauenfeldi* females are also common in females of other *Bactrocera* species, although ratios vary amongst species.

The differences between the composition of rectal glands and volatile emissions observed in this study are not likely due to sampling and/or sensitivity of the instrument because allowing for those factors, the ratios in the rectal gland samples would be matched in the headspace collections. This is not the case. In female rectal glands compounds **5** and **6** have similar relative abundance (< 1%) but only compound **6** was detected in the headspace samples. Similarly, compounds **16** and **19** were more abundant than compound **6** in female rectal glands (both > 5%) but were not detected in headspace samples. For the compounds with longer chain length, and hence lower volatility including the C14, C16, and C18 esters, compared to the C12 ester, it is likely that the relative abundance of these compounds is lower in headspace than rectal glands. The differences between the composition of rectal glands and volatile emissions could be due to the disproportionate release of some compounds.

The proportion of compounds in a blend can be critical for triggering behavioural responses, and the differences might be responsible for sex-specific behavioural responses. For example, in closely related moth species, pheromone blends often contain the same compounds but in different ratios [64,65]. In the present study we found sex differences in the proportion of some compounds. For instance, the spiroacetals **3** and **4** are present in very different ratios in the rectal gland of males and females; 19:81 in males vs. 99:1 in females. If the presence of the compounds was solely responsible for behavioural response, females should have had a preference for female glands in Y-maze bioassays. The fact that this was not the case suggests natural proportions of the compounds may be important in triggering sex-specific behavioural responses.

Male production of sex-attracting pheromones has been reported in diverse tephritid fruit flies [25,34,66]. For example, the male Caribbean fruit fly, *Anastrepha sunspensa* (Loew), releases volatile pheromone components including (*Z*)-non-3-en-1-ol and (3*Z*,6*Z*)-nona-3,6-dien-1-ol, anastrephin, and epianastrephin, which attract female conspecifics [25,66]. The male Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), releases a cyclic imine, l-pyrroline, that is highly attractive to virgin females [34,67]. In *B. tryoni*, male glandular blends include the amides, *N*-3-methylbutylpropanamide, *N*-3-methylbutylacetamide, *N*-(3-methylbutyl)-2-methylpropanamide, *N*-2-methylbutylpropanamide, *N*-2-methylbutylacetamide, and *N*-(2-methylbutyl)-2-methylpropanamide, which are thought to function as short-range stimulants for females [31,34]. Our Y-maze olfactometer results showed that female *B. frauenfeldi* are attracted to male gland odour, whereas males did not exhibit a significant preference for male or female gland odours during the period of peak mating activity. This suggests that a specific compound or specific compounds produced in the rectal gland of *B. frauenfeldi* males elicits attraction of females. Our GC-EAD/EPD results showed that (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane elicits antennal and palpal responses in both males and females. Although (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane elicits an antennal response only in females, it was detected by palps in both males and females. Our findings suggest that in *B. frauenfeldi* males are the main sex pheromone producer and (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane are sex pheromone candidates. This chemical

information about the sexual communication of *B. frauenfeldi* provides a valuable foundation for the synthesis and development of new nature-inspired attractants for the control of this pest species.

Supplementary Materials: The supplementary materials are available online. Synthesis of *N*-(2-methylbutyl)acetamide, *N*-(3-methylbutyl)acetamide, 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, ethyl palmitoleate, and ethyl elaidate. Figure S1. Y-maze apparatus used in this study.

Author Contributions: Conceptualization, S.N., P.T., I.J. and J.J.; Investigations, S.N., J.P., D.H., S.J.P. and V.M.A.; Data analysis, S.N.; Writing—Original draft preparation, S.N.; Writing—Review and editing, P.T., J.J., I.J., J.P. and S.J.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Australian Research Council Industrial Transformation Training Centre (ITTC) for Fruit Fly Biosecurity Innovation (Project IC50100026), funded by the Australian Government.

Acknowledgments: We gratefully acknowledge the Queensland Department of Agriculture and Fisheries (QDAF), especially Sybilla Oczkowicz and Peter Leach, for providing *B. frauenfeldi* from their colonies for this research.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Vijaysegaran, S. Fruit fly research and development in tropical Asia. In *Management of Fruit Flies in the Pacific*; Allwood, A., Drew, R.E., Eds.; Australian Centre for International Agricultural Research Proceedings: Canberra, Australia, 1997; pp. 21–29.
2. Hickey, P.; Woods, B.; Windle, B.; Ransom, L.; Gunasekera, D.; Green, A.; Plowman, D.; Panitz, M.; Scott, N.S.; Chapman, J. *National Fruit Fly Strategy Implementation Action Plan*; Plant Health Australia: Canberra, Australia, 2010.
3. Clarke, A.R.; Powell, K.S.; Weldon, C.W.; Taylor, P.W. The ecology of *Bactrocera tryoni* (Diptera: Tephritidae): what do we know to assist pest management? *Ann. Appl. Biol.* **2011**, *158*, 26–54. [CrossRef]
4. Benelli, G.; Daane, K.M.; Canale, A.; Niu, C.-Y.; Messing, R.H.; Vargas, R.I. Sexual communication and related behaviours in Tephritidae: current knowledge and potential applications for integrated pest management. *J. Pest. Sci.* **2014**, *87*, 385–405. [CrossRef]
5. McAuslane, H.J.; Metcalf, R.L.; Metcalf, E.R. Plant kairomones in insect ecology and control. *Fla. Èntomol.* **1992**, *75*, 613. [CrossRef]
6. Hancock, D.L.; Hamacek, E.L.; Lloyd, A.C.; Elson-Harris, M.M. *The Distribution and Host Plants of Fruit Flies (Diptera: Tephritidae) in Australia*; Queensland Department of Primary Industries: Brisbane, Australia, 2000.
7. Leblanc, L.; William, J.; Allwood, A.J. Host fruit of mango fly (*Bactrocera frauenfeldi* (Schiner)) (Diptera: Tephritidae) in the Federated States of Micronesia. *Micronesica* **2004**, *37*, 21–31.
8. Leblanc, L.; Allwood, A.J. Mango fruit fly (*Bactrocera frauenfeldi*): why so many in federated states of Micronesia. In *Management of Fruit Flies in the Pacific*; Allwood, A.J., Drew, R.A.I., Eds.; Australian Centre for International Agricultural Research: Canberra, Australia, 1997; pp. 125–130.
9. Hardy, D.E.; Adachi, M. Diptera: Tephritidae. In *Insects of Micronesia*; Bishop Museum Bulletin: Honolulu, Hawaii, 1956; pp. 1–28.
10. Allwood, A.J.; Leblanc, L. Losses caused by fruit flies (Diptera: Tephritidae) in seven Pacific Island countries. In *Management of Fruit Flies in the Pacific*; Australian Centre for International Agricultural Research: Canberra, Australia, 1997; pp. 208–211.
11. Mararuai, A.N. Market Access of Papua New Guinea Bananas (Musa spp.) with Particular Respect to Banana Fly (*Bactrocera musae* (Tryon)) (Diptera: Tephritidae). Ph.D. Thesis, School of Natural Resource Sciences, Queensland University of Technology, Queensland, Australia, 2010.
12. Schutze, M.; McMahon, J.; Krosch, M.; Strutt, F.; Royer, J.; Bottrill, M.; Woods, N.; Cameron, S.; Woods, B.; Blacket, M. *The Australian Handbook for the Identification of Fruit Flies*; Version 3.1; Plant Health Australia: Canberra, ACT, Australia, 2018; ISBN 9780648245605.
13. Osborne, R.; Meats, A.; Frommer, M.; Sved, J.A.; Drew, R.A.I.; Robson, M.K. Australian Distribution of 17 Species of Fruit Flies (Diptera: Tephritidae) Caught in Cue Lure Traps in February 1994. *Aust. J. Èntomol.* **1997**, *36*, 45–50. [CrossRef]
14. Drew, R.A.I.; Hancock, D.L.; Romig, M.C. New species and records of fruit flies (Diptera: Tephritidae: Dacinae) from north Queensland. *Aust. Entomol.* **1999**, *26*, 1–12.

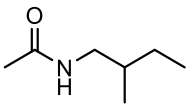
15. Royer, J.; Wright, C.L.; Hancock, D.L. *Bactrocera frauenfeldi* (Diptera: Tephritidae), an invasive fruit fly in Australia that may have reached the extent of its spread due to environmental variables. *Austral. Èntomol.* **2015**, *55*, 100–111. [CrossRef]
16. Tan, K.H.; Nishida, R.; Jang, E.B.; Shelly, T.E. Pheromones, male lures, and trapping of Tephritid fruit flies. In *Trapping and the Detection, Control, and Regulation of Tephritid Fruit Flies*; Lures, Area-Wide Programs, and Trade Implications; Shelly, T., Epsky, N., Jang, E.B., Reyes-Flores, J., Vargas, R., Eds.; Springer: Dordrecht, The Netherlands, 2014; pp. 15–74.
17. Epsky, N.D.; Kendra, P.; Schnell, E.Q. History and Development of Food-Based Attractants. In *Trapping and the Detection, Control, and Regulation of Tephritid Fruit Flies*; Springer: Dordrecht, The Netherlands, 2014; pp. 75–118.
18. Royer, J.E. Responses of fruit flies (Tephritidae: Dacinae) to novel male attractants in north Queensland, Australia, and improved lures for some pest species. *Austral. Èntomol.* **2015**, *54*, 411–426. [CrossRef]
19. Marco, J.; Barberà, O.; Rodriguez, S.; Domingo, C.; Adell, J. Flavonoids and other phenolics from *Artemisia hispanica*. *Phytochemistry* **1988**, *27*, 3155–3159. [CrossRef]
20. Keng-Hong, T.; Nishida, R. Synomone or kairomone?—*Bulbophyllum apertum* flower releases raspberry ketone to attract *Bactrocera* fruit flies. *J. Chem. Ecol.* **2005**, *31*, 497–507. [CrossRef]
21. Ayer, W.A.; Singer, P.P. Phenolic metabolites of the bird's nest fungus *Nidula niveo-tomentosa*. *Phytochemistry* **1980**, *19*, 2717–2721. [CrossRef]
22. Nishida, R.; Iwahashi, O.; Tan, K.H. Accumulation of *Dendrobium superbum* (orchidaceae) fragrance in the rectal glands by males of the melon fly, *Dacus cucurbitae*. *J. Chem. Ecol.* **1993**, *19*, 713–722. [CrossRef] [PubMed]
23. Tan, K.H.; Nishida, R. Zingerone in the floral synomone of *Bulbophyllum baileyi* (Orchidaceae) attracts *Bactrocera* fruit flies during pollination. *Biochem. Syst. Ecol.* **2007**, *35*, 334–341. [CrossRef]
24. Kumaran, N.; Hayes, R.A.; Clarke, A.R. Cuelure but not zingerone make the sex pheromone of male *Bactrocera tryoni* (Tephritidae: Diptera) more attractive to females. *J. Insect Physiol.* **2014**, *68*, 36–43. [CrossRef] [PubMed]
25. Nation, J.L. Courtship behavior and evidence for a sex attractant in the male Caribbean fruit fly, *Anastrepha suspensa*. *Ann. Èntomol. Soc. Am.* **1972**, *65*, 1364–1367. [CrossRef]
26. Perkins, M.V. *Characterisation and Synthesis of Bactrocera Fruit Fly Pheromones*; University of Queensland Library: Queensland, Australia, 2015.
27. Freidberg, A.; Kaneshiro, K.; Sivinski, J.; Aluja, M.; Landolt, P.; Dodson, G.; Headrick, D. Topics in the Evolution of Sexual Behavior in the Tephritidae. In *Fruit Flies (Tephritidae)*; CRC Press: Boca Raton, FL, USA, 1999; pp. 751–792.
28. Cruz-López, L.; Malo, E.A.; Rojas, J.C. Sex Pheromone of *Anastrepha striata*. *J. Chem. Ecol.* **2015**, *41*, 458–464. [CrossRef]
29. Sivinski, J.M.; Calkins, C. Use of pheromones in tropical crops: Pheromones and parapheromones in the control of tephritids. *Florida Entomol.* **1986**, *69*, 157–168. [CrossRef]
30. Hendrichs, J.; Robinson, A.S.; Cayol, J.P.; Enkerlin, W.; Society, F.E. Medfly areawide sterile insect technique programmes for prevention, supression or eradication: The importance of mating behavior studies. *Fla. Èntomol.* **2002**, *85*, 1–13. [CrossRef]
31. Bellas, T.E.; Fletcher, B.S. Identification of the major components in the secretion from the rectal pheromone glands of the Queensland fruit flies *Dacus tryoni* and *Dacus neohumeralis* (Diptera: Tephritidae). *J. Chem. Ecol.* **1979**, *5*, 795–803. [CrossRef]
32. Kitching, W.; Lewis, J.A.; Fletcher, M.T.; Drew, R.A.I.; Moore, C.J.; Francke, W. Spiroacetals in rectal gland secretions of Australasian fruit fly species. *J. Chem. Soc. Chem. Commun.* **1986**, *11*, 853. [CrossRef]
33. Kitching, W.; Lewis, J.A.; Perkins, M.V.; Drew, R.; Moore, C.J.; Schurig, V.; Koenig, W.A.; Francke, W. Chemistry of fruit flies. Composition of the rectal gland secretion of (male) *Dacus cucumis* (cucumber fly) and *Dacus halfordiae*. Characterization of (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. *J. Org. Chem.* **1989**, *54*, 3893–3902. [CrossRef]
34. Fletcher, M.T.; Kitching, W. Chemistry of fruit flies. *Chem. Rev.* **1995**, *95*, 789–828. [CrossRef]
35. Hayes, P.; Fletcher, M.T.; Moore, C.J.; Kitching, W. Synthesis and absolute stereochemistry of a constitutionally new spiroacetal from an insect. *J. Org. Chem.* **2001**, *66*, 2530–2533. [CrossRef] [PubMed]

36. Perkins, M.V.; Fletcher, M.T.; Kitching, W.; Drew, R.A.I.; Moore, C.J. Chemical studies of rectal gland secretions of some species of *Bactrocera dorsalis* complex of fruit flies (diptera: Tephritidae). *J. Chem. Ecol.* **1990**, *16*, 2475–2487. [CrossRef] [PubMed]
37. Ohinata, K.; Jacobson, M.; Kobayashi, R.M.; Chambers, D.L.; Fujimoto, M.S.; Higa, H.H. Oriental fruit fly and melon fly: Biological and chemical studies of smoke produced by males. *J. Environ. Sci. Heal. Part. A Environ. Sci. Eng.* **1982**, *17*, 197–216. [CrossRef]
38. Baker, R.; Herbert, R.H. Isolation and synthesis of 1,7-dioxaspiro[5.5]undecane and 1,7-dioxaspiro[5.5]undecan-3-and -4-ols from the olive fly (*Dacus oleae*). *J. Chem. Soc. Perkin Trans. 1* **1987**, *1123*. [CrossRef]
39. Tokushima, I.; Orankanok, W.; Tan, K.H.; Ono, H.; Nishida, R. Accumulation of phenylpropanoid and sesquiterpenoid volatiles in male rectal pheromonal glands of the guava fruit fly, *Bactrocera correcta*. *J. Chem. Ecol.* **2010**, *36*, 1327–1334. [CrossRef]
40. El-Sayed, A.M. The Pherobase: Database of Pheromones and Semiochemicals. 2011. Available online: http://www.pherobase.com/ (accessed on 20 November 2011).
41. Levi-Zada, A.; Nestel, D.; Fefer, D.; Nemni-Lavy, E.; Deloya-Kahane, I.; David, M. Analyzing diurnal and age-related pheromone emission of the olive fruit fly, *Bactrocera oleae* by Sequential SPME-GCMS Analysis. *J. Chem. Ecol.* **2012**, *38*, 1036–1041.
42. Haniotakis, G.E.; Mazomenos, B.E.; Tumlinson, J.H. A sex attractant of the olive fruit fly, *Dacus oleae* and its biological activity under laboratory and field conditions. *Èntomol. Exp. Appl.* **1977**, *21*, 81–87. [CrossRef]
43. Benelli, G.; Bonsignori, G.; Stefanini, C.; Raspi, A.; Canale, A. The production of female sex pheromone in *Bactrocera oleae* (Rossi) young males does not influence their mating chances. *Èntomol. Sci.* **2012**, *16*, 47–53. [CrossRef]
44. Baker, R.; Herbert, R.H.; Lomer, R.A. Chemical components of the rectal gland secretions of male *Dacus cucurbitae*, the melon fly. *Cell. Mol. Life Sci.* **1982**, *38*, 232–233. [CrossRef]
45. Baker, R.; Bacon, A.J. The identification of spiroacetals in the volatile secretions of two species of fruit fly (*Dacus dorsalis*, *Dacus curcurbitae*). *Cell. Mol. Life Sci.* **1985**, *41*, 1484–1485. [CrossRef]
46. Nishida, R.; Tan, K.H.; Serit, M.; Lajis, N.H.; Sukari, A.M.; Takahashi, S.; Fukami, H. Accumulation of phenylpropanoids in the rectal glands of males of the Oriental fruit fly, *Dacus dorsalis*. *Cell. Mol. Life Sci.* **1988**, *44*, 534–536. [CrossRef]
47. Nishida, R.; Tan, K.H.; Fukami, H. Cis-3,4-dimethoxycinnamyl alcohol from the rectal glands of male oriental fruit fly, *Dacus dorsalis*. *Chem. Express* **1988**, *3*, 207–210.
48. Witzgall, P.; Kirsch, P.; Cork, A. Sex pheromones and their impact on pest management. *J. Chem. Ecol.* **2010**, *36*, 80–100. [CrossRef]
49. Canale, A.; Benelli, G.; Germinara, G.S.; Fusini, G.; Romano, D.; Rapalini, F.; Desneux, N.; Rotundo, G.; Raspi, A.; Carpita, A. Behavioural and electrophysiological responses to overlooked female pheromone components in the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae). *Chemoecology* **2014**, *25*, 147–157. [CrossRef]
50. Biasazin, T.D.; Chernet, H.T.; Herrera, S.L.; Bengtsson, M.; Karlsson, M.F.; Lemmen-Lechelt, J.K.; Dekker, T. Detection of volatile constituents from food lures by tephritid fruit flies. *Insects* **2018**, *9*, 119. [CrossRef]
51. Zhang, X.; Wei, C.; Miao, J.; Zhang, X.; Wei, B.; Dong, W.-X.; Xiao, C. Chemical compounds from female and male rectal pheromone glands of the guava fruit fly, *Bactrocera correcta*. *Insects* **2019**, *10*, 78. [CrossRef]
52. Steiner, L.F.; Mitchell, S. Tephritid Fruit Flies. In *Insect Colonization and Mass Production*; Academic Press: Waltham, MA, USA, 1966; pp. 555–583.
53. Pérez, J.; Park, S.J.; Taylor, P. Domestication modifies the volatile emissions produced by male Queensland fruit flies during sexual advertisement. *Sci. Rep.* **2018**, *8*, 16503. [CrossRef]
54. Kitching, W.; Lewis, J.A.; Fletcher, M.T.; De Voss, J.J.; Drew, R.A.I.; Moore, C.J. Spiroacetals from dienones and hydroxyenones by mercury(II) cyclisation. *J. Chem. Soc. Chem. Commun.* **1986**, 855–856. [CrossRef]
55. El-Sayed, A.M.; Byers, J.A.; Manning, L.M.; Jürgens, A.; Mitchell, V.J.; Suckling, D.M. Floral scent of Canada thistle and its potential as a generic insect attractant. *J. Econ. Entomol.* **2008**, *101*, 720–727. [CrossRef] [PubMed]
56. Booth, Y.K.; Hayes, P.Y.; Moore, C.J.; Lambert, L.K.; Kitching, W.; De Voss, J.J. Synthesis and absolute configuration of a constitutionally-new [5.6] spiroacetal from *B. tryoni* (Queensland fruit fly). *Org. Biomol. Chem.* **2007**, *5*, 1111. [CrossRef] [PubMed]

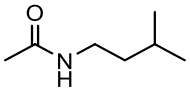
57. Francke, W.; Kitching, W. Spiroacetals in Insects. *Curr. Org. Chem.* **2001**, *5*, 233–251. [CrossRef]
58. El-Sayed, A.; Venkatesham, U.; Unelius, C.R.; Sporle, A.; Pérez, J.; Taylor, P.W.; Suckling, D.M. Chemical composition of the rectal gland and volatiles released by female Queensland Fruit Fly, *Bactrocera tryoni* (Diptera: Tephritidae). *Environ. Èntomol.* **2019**, *48*, 807–814. [CrossRef] [PubMed]
59. Fletcher, M.T.; Wells, J.A.; Jacobs, M.F.; Krohn, S.; Francke, W.; Kitching, W.; Drew, R.A.I.; Moore, C.J. Chemistry of fruit-flies. Spiroacetal-rich secretions in several *Bactrocera* species from the South-West Pacific region. *J. Chem. Soc. Perkin Trans. 1* **1992**, 2827–2831. [CrossRef]
60. Booth, Y.K.; Schwartz, B.; Fletcher, M.T.; Lambert, L.K.; Kitching, W.; De Voss, J.J. A diverse suite of spiroacetals, including a novel branched representative, is released by female *Bactrocera tryoni* (Queensland fruit fly). *Chem. Commun.* **2006**, 3975. [CrossRef]
61. Noushini, S.; Pérez, J.; Park, S.; Holgate, D.; Jamie, I.; Jamie, J.; Taylor, P. Rectal gland chemistry, volatile emissions, and antennal responses of male and female banana fruit fly, *Bactrocera musae*. *Insects* **2019**, *11*, 32. [CrossRef]
62. Symonds, M.R.E.; Moussalli, A.; Elgar, M. The evolution of sex pheromones in an ecologically diverse genus of flies. *Boil. J. Linn. Soc.* **2009**, *97*, 594–603. [CrossRef]
63. Perkins, M.V.; Kitching, W.; Drew, R.A.I.; Moore, C.J.; König, W.A. Chemistry of fruit flies: Composition of the male rectal gland secretions of some species of South-East Asian Dacinae. Re-examination of *Dacus cucurbitae* (melon fly). *J. Chem. Soc. Perkin Trans. 1* **1990**, 1111–1117. [CrossRef]
64. Wu, H.; Hou, C.; Huang, L.-Q.; Yan, F.-S.; Wang, C.-Z. Peripheral coding of sex pheromone blends with reverse ratios in two *Helicoverpa* species. *PLoS ONE* **2013**, *8*, e70078. [CrossRef]
65. Arn, H.; Tóth, M.; Priesner, E. *List of Sex Pheromones of Lepidoptera and Related Attractants*, 2nd ed.; International Organization for Biological Control, West Palearctic Regional Section: Montfavet, France, 1992.
66. Nation, J.L. Biology of pheromone release by male Caribbean fruit flies, *Anastrepha suspensa* (Diptera: Tephritidae). *J. Chem. Ecol.* **1990**, *16*, 553–572. [CrossRef] [PubMed]
67. Baker, R.; Herbert, R.H.; Grant, G.G. Isolation and identification of the sex pheromone of the Mediterranean fruit fly, *Ceratitis capitata* (Wied). *J. Chem. Soc. Chem. Commun.* **1985**, *12*, 824–825.

Sample Availability: Samples of the compounds are not available from the authors.

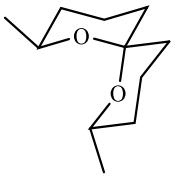
Figure 5. Structure of compounds identified in *Bactrocera frauenfeldi* chemical profile.



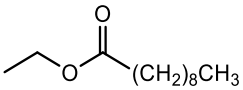
N-(2-Methylbutyl)acetamide



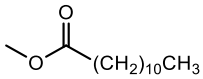
N-(3-methylbutyl)acetamide



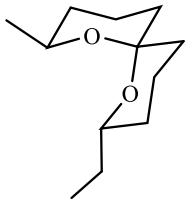
(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane



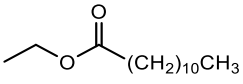
Ethyl caprate



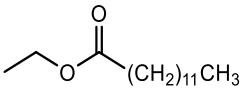
Methyl laurate



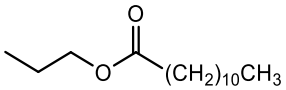
(*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane



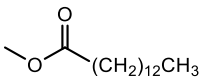
Ethyl laurate



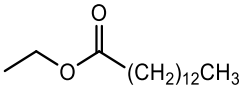
Ethyl tridecanoate



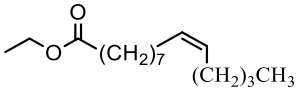
Propyl laurate



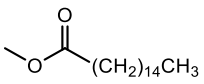
Methyl myristate



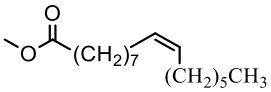
Ethyl myristate



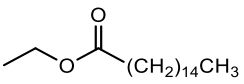
Ethyl myristoleate



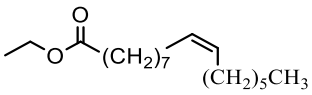
Methyl palmitate



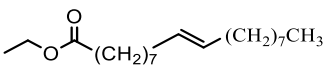
Methyl palmitoleate



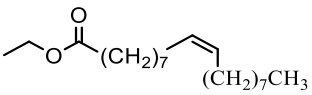
Ethyl palmitate



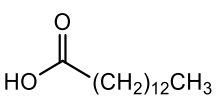
Ethyl palmitoleate



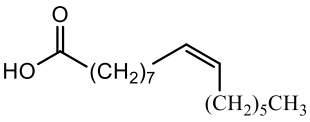
Ethyl elaidate



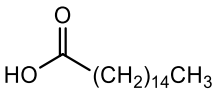
Ethyl oleate



Myristic acid



Palmitoleic acid



Palmitic acid

Chapter Four

Rectal gland exudates and emissions of *Bactrocera bryoniae*: chemical identification, electrophysiological and pheromonal functions

Accepted in Chemoecology

(5/11/2020)

***Bactrocera bryoniae* mating**
photo by author

Author contributions:
All experimental and data analysis were carried out by S. Noushini. S. J. Park helped with Y-tube observations. The manuscript drafted by S. Noushini. All authors read the manuscript and provided critical feedback.

At the begging of this chapter there is a short summery of phylogenetic situation of *Bactrocera bryoniae* and species which are known as close relatives to this species.

At the end of this chapter, after the manuscript figure 4 has been provided summarising structure of all identified compounds in male and female *Bactrocera bryoniae*.

Preamble

Bactrocera bryoniae is a polyphagous pest species belongs to order Diptera and family Tephritidae.[1,2] They are similar to *Bactrocera dorsalis* complex species in having a black scutum and T on the abdomen and a very broad costal band to R_{4+5}.[1] *Bactrocera bryoniae* is also similar to *Bactrocera trivialis* (Drew) except it has a distinct wraparound T pattern on terga III-V and a very broad costal band.[1]

All molecular markers including COI, EIF3L, POP4, RPA2 and DDOSTS2 consistently separate *B. bryoniae* from the abovementioned morphologically similar species.[1,3]

Among the abovementioned species pheromone profile of *B. dorsalis* (Hendel) has been studied. [4–8] This has been discussed in detail in chapter 2 on page 31.

References:

1. Schutze, M. *et al. The Australian Handbook for the Identification of Fruit Flies. Version 3.1*. (Plant Health Australia, 2018). doi:10.1016/j.jasms.2007.01.008
2. Leblanc, L., Tora, E., Drew, R. A. I. & Allwood, A. J. Host plant records for fruit flies (Diptera: Tephritidae: Dacini) in the Pacific Islands. *Proc. Hawaiian Entomol. Soc.* **44**, 11–53 (2013).
3. Schutze, M. *et al.* Fruit fly identification Australia. (2019). Available at: https://fruitflyidentification.org.au/species/bactrocera-bryoniae-2/. (Accessed: 24th August 2020)
4. Shen, J. *et al.* Allyl-2,6-dimethoxyphenol, a female-biased compound, is robustly attractive to conspecific males of *Bactrocera dorsalis* at close range. *Entomol. Exp. Appl.* **167**, 811–819 (2019).
5. Ohinata, K. *et al.* Oriental fruit fly and melon fly: Biological and chemical studies of smoke produced by males. *J. Environ. Sci. Heal. Part A Environ. Sci. Eng.* **17**, 197–216 (1982).
6. Nishida, R. *et al.* Accumulation of phenylpropanoids in the rectal glands of males of the Oriental fruit fly, *Dacus dorsalis*. *Experientia* **44**, 534–536 (1988).
7. Baker, R. & Bacon, A. J. The identification of spiroacetals in the volatile secretions of two species of fruit fly (*Dacus dorsalis, Dacus curcurbitae*). *Experientia* **41**,

1484–1485 (1985).

8. Perkins, M. V., Fletcher, M. T., Kitching, W., Drew, R. A. I. & Moore, C. J. Chemical studies of rectal gland secretions of some species of *Bactrocera dorsalis* complex of fruit flies (diptera: Tephritidae). *J. Chem. Ecol.* **16**, 2475–2487 (1990).

Rectal Gland Exudates and Emissions of *Bactrocera bryoniae*: Chemical Identification, Electrophysiological and Pheromonal Functions

Saeedeh Noushini[1,3*], Soo Jean Park[2,3], Ian Jamie[1,3], Joanne Jamie[1], Phillip Taylor[2,3]

[1] *Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia.*

[2] *Applied BioSciences, Macquarie University, Sydney, NSW 2109, Australia.*

[3] *Australian Research Council Industrial Transformation Training Centre for Fruit Fly Biosecurity Innovation, Macquarie University, Sydney, NSW 2109, Australia.*

Corresponding author:
Saeedeh Noushini, Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia
E-mail:
ORCID: 0000-0001-5558-1656

Abstract

Bactrocera bryoniae is a polyphagous and economically significant fruit fly found in Indonesia, Papua New Guinea and Australia. To understand chemical-mediated sexual communication, and the potential for novel pheromone-based attractants for monitoring and mass-trapping of *B. bryoniae*, rectal gland exudates and emissions from sexually mature males and females were investigated. Gas chromatography-mass spectrometry showed that male rectal glands contained six compounds, of which 1,7-dioxaspiro[5,5]undecane elicited electroantennographic (EAD) and electropalpographic (EPD) responses in both sexes, ethyl 3-acetoxybutanoate elicited EPD responses in both sexes, *N*-(3-methylbutyl)acetamide elicited EAD response from males and 4-hydroxy-1,7-dioxaspiro[5.5]undecane elicited EAD responses in males and females and EPD responses in females. Female rectal glands contained 23 compounds with the esters ethyl laurate and ethyl myristate as major components. Amongst the female rectal gland constituents, ethyl laurate, ethyl myristate and ethyl palmitate elicited EAD responses in males and females, *N*-(3-methylbutyl)acetamide elicited EAD responses in males only, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane elicited EAD responses in males and EPD responses in females, and 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, (*E*,*E*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane, (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane and ethyl caprate elicited EPD responses in females only. Y-tube bioassays indicated that male rectal gland extracts and headspace volatiles attracted females and males, while female rectal gland extracts and headspace volatiles only attracted males. The results suggest that ethyl 3-acetoxybutanoate, 1,7-dioxaspiro[5,5]undecane and 4-hydroxy-1,7-dioxaspiro[5.5]undecane may be components of male-produced sex pheromone in *B. bryoniae* while (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, *N*-(3-methylbutyl)acetamide, ethyl laurate, ethyl myristate and ethyl palmitate may be components of female-produced sex pheromone. Ethyl 3-acetoxybutanoate, *N*-(3-methylbutyl)acetamide, 1,7-dioxaspiro[5,5]undecane and 4-hydroxy-1,7-dioxaspiro[5.5]undecane may be components of male aggregation pheromone. These findings contribute to the understanding of pheromone communication in *B. bryoniae* and provide a foundation for developing pheromone-based monitoring and control methods.

Keywords: Fruit Fly, Pheromone, Attractants, Rectal Gland, Olfaction, GC–MS, EAG, EPG

Introduction

Many *Bactrocera* fruit flies (Tephritidae) are economically important pests of fruits and vegetables, and some pose serious quarantine risks with potential to cause major disruptions in the international trade of fresh fruits and vegetables (Vijaysegaran 1997; Hickey et al. 2010; Clarke et al. 2011; Benelli et al. 2014; Dominiak and Mapson 2017). Diverse methods have been used in fruit fly control programs, including cover sprays, sterile insect technique (SIT), bait sprays, male annihilation technique (MAT), and biological control (Clarke et al. 2011; Ero et al. 2011; Zamek et al. 2012; Lauzon and Potter 2012; Dominiak and Ekman 2013). Fruit fly management relies on effective monitoring tools, as well as attractants for lure-and-kill methods. Sex and aggregation pheromones are central to the mating systems of many *Bactrocera* species and have been considered as potential attractants for management of some species. For example, 1,7-dioxaspiro[5,5]undecane, a female-produced sex pheromone, has been used for monitoring and mass trapping of *Bactrocera oleae* (Rossi) (Haniotakis et al. 1977).

The rectal gland is well known as a sex pheromone-secreting organ in fruit flies (Fletcher 1968, 1969; Piccardi 1980; Perkins et al. 1990a; Wee and Tan 2005; Tokushima et al. 2010). The volatile compounds emitted during calling and courtship, especially by males, are known as short and long range attractants for the opposite sex in some species (Nation 1972; Perkins 1990; Sivinski et al. 2000; Cruz-López et al. 2015). Male-produced volatiles are also known to function as mating aggregation pheromones in some species (Sivinski and Calkins 1986; Nishida et al. 1988b; Hendrichs et al. 2002). Among the more than 450 species of *Bactrocera*, the sex and aggregation pheromones of only a few pest species have been investigated (Doorenweerd et al. 2018). Some of the first investigations described production and release of sex pheromones by male *Bactrocera tryoni* (Froggatt) (Fletcher 1968, 1969; Bellas and Fletcher 1979). Males are thought to be the main pheromone producers in most fruit flies (Heath et al. 2000; El-Sayed 2019), and studies of volatile compounds in *Bactrocera* have mainly focused on males (Bellas and Fletcher 1979; Kitching et al. 1986, 1989; Perkins et al. 1990b; Krohn et al. 1991; Hayes et al. 2001; Tokushima et al. 2010). However, there are at least three examples of fruit fly species in which females are also known to produce sex pheromones. In the olive fruit fly, *Bactrocera oleae,* sex pheromones are mainly produced by females (Haniotakis 1974;

Mazomenos and Haniotakis 1981, 1985) while males produce a compound, (*Z*)-9-tricosene, that only acts as a close range attractant for females (Carpita et al. 2012; Canale et al. 2013). Similarly, in the melon fly, *Zeugodacus cucurbitae* (Coquillett) and the oriental fruit fly, *Bactrocera dorsalis* (Hendel), both male and female volatile emissions have been reported to attract the opposite sex (Baker et al. 1982a; Baker and Bacon 1985; Nishida et al. 1988a, b).

Bactrocera bryoniae (Tryon) (Diptera: Tephritidae) is an economically important pest species in Indonesia (Papua, formerly part of Irian Jaya), Papua New Guinea (every province except Bougainville and Manus) and Australia (South East Queensland, Central Queensland, Northern Queensland, Northern Western Australia, Northern Territory, east coast south to Sydney, New South Wales, and the Torres Strait Islands) (Drew and Romig 2013; Leblanc et al. 2013; Schutze et al. 2018). *Bactrocera bryoniae* is polyphagous, having been reported to infest nine fruits and vegetables from five families including Cucurbitaceae, Loganiaceae, Musaceae, Passifloraceae and Solanaceae. Chilli pepper is the main commercial host of *B. bryoniae* (Leblanc et al. 2013). Chemical communication of *B. bryoniae* has not been investigated previously. The present study identifies and characterizes rectal gland secretions and volatiles released by both males and females, and evaluates the attractiveness of rectal gland volatiles to the opposite and same sex. This information about the chemistry of *B. bryoniae* not only provides insight into the functional role of rectal gland volatiles but also the potential application of *B. bryoniae* volatiles as attractants for monitoring and control.

Methods and Material

Bactrocera bryoniae rearing

A laboratory-reared population of *B. bryoniae* (G68) was obtained from the Queensland Department of Agriculture and Fisheries (Cairns, Queensland). Approximately 500 pupae were placed in a 47.5 × 47.5 × 47.5 cm fine mesh cage (Megaview Bugdorm 4S4545, Taiwan) for emergence and kept in a controlled-environment room at 25 ± 0.5 °C, 65 ± 5% relative humidity (RH) and 11.5:0.5:11.5:0.5 hour light/dusk/dark/dawn photoperiod at Macquarie University. Adult flies were provided sugar and yeast hydrolysate (MP Biomedicals LLC) as food in separate dishes, and were provided tap water through a

soaked sponge. Flies were separated by sex within 3 days of emergence and transferred to 12.5 L clear plastic cages that had two 10 cm diameter mesh-covered openings for ventilation (180 flies per cage). No mating was observed before separating the flies. All cages were maintained with the same diet and environmental conditions described above. All experiments used 13-18 days old virgin flies.

Rectal gland extraction

Gland extracts were obtained from sexually mature males and females of *B. bryoniae*. Handling of the flies and the gland extractions followed the procedure of Kitching et al. (1989). Flies were chilled on dry ice to kill them. The abdomen was gently squeezed with tweezers such that the glands protruded slightly. The glands were then gently pulled out with tweezers, and the secretory sac separated. Glands were carefully placed in a 1.1 mL tear-drop vial in dry ice. Once 10 glands were collected, the vials were removed from the dry ice and the contents were extracted into 100 μL of *n*-hexane (HPLC grade, Sigma-Aldrich) by saturating the glands with solvent and leaving them to stand at room temperature for 10 minutes. Ten replicates per sex were collected using 10 glands per replicate. Samples were stored at –20 °C until analysed.

Collection of airborne volatiles

Headspace collections were conducted during the dusk period in the controlled-environment room. This time of the day was selected based on our observations of male calling behaviour and mating. Ten sexually mature males or females were separately placed into a glass chamber (150 mm long × 40 mm ID) 30 minutes before dusk and charcoal-filtered air at a flow rate of 0.5 L per minute was drawn over the flies for a period of one hour, starting from beginning of dusk. Released volatiles were adsorbed onto 50 mg of Tenax adsorbent (Scientific Instrument Services, Inc, Tenax-GR Mesh 60/80) packed into glass cartridges (6 mm ID × 50 mm) and fitted with glass wool plugs. Volatiles were subsequently extracted from the absorbent with 1 mL of *n*-hexane. Samples were stored at –20 °C until analysis. Nine replicates per sex were collected. To identify any possible contaminants, an air control sample comprising an empty glass chamber, was run and analyzed along with every volatile collection. Tenax traps were conditioned at 200 °C for three hours under a nitrogen stream (75 mL/min) prior to each headspace collection. The glass chambers were washed with 5% Extran aqueous solution, rinsed with hot tap water,

and heated at 200 °C for 18 hours. Activated charcoal filters were thermally conditioned by heating them at 200 °C for 18 hours prior to each headspace collection (El-Sayed et al. 2008).

Analysis of rectal gland extracts and headspace collections

Mass spectra were recorded by gas chromatography-mass spectrometry (GC-MS) on a Shimadzu GCMS-TQ8040 instrument equipped with a split/splitless injector and SH Rtx-5MS (30 m × 0.25 mm ID × 0.25 μm film thickness) fused silica capillary column as the stationary phase. The carrier gas was helium (99.999%) (BOC, North Ryde, NSW, Australia) at a constant flow rate of 1 mL/min. The injection port temperature was 270 °C. The initial column temperature was 40 °C, held for 1 minute, followed by an increase to 250 °C at a rate of 10 °C/min. The final temperature was held for 3 minutes. The ionisation method was electron impact at a voltage of 70 eV, and the spectra were obtained with scanning from 45 to 500 *m/z*. The ion source and transfer line temperatures were 200 °C and 290 °C, respectively. The relative percentage of each compound in the rectal gland blend or headspace was obtained by dividing its individual peak area by the total peak area and multiplying the result by 100. All compounds were identified through comparison with retention times and mass spectra of authentic samples, where available, or NIST library (NIST17-1, NIST17-2 and NIST17s) and mass spectra published in the literature, where authentic samples were not available.

Electrophysiology

Electrophysiological recordings were performed using antennae and maxillary palps of sexually mature virgin females and males using the rectal gland extracts of both sexes as stimuli. Male rectal gland extracts and female rectal gland extracts were separately subjected to both female and male *B. bryoniae* to detect active compounds.

The responses were evaluated by gas chromatography-electroantennogram detection (GC-EAD) or gas chromatography-electropalpogram detection (GC-EPD). The system comprised of an Agilent 7890B gas chromatograph equipped with an SH-Rtx-35 (30 m × 0.25 mm ID × 0.25 μm film thickness) fused silica capillary and FID detector. The carrier gas was hydrogen (99.999% pure) with a constant flow of 2.5 mL/min. The injection port temperature was 270 °C. The initial temperature of the column was 50 °C,

held for 1 minute, ramped to 250 °C at a rate of 10 °C/min, and held for 3 minutes. The detector temperature was 290 °C. The effluent of the column was mixed with 30 mL/min make-up nitrogen gas and split at 1:1.5 (*v/v*) ratio, with one part going to the internal FID and the other through a heated transfer line (TC-02, Syntech, Hilversum, The Netherlands), kept at a constant temperature of 200 °C.

The head of a male or female fly was mounted between two silver wires with capillary electrodes filled with phosphate-buffer saline and electrically conductive gel (Spectra 360). In both the GC-EAD and GC-EPD experiments, the electrode with phosphate-buffered saline was placed at the tip of an antenna or a maxillary palp as the recording electrode and the other electrode, filled with electrically conductive gel, at the back of the head as the reference electrode. The mounted heads were subjected to a charcoal-filtered and humidified air-flow (400 mL/min) controlled by a flow controller (Syntech Stimulus Controller CS-55, Syntech, Hilversum, The Netherlands). All signals were captured and processed with a data acquisition controller (IDAC-4, Syntech, Hilversum, The Netherlands) and analysed using GC-EAD 2014 software version 1.2.5. Before injection of a sample, the antenna or maxillary palp were stimulated with 1-hexanol to check sensitivity, then 1 µL of the rectal gland extract from the opposite sex as well as the same sex was injected. Nine GC-EAD and nine GC-EPD recordings per sex were obtained. Responses were considered genuine if present in at least six of the nine replicates collected. The identity of each compound eliciting an electrophysiological response was confirmed by comparing retention time with that of the GC-MS chromatograms.

Behavioural assays

The response of sexually mature virgin (13-18 days old) *B. bryoniae* males and females toward volatiles released from the rectal glands of the same and opposite sex was evaluated using Y-tube olfactometers. The Y-tube olfactometer comprised of a clear acrylic Y shaped tube with one central arm (6.5 cm × 4.5 cm × 5 cm) in which the release chamber (5 cm × 5 cm × 5 cm) was located, and two upwind lateral arms (12.5 cm × 4.5 cm × 5 cm), each of them connected to a rectangular chamber (7.5 cm × 5 cm × 5 cm) (see Online Resource). The Y-tube olfactometer was positioned horizontally on a white table and a humidified and charcoal-filtered air stream was passed through the Y-tube at a flow rate of 140 ± 5 mL/min. For the response of flies toward the rectal gland volatiles, the stimulus cartridge was prepared by crushing 15 rectal glands of male or female *B. bryoniae* on a 1

210 cm^2 section of filter paper (Advantec, Japan) inserted in a glass Pasteur pipette (145 mm
211 long). The control cartridge was prepared using 1 cm^2 filter paper inserted in the same type
212 of glass Pasteur pipette. One cartridge of each type was fitted to one of the Y-tube upwind
213 arms using Tygon tubing (Tygon® formula E-3603, Sigma-Aldrich). For the response of
214 flies towards the natural blend of volatile compounds released from live flies, four *B.*
215 *bryoniae* males or females were separately placed into a glass chamber (150 mm long × 40
216 mm ID) 30 minutes before experiments started at dusk in a controlled-environment room,
217 under the same conditions the flies had been maintained in. The control unit was prepared
218 using an empty glass chamber. One chamber of each type was fitted to one of the Y-tube
219 upwind arms using 12 cm of Tygon tubing. For both experiments, an individual fly was
220 placed in the release chamber to acclimatize 30 minutes before dusk. Every trial lasted 30
221 minutes. Once the two cartridges (stimulus and control) or chambers (flies and control)
222 were connected to the upwind arms, the system was equilibrated for two minutes and then
223 the barriers of the two upwind arms and the release chamber were removed. Behaviours of
224 flies were observed every 5 minutes and the arm in which the fly was located was
225 recorded. Overall, six observations were made until dark and the arm in which a fly spent
226 most time was recorded as a final choice. Flies that did not make any choice, *i.e.*, remained
227 in the release chamber and did not reach one of the two upwind arms, were not counted.
228 For the rectal gland attraction experiments, at least 56 replicates/treatment and for
229 headspace attraction 30-35 replicates/treatment were carried out (non-responsive flies were
230 not counted). To compare the number of flies choosing the stimulus over the control, a
231 binomial test was used ($\alpha = 0.05$).
232
233 The position (left or right) of the stimulus and the control was alternated every trial
234 to counter potential positional effects. Each fly was tested only once and fresh rectal glands
235 were used each day. Before each replicate, the Y-tube olfactometer and tubes were washed
236 with 5% Extran aqueous solution, rinsed with hot tap water and air-dried. The glass
237 chambers were washed with 5% Extran aqueous solution, rinsed with hot tap water, and
238 heated at 200 °C for 3 hours.
239
240 Chemicals
241
242 The following chemicals were purchased from Sigma-Aldrich (St Louis, MO, US), Alfa-
243 Aesar (Ward Hill, MA, US) and Chem-Supply (Bedford St, Gillman, SA), with the purities
244 noted in parentheses, and were used without further purification: ethyl 3-acetoxybutanoate

(98%), ethyl caprate (98%), methyl laurate (98%), ethyl laurate (98%), ethyl tridecanoate (99%), methyl myristate (99%), ethyl myristate (98%), methyl palmitate (99%), ethyl palmitate (99%), ethyl oleate (98%), lauric acid (98%), palmitoleic acid (98.5%), palmitic acid (98%), oleic acid (99%) and 1,7-dioxaspiro[5,5]undecane (97%). Propyl laurate, ethyl palmitoleate, *N*-(2-methylbutyl)acetamide, *N*-(3-methylbutyl)acetamide, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and (*E*,*E*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane were synthesised (see Online Resource for synthesis details).

Results

Rectal gland and released volatile components of *B. bryoniae*

GC-MS analyses identified a total of 26 compounds that were produced and released by male and female *B. bryoniae* (Table 1). All compounds were tentatively identified based on their mass spectral fragmentation patterns. Identities of compounds **1**, **3**-**7**, **12**-**14** and **15**-**26** were confirmed by comparison of GC retention times and mass spectral fragmentation patterns with authentic samples. These were identified as ethyl 3-acetoxybutanoate (**1**), 1,7-dioxaspiro[5,5]undecane (**3**), (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**4**), (*E*,*E*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**5**), *N*-(2-methylbutyl)acetamide (**6**), *N*-(3-methylbutyl)acetamide (**7**), ethyl caprate (**12**), methyl laurate (**13**), lauric acid (**14**), ethyl laurate (**15**), ethyl tridecanoate (**16**), propyl laurate (**17**), methyl myristate (**18**), ethyl myristate (**19**), ethyl myristate (**20**), palmitoleic acid (**21**), palmitic acid (**22**), ethyl palmitoleate (**23**), ethyl palmitate (**24**), oleic acid (**25**) and ethyl oleate (**26**). Five compounds, 2,7-dimethyl-1,6-dioxaspiro[4.5]decane (**2**), (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**8**), (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**9**), (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**10**) and 4-hydroxy-1,7-dioxaspiro[5.5]undecane (**11**), which were not commercially available or synthesized, were tentatively identified based on the literature mass spectral fragmentation patterns (Baker et al. 1982b; Perkins 1990; Fletcher et al. 1992; Booth et al. 2006, 2007; Schwartz et al. 2008; Mitchell et al. 2017). For both males and females, all compounds that were detected in the headspace were also detected in the rectal gland extracts.

Six compounds were identified in male rectal glands, including ester **1**, spiroacetals **3** and **4** and **11**, and the amides **6** and **7**. Of these, all compounds except the spiroacetal

(*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**4**) were present in the male headspace samples. The most abundant compound in male gland extracts and headspace samples, being found in a similar proportion (~85%), was spiroacetal **4**. Females produced a more complex blend than males. Of the 23 compounds that were found in females, eight compounds were detected only in rectal gland extracts (Table 1). This included 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, *N*-(2-methylbutyl)acetamide, lauric acid, palmitoleic acid, palmitic acid, oleic acid, methyl palmitate and ethyl oleate. The main compounds produced and released by females were ethyl laurate (~40% and 67%, respectively) and ethyl myristate (~32% and 21%, respectively). More compounds were detected in female rectal glands than in female headspace collections. Compounds found in both rectal gland extracts and headspace include spiroacetals **4**, **5**, **8**, **9** and **10**, amide **7** and esters **12**, **13**, **15**, **16**, **17**, **18**, **19**, **23** and **24**.

Electrophysiological responses of antennae

Males and females shared the EAD response to five compounds; 1,7-dioxaspiro[5,5]undecane (**3**) and 4-hydroxy-1,7-dioxaspiro[5.5]undecane (**11**) from male rectal glands (Fig. 1) and ethyl laurate (**15**), ethyl myristate (**19**) and ethyl palmitate (**24**) from female rectal glands (Fig. 2). Other compounds only elicited responses in the antennae of one sex; *N*-(3-methylbutyl)acetamide (**7**) from male and female rectal glands elicited EAD responses in males and (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**4**) from female rectal glands elicited EAD responses in males. There were no compounds that only females responded to in the rectal glands of either males or females.

Electrophysiological responses of maxillary palps

Males and females shared EPD responses to two compounds from the rectal glands of males (Fig. 1); ethyl 3-acetoxybutanoate (**1**) and 1,7-dioxaspiro[5,5]undecane (**3**). These are the only compounds that elicited EPD responses in males from male rectal glands. 4-Hydroxy-1,7-dioxaspiro[5.5]undecane (**11**) from male rectal gland extracts elicited EPD response only in females.

There were no compounds in female rectal glands that elicited EPD response in males. In contrast, seven compounds in female rectal glands elicited EPD responses in females; 2,7-dimethyl-1,6-dioxaspiro[4.5]decane (**2**), (*E*,*E*)-2,8-dimethyl-1,7-

dioxaspiro[5.5]undecane (**4**), (*E*,*E*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**5**), (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**8**), (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**9**), (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**10**) and ethyl caprate (**12**). The ten EPD active compounds in females belong to two functional groups; eight spiroacetals and two esters.

Behavioural assays

Sexually mature males were attracted to emissions from female rectal glands ($P = 0.001$), while females did not show any preference for female rectal gland emissions over the control ($P > 0.05$). The percentage of non-responders was 5.3% and 9.2% when males and females, respectively, were presented with emissions from female rectal glands. Both males and females were attracted to male rectal gland emissions ($P = 0.03$ and $P < 0.001$, respectively). The percentage of non-responders was 9.3% and 6.6% when males and females, respectively, were presented with male rectal gland emissions (Fig. 3A).

The behavioural responses of sexually mature male and female *B. bryoniae* to headspace volatiles from live conspecific males and females were very similar to responses to rectal gland emissions (see above). Females were attracted to the volatiles released by males ($P < 0.001$), but were not attracted to the volatiles released by females ($P > 0.05$). Males were attracted to the volatiles released by males and females ($P = 0.005$ and $P = 0.04$, respectively) (Fig. 3B).

Discussion

We identified the composition of rectal gland secretions in male and female *B. bryoniae* and evaluated electrophysiological and behavioural responses of both sexes to the volatiles released by males and females of this species for the first time. Chemical analyses revealed that female *B. bryoniae* produced and released a more complex blend than males. The volatiles from female *B. bryoniae* consisted of two aliphatic amides, six spiroacetals, eleven saturated/unsaturated esters and four fatty acids of which all except *N*-(2-methylbutyl)acetamide, *N*-(3-methylbutyl)acetamide and (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane are female specific.

The aliphatic amides found in this study, *N-(*2-methylbutyl)acetamide and *N*-(3-methylbutyl)acetamide, have been reported as part of rectal gland compositions of other species, including *B. tryoni* (Bellas and Fletcher 1979; El-Sayed et al. 2019), *B. dorsalis, Z. cucurbitae* (Baker and Bacon 1985), *Zeugodacus cucumis* (French) (Fletcher and Kitching 1995) and *Bactrocera zonata* (Saunders) (Levi-zada et al. 2020). Different isomers of the spiroacetals were also identified in rectal glands of other *Bactrocera* and species from the closely related genus *Zeugodacus*. For instance, 2,7-dimethyl-1,6-dioxaspiro[4.5]decane has been previously reported in the *n*-pentane extract of female *B. tryoni* (Booth et al. 2006). Isomers of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane have been reported in the previous investigation of rectal glands of *B. dorsalis* (*E*,*E* isomer), *Bactrocera nigrotibialis* (Perkins) (*E*,*E* isomer), *Bactrocera albistrigata* (Meijere) (*E*,*E* isomer), *Bactrocera jarvisi* (Tryon) (*E*,*E* isomer), *Bactrocera kirki* (Froggatt) (*E*,*E* isomer), *Bactrocera kraussi* (Hardy) (*E*,*E* isomer), *Z. cucumis* (*E*,*E*, *E*,*Z*, *Z*,*Z* isomers), *B. tryoni* (*E*,*E* isomer) and *B. musae* (Tryon) (*E*,*E* isomer) (Baker and Bacon 1985; Kitching et al. 1989; Fletcher et al. 1992; Fletcher and Kitching 1995; Booth et al. 2006; El-Sayed et al. 2019; Noushini et al. 2020). 2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane has been reported in the rectal gland of male *Bactrocera kraussi* (Hardy) (*E*,*E* or *E*,*Z* isomer, unidentified) and female *B. tryoni* (*E*,*Z* isomer) (Fletcher et al. 1992; Booth et al. 2006). (*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane has been previously reported as part of male emanations of *B. nigrotibialis*, *Bactrocera halfordiae* (Tryon), *B. dorsalis*, *B. kirki*, *Bactrocera latifrons* (Hendel) and *Bactrocera occipitalis* (Bezzi) as well as female of *B. tryoni* and *B. musae* (Perkins et al. 1990b; Symonds et al. 2009; Benelli et al. 2014; El-Sayed et al. 2019; Noushini et al. 2020). 2-Propyl-8-methyl-1,7-dioxaspiro[5.5]undecane has been identified in *n*-pentane extracts of the abdomen of female *B. tryoni* (Booth et al. 2006). With respect to fatty acid esters, ethyl caprate (**12**), methyl laurate (**13**), ethyl laurate (**15**), methyl myristate (**18**), ethyl myristate (**19**), methyl palmitate (**20**), ethyl palmitoleate (**23**), ethyl palmitate (**24**) and ethyl oleate (**26**) have also been found in *B. oleae, B. tryoni* and *B. correcta* female rectal gland extracts (Canale et al. 2015; El-Sayed et al. 2019; Zhang et al. 2019). Of these, ethyl caprate and methyl palmitate have been reported as attractants for both *B. oleae* males and females (Canale et al. 2015).

Mature male *B. bryoniae* produced a simple blend of six volatiles (Table 1) with 1,7-dioxaspiro[5,5]undecane (**3**) as the major component and being detected by both antenna and maxillary palps of both males and females. The headspace samples contained five of the same components, **1**, **3**, **6**, **7** and **11**, with the same dominant compound,

1,7-dioxaspiro[5,5]undecane, which has been identified as the major component of the female sex pheromone of *B. oleae* (Mazomenos and Haniotakis 1981). Although young male *B. oleae* also produce this spiroacetal in their rectal gland as a male aggregation pheromone, 1,7-dioxaspiro[5,5]undecane does not attract female *B. oleae* (Haniotakis et al. 1986). This spiroacetal has also been reported as the major volatile component of *Bactrocera cacuminata* (Hering) males (Kitching et al. 1986). The male specific compound, ethyl 3-acetoxybutanoate, elicited electrophysiological responses in palps of both males and females. Ethyl 3-acetoxybutanoate has not been identified in rectal glands or emissions of other tephritids but is found in pineapple (Zheng et al. 2012). 4-Hydroxy-1,7-dioxaspiro[5.5]undecane (**11**) was unique to males and elicited EAD responses in males and females as well as EPD responses in females. 4-Hydroxy-1,7-dioxaspiro[5.5]undecane has also been isolated from rectal glands of female *B. oleae* (Baker et al. 1982b).

Although males of many tephritid fruit flies have been reported as sex pheromone producers (Nation 1972, 1990; Baker et al. 1985; Fletcher and Kitching 1995), in some species such as *Z. cucurbitae* and *B. dorsalis*, both male and female volatile emissions have been reported to attract the opposite sex (Baker et al. 1982a; Baker and Bacon 1985; Nishida et al. 1988a, b). Our Y-maze olfactometer results demonstrated that males and females of *B. bryoniae* are attracted to the rectal gland emissions of opposite sex conspecifics. This suggests that rectal gland secretions of both sexes may function as mate-attracting sex pheromones in this species. We showed that males are attracted to male rectal gland odour whereas females did not exhibit a significant preference for female rectal gland odour. This suggests rectal gland secretions may play a role in aggregation of males only. In some fruit fly species, male rectal gland secretions function both as sex pheromones and as aggregation pheromones. For example, in *B. dorsalis* volatiles produced by males are known to act as aggregation pheromones for males as well as attractant for females (Nishida et al. 1988b). Similarly in *Anastrepha suspensa* (Loew) male volatiles attract both males and females (Perdomo et al. 1976).

Our GC-EAD and GC-EPD results demonstrate differences in the detection of compounds between antennae and maxillary palps, suggesting different olfactory function of antenna from that of palps. Independent olfactory roles of maxillary palps and antenna have been reported in other tephritid fruit flies. For example, in male *B. tryoni* and *Bactrocera depressa* (Shiraki) the palps exhibit stronger electrophysiological responses to

cuelure than the antennae (Verschut et al. 2018; Oh et al. 2019). In combination with our findings from Y-tube olfaction studies, the electrophysiological results suggest that ethyl 3-acetoxybutanoate, 1,7-dioxaspiro[5,5]undecane and 4-hydroxy-1,7-dioxaspiro[5.5]undecane are likely components of male-produced sex pheromone in *B. bryoniae* and ethyl 3-acetoxybutanoate, *N*-(3-methylbutyl)acetamide, 1,7-dioxaspiro[5,5]undecane and 4-hydroxy-1,7-dioxaspiro[5.5]undecane are likely components of male aggregation pheromone. Our findings also suggest (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, *N*-(3-methylbutyl)acetamide, ethyl laurate, ethyl myristate and ethyl palmitate as components of female-produced sex pheromone in *B. bryoniae*. Further behavioural studies are needed to clarify the function of each these compounds.

Acknowledgements

We are grateful to the Queensland Department of Agriculture and Fisheries (QDAF), especially Sybilla Oczkowicz for providing *B. bryoniae* for this research.

Declarations

Funding: This research was funded by Australian Research Council Industrial Transformation Training Centre (ITTC) for Fruit Fly Biosecurity Innovation (Project IC50100026), funded by the Australian Government

Conflict of interest: The authors declare that they have no conflict of interest.

Availability of data and material: The authors confirm that data supporting the findings of this study are available within the manuscript and Online Resource.

Authors' contributions: S.N., P.T., J.J., I.J., designed the experiment. S.N. and S.J.P. performed the experiments. S.N. analysed the data and wrote the original draft. P.T., J.J., I.J. and S.J.P reviewed and edited the manuscript. All authors read and approved the final manuscript.

References

Baker R, Bacon AJ (1985) The identification of spiroacetals in the volatile secretions of two species of fruit fly (*Dacus dorsalis, Dacus curcurbitae*). Experientia 41:1484–1485. doi: 10.1007/BF01950049

Baker R, Herbert RH, Grant GG (1985) Isolation and identification of the sex pheromone of the Mediterranean fruit fly, *Ceratitis capitata* (Wied). J Chem Soc Chem Commun 824–825

Baker R, Herbert RH, Lomer RA (1982a) Chemical components of the rectal gland secretions of male *Dacus cucurbitae*, the melon fly. Experientia 38:232–233. doi: 10.1007/BF01945082

Baker R, Herbert RH, Parton AH (1982b) Isolation and synthesis of 3- and 4-hydroxy-1,7-dioxaspiro[5.5]undecanes from the olive fly (*Dacus oleae*). J Chem Soc Chem Commun 601. doi: 10.1039/c39820000601

Bellas TE, Fletcher BS (1979) Identification of the major components in the secretion from the rectal pheromone glands of the Queensland fruit flies *Dacus tryoni* and *Dacus neohumeralis* (Diptera: Tephritidae). J Chem Ecol 5:795–803. doi: 10.1007/BF00986564

Benelli G, Daane KM, Canale A, et al (2014) Sexual communication and related behaviours in Tephritidae: current knowledge and potential applications for integrated pest management. J Pest Sci 87:385–405. doi: 10.1007/s10340-014-0577-3

Booth YK, Hayes PY, Moore CJ, et al (2007) Synthesis and absolute configuration of a constitutionally-new [5.6] spiroacetal from *B. tryoni* (Queensland fruit fly). Org Biomol Chem 5:1111–1117. doi: 10.1039/B701833A

Booth YK, Schwartz BD, Fletcher MT, et al (2006) A diverse suite of spiroacetals, including a novel branched representative, is released by female Bactrocera tryoni (Queensland fruit fly). Chem Commun 3975–3977. doi: 10.1039/B611953K

Canale A, Benelli G, Germinara GS, et al (2015) Behavioural and electrophysiological responses to overlooked female pheromone components in the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae). Chemoecology 25:147–157. doi: 10.1007/s00049-014-0183-0

Canale A, Germinara SG, Carpita A, et al (2013) Behavioural and electrophysiological responses of the olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), to male- and female-borne sex attractants. Chemoecology 23:155–164. doi: 10.1007/s00049-013-0131-4

Carpita A, Canale A, Raffaelli A, et al (2012) (*Z*)-9-tricosene identified in rectal gland extracts of *Bactrocera oleae* males: first evidence of a male-produced female attractant in olive fruit fly. Naturwissenschaften 99:77–81. doi: 10.1007/s00114-011-0868-y

Clarke AR, Powell KS, Weldon CW, Taylor PW (2011) The ecology of *Bactrocera tryoni*

(Diptera: Tephritidae): what do we know to assist pest management? Ann Appl Biol 158:26–54. doi: 10.1111/j.1744-7348.2010.00448.x

Cruz-López L, Malo EA, Rojas JC (2015) Sex pheromone of *Anastrepha striata*. J Chem Ecol 41:458–464. doi: 10.1007/s10886-015-0581-y

Dominiak BC, Ekman JH (2013) The rise and demise of control options for fruit fly in Australia. Crop Prot 51:57–67. doi: 10.1016/j.cropro.2013.04.006

Dominiak BC, Mapson R (2017) Revised distribution of *Bactrocera tryoni* in eastern Australia and effect on possible incursions of Mediterranean fruit fly: development of Australia's eastern trading block. J Econ Entomol 110:2459–2465. doi: 10.1093/jee/tox237

Doorenweerd C, Leblanc L, Norrbom AL, et al (2018) A global checklist of the 932 fruit fly species in the tribe Dacini (Diptera, Tephritidae). Zookeys 730:19–56. doi: 10.3897/zookeys.730.21786

Drew RAI, Romig MC (2013) Tropical Fruit Flies of South-East Asia. CAB International, Wallingford

El-Sayed AM (2019) The Pherobase: Database of pheromones and semiochemicals. http://www.pherobase.com/. Accessed 17 Mar 2019

El-Sayed AM, Byers JA, Manning LM, et al (2008) Floral scent of Canada thistle and its potential as a generic insect attractant. J Econ Entomol 101:720–727. doi: 10.1093/jee/101.3.720

El-Sayed AM, Venkatesham U, Unelius CR, et al (2019) Chemical composition of the rectal gland and volatiles released by female Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae). Environ Entomol. doi: 10.1093/ee/nvz061

Ero MM, Hamacek E, Clarke AR (2011) Foraging behaviours of *Diachasmimorpha kraussii* (Fullaway) (Hymenoptera: Braconidae) and its host *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) in a nectarine (*Prunus persica* (L.) Batsch var. *nectarina* (Aiton) Maxim) orch. Aust J Entomol no-no. doi: 10.1111/j.1440-6055.2011.00821.x

Fletcher BS (1968) Storage and release of sex pheromone by the Queensland fruit fly, *Dacus tryoni* (Diptera:Trypetidae). Nature 219:631–632. doi: 10.1038/219631a0

Fletcher BS (1969) The structure and function of the sex pheromone glands of the male Queensland fruit fly, *Dacus tryoni*. J Insect Physiol 15:1309–1322. doi: 10.1016/0022-1910(69)90193-0

Fletcher MT, Kitching W (1995) Chemistry of fruit flies. Chem Rev 95:789–828. doi: 10.1021/cr00036a001

525 Fletcher MT, Wells JA, Jacobs MF, et al (1992) Chemistry of fruit-flies. Spiroacetal-rich
526 secretions in several *Bactrocera* species from the South-West Pacific region. J Chem
527 Soc Perkin Trans 1 2827–2831. doi: 10.1039/P19920002827
528 Haniotakis G, Francke W, Mori K, et al (1986) Sex-specific activity of (*R*)-(−)- and (*S*)-
529 (+)-1,7-dioxaspiro[5.5]undecane, the major pheromone of *Dacus oleae*. J Chem Ecol
530 12:1559–1568. doi: 10.1007/BF01012372
531 Haniotakis GE (1974) Sexual attraction in the olive fruit fly, *Dacus oleae* (Gmelin).
532 Environ Entomol 3:82–86. doi: 10.1093/ee/3.1.82
533 Haniotakis GE, Mazomenos BE, Tumlinson JH (1977) A sex attractant of the olive fruit
534 fly, *Dacus oleae* and its biological activity under laboratory and field conditions.
535 Entomol Exp Appl 21:81–87. doi: 10.1111/j.1570-7458.1977.tb02660.x
536 Hayes P, Fletcher MT, Moore CJ, Kitching W (2001) Synthesis and absolute
537 stereochemistry of a constitutionally new spiroacetal from an insect. J Org Chem
538 66:2530–2533. doi: 10.1021/jo015502p
539 Heath RR, Landolt PJ, Robacker DC, et al (2000) Sexual pheromones of tephritid flies:
540 clues to unravel phylogeny and behavior. In: Aluja M, Norrbom AL (eds) Fruit Flies
541 (Tephritidae): Phylogeny and Evolution of Behavior. CRC Press, pp 793–809
542 Hendrichs J, Robinson AS, Cayol JP, Enkerlin W (2002) Medfly areawide sterile insect
543 technique programmes for prevention, suppression or eradication: the importance of
544 mating behavior studies. Fla Entomol 85:1–14. doi: 10.1653/0015-
545 4040(2002)085[0001:MASITP]2.0.CO;2
546 Hickey P, Woods B, Windle B, et al (2010) National fruit fly strategy implementation
547 action plan. Plant Health Australia, Canberra, Australia
548 Kitching W, Lewis JA, Fletcher MT, et al (1986) Spiroacetals in rectal gland secretions of
549 Australasian fruit fly species. J Chem Soc Chem Commun 11:853. doi:
550 10.1039/c39860000853
551 Kitching W, Lewis JA, Perkins M V, et al (1989) Chemistry of fruit flies. Composition of
552 the rectal gland secretion of (male) *Dacus cucumis* (cucumber fly) and *Dacus*
553 *halfordiae*. Characterization of (*Z,Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. J
554 Org Chem 54:3893–3902. doi: 10.1021/jo00277a028
555 Krohn S, Fletcher MT, Kitching W, et al (1991) Chemistry of fruit flies: Nature of
556 glandular secretion and volatile emission of *Bactrocera (bactrocera) cacuminatus*
557 (Héring). J Chem Ecol 17:485–495. doi: 10.1007/BF00994347
558 Lauzon CR, Potter SE (2012) Description of the irradiated and nonirradiated midgut of
559 *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) and *Anastrepha ludens* Loew

(Diptera: Tephritidae) used for sterile insect technique. J Pest Sci 85:217–226. doi: 10.1007/s10340-011-0410-1

Leblanc L, Tora E, Drew RAI, Allwood AJ (2013) Host plant records for fruit flies (Diptera: Tephritidae: Dacini) in the Pacific Islands. Proc Hawaiian Entomol Soc 44:11–53. doi: 10.1002/ana.21927.Dicer

Levi-zada A, Levy A, Rempoulakis P, et al (2020) Diel rhythm of volatile emissions of males and females of the peach fruit fly *Bactrocera zonata*. J Insect Physiol 120:103970. doi: 10.1016/j.jinsphys.2019.103970

Mazomenos BE, Haniotakis GE (1985) Male olive fruit fly attraction to synthetic sex pheromone components in laboratory and field tests. J Chem Ecol 11:397–405. doi: 10.1007/BF01411425

Mazomenos BE, Haniotakis GE (1981) A multicomponent female sex pheromone of *Dacus oleae* Gmelin: Isolation and bioassay. J Chem Ecol 7:437–444. doi: 10.1007/BF00995766

Mitchell RF, Curkovic T, Mongold-Diers JA, et al (2017) Evidence that cerambycid beetles mimic vespid wasps in odor as well as appearance. J Chem Ecol 43:75–83. doi: 10.1007/s10886-016-0800-1

Nation JL (1990) Biology of pheromone release by male Caribbean fruit flies, *Anastrepha suspensa* (Diptera: Tephritidae). J Chem Ecol 16:553–572. doi: 10.1007/BF01021786

Nation JL (1972) Courtship behavior and evidence for a sex attractant in the male Caribbean fruit fly, *Anastrepha suspensa*. Ann Entomol Soc Am 65:1364–1367. doi: 10.1093/aesa/65.6.1364

Nishida R, Tan KH, Fukami H (1988a) *Cis*-3,4-dimethoxycinnamyl alcohol from the rectal glands of male oriental fruit fly, *Dacus dorsalis*. Chem Express 3:207–210

Nishida R, Tan KH, Serit M, et al (1988b) Accumulation of phenylpropanoids in the rectal glands of males of the Oriental fruit fly, *Dacus dorsalis*. Experientia 44:534–536. doi: 10.1007/BF01958941

Noushini S, Perez J, Park SJ, et al (2020) Rectal gland chemistry, volatile emissions, and antennal responses of male and female banana fruit fly, Bactrocera musae. Insects 11:32. doi: 10.3390/INSECTS11010032

Oh H, Jeong SA, Kim J, Park KC (2019) Morphological and functional heterogeneity in olfactory perception between antennae and maxillary palps in the pumpkin fruit fly, *Bactrocera depressa*. Arch Insect Biochem Physiol 101:. doi: 10.1002/arch.21560

Perdomo AJ, Nation JL, Baranowski RM (1976) Attraction of female and male Caribbean fruit flies to food-B\baited and male-baited traps under field conditions. Environ

595 Entomol 5:1208–1210. doi: 10.1093/ee/5.6.1208
596 Perkins MV (1990) Characterisation and synthesis of Bactrocera fruit fly pheromones. PhD
597 Thesis, Department of Chemistry.The University of Queensland, Australia
598 Perkins MV, Fletcher MT, Kitching W, et al (1990a) Chemical studies of rectal gland
599 secretions of some species of *Bactrocera dorsalis* complex of fruit flies (diptera:
600 Tephritidae). J Chem Ecol 16:2475–2487. doi: 10.1007/BF01017470
601 Perkins MV, Kitching W, Drew RAI, et al (1990b) Chemistry of fruit flies: composition of
602 the male rectal gland secretions of some species of South-East Asian Dacinae. Re-
603 examination of *Dacus cucurbitae* (melon fly). J Chem Soc Perkin Trans 1 1111–1117.
604 doi: 10.1039/P19900001111
605 Piccardi P (1980) Insect sex-communication and prospects for pheromones in pest
606 management. Bolletino di Zool 47:397–408. doi: 10.1080/11250008009438696
607 Schutze M, McMahon J, Krosch M, et al (2018) The Australian Handbook for the
608 Identification of Fruit Flies. Version 3.1. Plant Health Australia, Canberra, ACT
609 Schwartz BD, Moore CJ, Rahm F, et al (2008) Spiroacetal biosynthesis in insects from
610 Diptera to Hymenoptera: The giant ichneumon wasp *Megarhyssa nortoni nortoni*
611 Cresson. J Am Chem Soc 130:14853–14860. doi: 10.1021/ja8036433
612 Sivinski J, Aluja M, Dodson GN, et al (2000) Topics in the evolution of sexual behavior in
613 the Tephritidae. In: Aluja M, Norrbom AL (eds) Fruit Flies (Tephritidae): Phylogeny
614 and Evolution of Behavior. CRC Press, pp 751–792
615 Sivinski JM, Calkins C (1986) Use of pheromones in tropical crops: pheromones and
616 parapheromones in the control of tephritids. Florida Entomol 69:157–168. doi:
617 10.2307/3494757
618 Symonds MRE, Moussalli A, Elgar MA (2009) The evolution of sex pheromones in an
619 ecologically diverse genus of flies. Biol J Linn Soc 97:594–603
620 Tokushima I, Orankanok W, Tan KH, et al (2010) Accumulation of phenylpropanoid and
621 sesquiterpenoid volatiles in male rectal pheromonal glands of the guava fruit fly,
622 *Bactrocera correcta*. J Chem Ecol 36:1327–1334. doi: 10.1007/s10886-010-9874-3
623 Verschut TA, Farnier K, Cunningham JP, Carlsson MA (2018) Behavioral and
624 physiological evidence for palp detection of the male-specific attractant cuelure in the
625 Queensland fruit fly (*Bactrocera tryoni*). Front Physiol 9:. doi:
626 10.3389/fphys.2018.00990
627 Vijaysegaran S (1997) Fruit fly research and development in tropical Asia. In: Allwood,
628 A., Drew, R E (ed) Management of Fruit Flies in the Pacific. Australian Centre for
629 International Agricultural Research Proceedings, Canberra, pp 21–29

630 Wee SL, Tan KH (2005) Female sexual response to male rectal volatile constituents in the
631 fruit fly, *Bactrocera carambolae* (Diptera: Tephritidae). Appl Entomol Zool 40:365–
632 372. doi: 10.1303/aez.2005.365
633 Zamek AL, Spinner JE, Micallef JL, et al (2012) Parasitoids of Queensland fruit fly
634 *Bactrocera tryoni* in Australia and prospects for improved biological control. Insects
635 3:1056–1083
636 Zhang X, Wei C, Miao J, et al (2019) Chemical compounds from female and male rectal
637 pheromone glands of the guava fruit fly, *Bactrocera correcta*. Insects 10:. doi:
638 10.3390/insects10030078
639 Zheng L-Y, Sun G-M, Liu Y-G, et al (2012) Aroma volatile compounds from two fresh
640 pineapple varieties in China. Int J Mol Sci 13:7383–7392. doi: 10.3390/ijms13067383
641
642

Table 1. Rectal gland (RG) and headspace (HS) volatile compounds of *B. bryoniae* adults. No = number, FRG = female rectal gland, FHS = female headspace, MRG = male rectal gland, MHS = male headspace, RI = retention index, MW = molecular weight, ND = not detected

No	Name	FRG (%)	FHS (%)	MRG (%)	MHS (%)	Characteristic EI ions *m/z* (%)	RI
1	Ethyl 3-acetoxybutanoate	ND	ND	2.88	0.49	174 (M^+, 0.02), 131 (M – CH_3C=O, 32.84), 129 (M – OC_2H_5,16.93), 117 (10.7), 114 (27.4), 85 (CH_3(CO) CH_2C=O, 25.26), 69 (100)	1109
2	2,7-Dimethyl-1,6-dioxaspiro[4.5]decane	0.01	ND	ND	ND	170 (M^+, 1.9), 155 (M – CH_3, 1.7), 126 (7.8), 115 (13.1), 111 (4.9), 101 (CH_3(C_4H_5O)=OH^+, 100), 98 (CH_3(C_4H_5O)=CH_2, 77.32), 83 (31.2), 69 (12.7), 55 (49.1)	1087
3	1,7-Dioxaspiro[5,5]undecane	ND	ND	87.335	84.81	156 (M+, 8.2%), 128 (7.5), 111 (12.7), 102 (6), 101 ((C_5H_7O)=OH^+, 100), 100 (64.7), 99 (7.8), 98 ((C_5H_7O)=CH_2, 85.1), 97 (3.6), 83 (33.4), 70 (5.9), 56 (16), 55 (36.52)	1135
4	(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane	4.85	5.78	0.374	ND	184 (M^+, 6.6), 169 (M – CH_3, 1.7), 140 (13.7), 125 (8.6), 115 (CH_3(C_5H_7O)=OH^+, 92), 112 (CH_3(C_5H_7O)=CH_2, 100), 97 (73.9), 69 (37), 55 (36.8)	1147
5	(*E*,*E*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane	0.03	0.12	ND	ND	184 (M^+, 3.3), 155 (M – C_2H_5, 30.2), 140 (11.8), 115 (M – $C_5H_9^-$, 100), 112 (M – C_4H_8O, 60.5), 97 (90.4), 85 (56.2), 69 (47.2), 55 (56.8)	1160
6	*N*-(2-Methylbutyl)acetamide	0.01	ND	0.11	0.057	129 (M^+, 9.8), 100 (M – C_2H_5, 61.2), 73 (β-cleavage product/H rearrangement, 76.8), 72 (M – C_4H_9, 100), 60 ($CH_3C(OH)NH^+$, 56.4)	1132
7	*N*-(3-Methylbutyl)acetamide	0.43	0.30	8.26	13.56	129 (M^+, 6.1), 114 (M – CH_3, 16.9), 86 (M – C_3H_7, 29.1), 73 (β-cleavage product/H rearrangement, 100), 72 (M – C_4H_9, 69.7), 60 ($CH_3C(OH)NH^+$, 25.3)	1136
8	(*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane	0.39	2.14	ND	ND	198 (M^+, 6.6), 169 (M - C_2H_5, 14.8), 140 (13.7), 129 (CH_3CH_2(C_5H_7O)=OH^+, 45.8), 126 (CH_3CH_2(C_5H_7O)=CH_2, 37.3), 115 CH_3(C_5H_7O)=OH^+, 92.8), 112 (CH_3(C_5H_7O)=CH_2, 100), 97 (84.9), 83 (70), 69 (60.9), 55 (71.2)	1236
9	(*Z*,*Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane	0.08	0.197	ND	ND	184 (M^+, 3.5), 169 (M – CH_3, 1.1), 140 (4.7), 125 (6), 115 (CH_3(C_5H_7O)=OH^+, 100), 114 (42.4) 112 (CH_3(C_5H_7O)=CH_2, 45.4), 97 (87.6), 69 (56.3), 55 (40)	1221
10	(*E*,*E*)-2-Propyl-8-methyl-1,7-dioxaspiro[5.5]undecane	0.09	0.52	ND	ND	212 (M^+, 4.9), 169 (M – C_3H_7, 16.7), 143 ($CH_3CH_2CH_2$(C_5H_6O)=OH^+, 28.2), 140 ($CH_3CH_2CH_2$(C_5H_6O)=$CH_2CH_2CH_3$, 29.4), 125 (47.1), 115 (CH_3(C_5H_7O)=OH^+, 100),112 (CH_3(C_5H_7O)=CH_2, 88), 97 (96.9), 83 (38.6), 82 (31.5), 69 (40.2), 55 (63.2)	1322
11	4-Hydroxy-1,7-dioxaspiro[5.5]undecane	ND	ND	0.81	0.47	172 (M^+, 0.4), 155 (M – OH, 11.1), 127 (31.6), 117 (OH(C_5H_7O)=OH^+, 96.2), 114 (OH(C_5H_7O)=CH_2, 39.4), 101 ((C_5H_7O)=OH^+, 100), 98 ((C_5H_7O)=CH_2, 57.1), 83 (22.6), 55 (35.8)	1357
12	Ethyl caprate	0.21	0.59	ND	ND	200 (M^+, 1.2), 171 (M – C_2H_5, 2.4), 157 (11.9), 155 (M – OC_2H_5, 9.9), 115 (7.7), 101 (36.5), 88 (McLafferty rearrangement product, 100), 73 ($COOC_2H_5$, 22.6), 70 (27.1), 55 (24.5)	1395

13	Methyl laurate	0.21	0.42	ND	ND	214 (M^+, 2.1), 183 (M – OCH_3, 4.3), 171 (8.4), 143 (11.8), 87 (59.3), 74 (McLafferty rearrangement product, 100), 69 (11.6), 59 ($COOCH_3$, 9.6), 55 (24.8)	1524
14	Lauric acid	0.29	ND	ND	ND	200 (M^+, 5.6), 171 (9.2), 157 (25), 129 (35.1), 115 (18.8), 101 (15.7), 97 (17.1), 85 (35.2), 73 (100), 69 (28.7), 57 (54.7), 55 (63.3)	1559
15	Ethyl laurate	39.9	66.59	ND	ND	228 (M^+, 3.3), 199 (M – C_2H_5, 3.7), 183 (M – OC_2H_5, 9.5), 157 (14.8), 101 (48.4), 88 (McLafferty rearrangement product, 100), 73 ($COOC_2H_5$, 20), 70 (23.4), 61 (11.3), 55 (22)	1594
16	Ethyl tridecanoate	0.03	0.08	ND	ND	242 (M^+, 2.8), 213 (M – C_2H_5, 5.1), 199 (6.8), 197 (M – OC_2H_5, 2.1), 157 (13.7), 101 (58.6), 88 (McLafferty rearrangement product, 100), 83 (27), 73 ($COOC_2H_5$, 13.9), 57 (18.8), 55 (30.8)	1665
17	Propyl laurate	0.09	0.10	ND	ND	242 (M^+, 2.4), 201 (23.4), 199 (M – C_3H_7, 1.6), 183 (M – OC_3H_7, 22.6), 157 (8.3), 129 (9.8), 115 (17.9), 102 (McLafferty rearrangement product, 23.9), 87 ($COOC_3H_7$, 10.4), 61 (100), 60 (38.5), 59 (6.1), 55 (30.4)	1690
18	Methyl myristate	0.28	0.25	ND	ND	242 (M^+, 2.8), 211 (M – OCH_3, 2.4), 199 (8.1), 143 (16.8), 125 (7.3), 129 (5.8), 101 (7.9), 87 (65.9), 74 (McLafferty rearrangement product, 100), 69 (13.5), 59 ($COOCH_3$, 8.7), 55 (26.5)	1725
19	Ethyl myristate	31.99	20.78	ND	ND	256 (M^+, 5.7), 213 (10.9), 211 (M – OC_2H_5, 6.6), 157 (18.8), 101 (52.5), 88 (McLafferty rearrangement product, 100), 73 ($COOC_2H_5$, 18.2), 70 (21.7), 69 (12.3), 55 (22.1)	1794
20	Methyl palmitate	0.15	ND	ND	ND	270 (M^+, 5.1), 227 (6.9), 143 (15.7), 87 (68.4), 74 (McLafferty rearrangement product, 100), 69 (15.9), 59 ($COOCH_3$, 8.8), 55 (29.4)	1926
21	Palmitoleic acid	0.24	ND	ND	ND	254 (M^+, 1.7), 236 (5.8), 152 (7.2), 111 (22.2), 98 (30.1), 97 (45.8), 96 (32.7), 83 (54.7), 73 (17.6), 69 (75.5), 60 (McLafferty rearrangement product, 12.8), 57 (33.7), 55 (100)	1943
22	Palmitic acid	0.39	ND	ND	ND	256 (M^+, 14.4), 227 (5.6), 213 (M – COOH, 15.9), 185 (15.1), 157 (20.6), 129 (41.3), 115 (22.8), 97 (25.5), 87 (37.4), 85 (35), 83 (38.3), 73 (100), 69 (45.6), 60 (McLafferty rearrangement product, 89.3), 57 (80.9), 55 (83.1)	1960
23	Ethyl palmitoleate	2.46	0.19	ND	ND	282 (M^+, 2.8), 237 (M – OC_2H_5, 10.1), 236 (M – C_2H_5OH, 10.2), 194 (11.9), 152 (17.6), 88 (McLafferty rearrangement product, 52.1), 73 ($COOC_2H_5$, 15.3), 69 (69.8), 55 (100)	1975
24	Ethyl palmitate	15.9	0.96	ND	ND	284 (M^+, 7.8), 255 (M – C_2H_5, 2.9), 241 (9.1), 239 (M – OC_2H_5, 5.3), 157 (17.3), 101 (55.2), 88 (McLafferty rearrangement product, 100), 73 ($COOC_2H_5$, 17.3), 55 (23.5)	1994
25	Oleic acid	0.25	ND	ND	ND	282 (M^+, 2.3), 264 (10), 221 (2.9), 180 (4), 165 (5.6), 137 (7.6), 1151 (29.8), 97 (61.5), 87 (69.9), 69 (77.6), 60 (McLafferty rearrangement product, 10.6), 57 (40.3), 55 (100)	2141
26	Ethyl oleate	1.5	ND	ND	ND	310 (M^+, 3.4), 265 (M – OC_2H_5, 12.4), 264 (M – C_2H_5OH, 14.8), 222 (9.6), 180 (10), 123 (15.9), 110 (23.2), 97 (56.5), 88 (McLafferty rearrangement product, 57), 83 (61.9), 73 ($COOC_2H_5$, 15.9), 69 (68.1), 55 (100)	2171

Figures Legend

Fig. 1 Flame ionization detector (FID) response and electrophysiological responses of antennae (EAD) and palps (EPD) of *Bactrocera bryoniae* males and females to the rectal gland extracts from conspecific males. Numbered peaks indicate EAD- and/or EPD-active compounds: ethyl 3-acetoxybutanoate (**1**), 1,7-dioxaspiro[5,5]undecane (**3**), *N*-(3-methylbutyl)acetamide (**7**), 4-hydroxy-1,7-dioxaspiro[5.5]undecane (**11**)

Fig. 2 Flame ionization detector (FID) response and electrophysiological responses of antennae (EAD) and palps (EPD) of *Bactrocera bryoniae* males and females to the rectal gland extracts from conspecific females. Numbered peaks indicate EAD- and/or EPD-active compounds: 2,7-dimethyl-1,6-dioxaspiro[4.5]decane (**2**), (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**4**), (*E*,*E*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**5**), *N*-(3-methylbutyl)acetamide (**7**), (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**8**), (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**9**), and (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**10**), ethyl caprate (**12**), ethyl laurate (**15**), ethyl myristate (**19**) and ethyl palmitate (**24**)

Fig. 3 Behavioural response of virgin adults of *Bactrocera bryoniae* males and females to (A) the rectal gland volatiles and (B) the headspace volatiles of males and females in a Y-tube olfactometer. N total number of responders, NC number of non-responders (excluded from statistical analysis), ns $P > 0.05$, * $0.01 < P < 0.05$, ** $0.001 < P < 0.01$, *** $P < 0.001$

Fig. 1

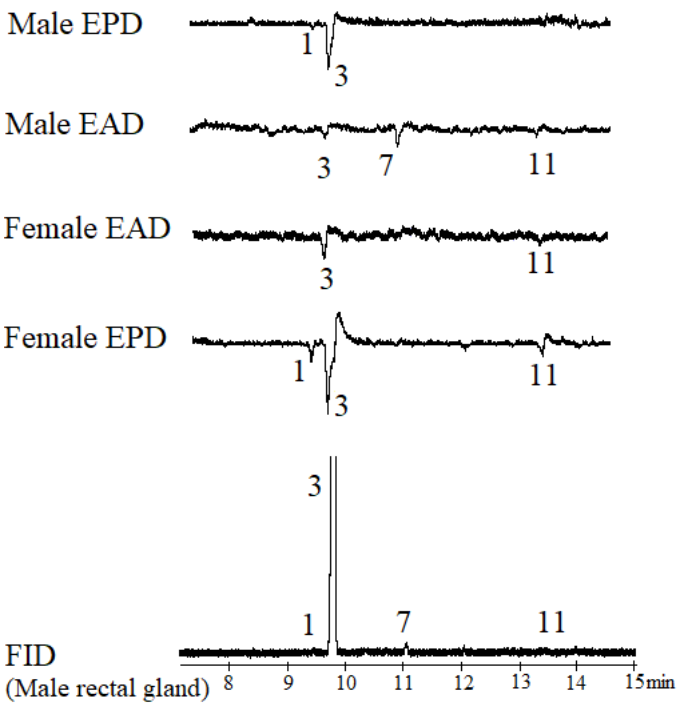

Male EPD
1
3
Male EAD
3
7
11
Female EAD
3
11
Female EPD
1
3
11
3
1
7
11
FID
(Male rectal gland)
8
9
10
11
12
13
14
15min

Fig. 2

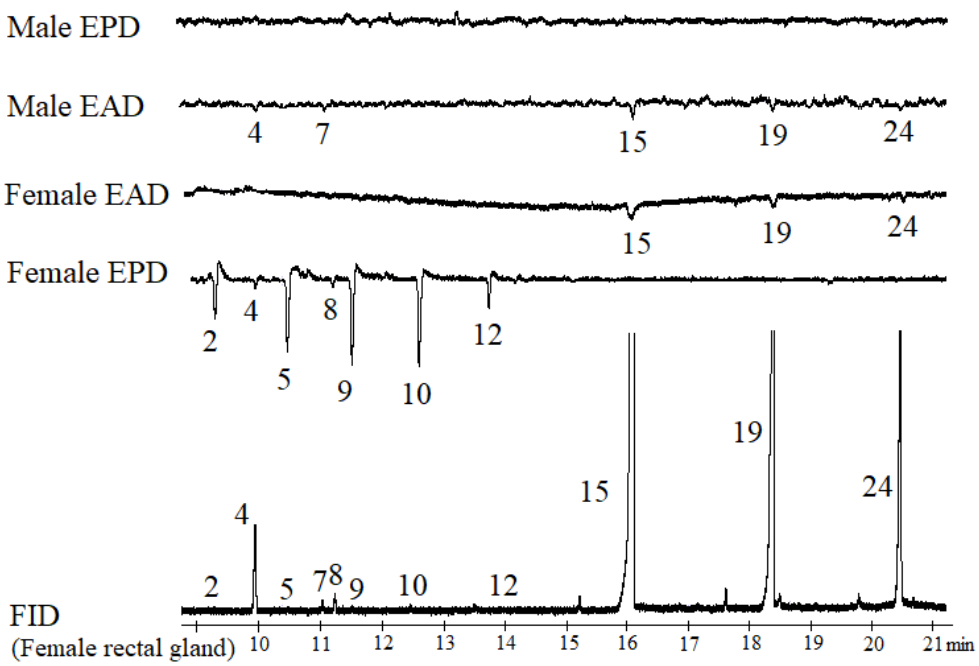

Male EPD
Male EAD
4
7
15
19
24
Female EAD
15
19
24
Female EPD
2
4
8
12
5
9
10
19
15
24
4
2
5
7
8
9
10
12
FID
(Female rectal gland)
10
11
12
13
14
15
16
17
18
19
20
21min

Fig. 3

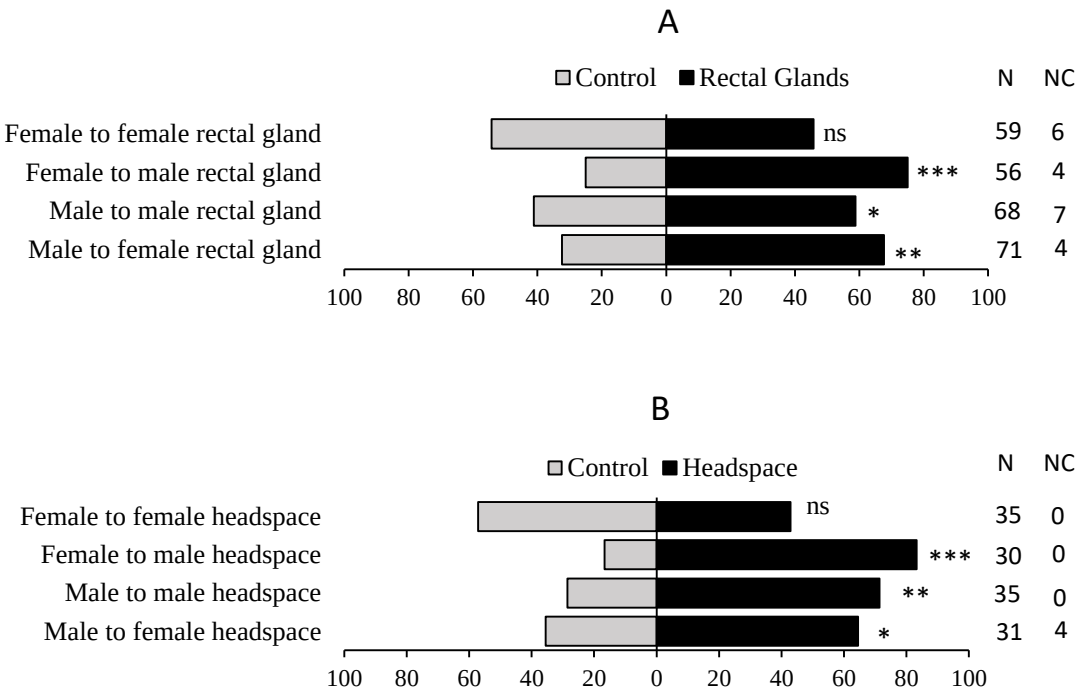
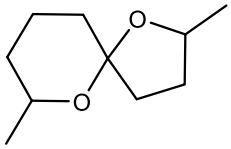
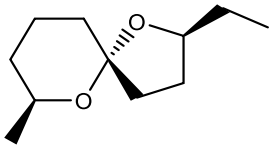

A
Control
Rectal Glands
N
NC
Female to female rectal gland
Female to male rectal gland
Male to male rectal gland
Male to female rectal gland
ns
***
*
**
59
6
56
4
68
7
71
4
100
80
60
40
20
0
20
40
60
80
100
B
Control
Headspace
N
NC
Female to female headspace
Female to male headspace
Male to male headspace
Male to female headspace
ns
***
**
*
35
0
30
0
35
0
31
4
100
80
60
40
20
0
20
40
60
80
100

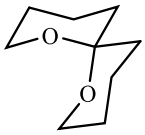
Figure 4. Structure of volatiles identified in chemical profile of male and female *Bactrocera bryoniae.*



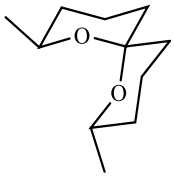
2,7-Dimethyl-1,6-dioxaspiro[4.5]decane



(*E*,*E*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane

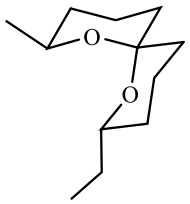


1,7-Dioxaspiro[5,5]undecane

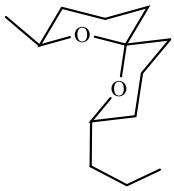


(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane

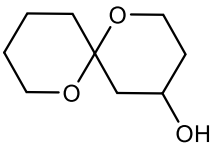
(*Z*,*Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane



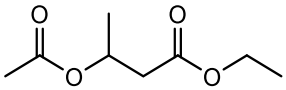
(*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane



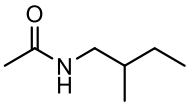
(*E*,*E*)-2-Propyl-8-methyl-1,7-dioxaspiro[5.5]undecane



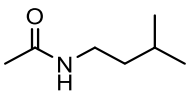
4-Hydroxy-1,7-dioxaspiro[5.5]undecane



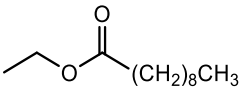
Ethyl 3-acetoxybutanoate



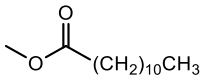
N-(2-Methylbutyl)acetamide



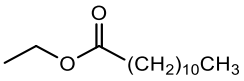
N-(3-methylbutyl)acetamide



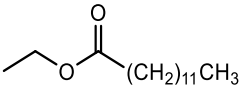
Ethyl caprate



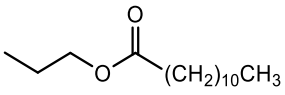
Methyl laurate



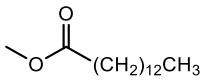
Ethyl laurate



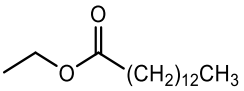
Ethyl tridecanoate



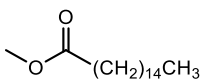
Propyl laurate



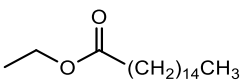
Methyl myristate



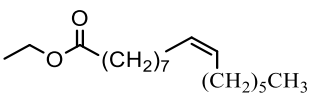
Ethyl myristate



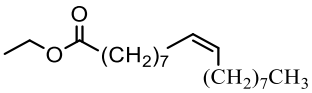
Methyl palmitate



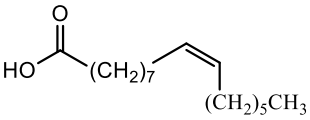
Ethyl palmitate



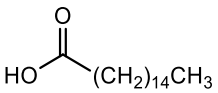
Ethyl palmitoleate



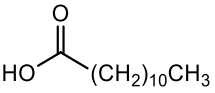
Ethyl oleate



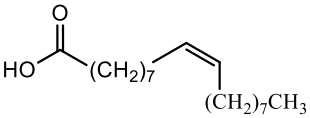
Palmitoleic acid



Palmitic acid



Lauric acid

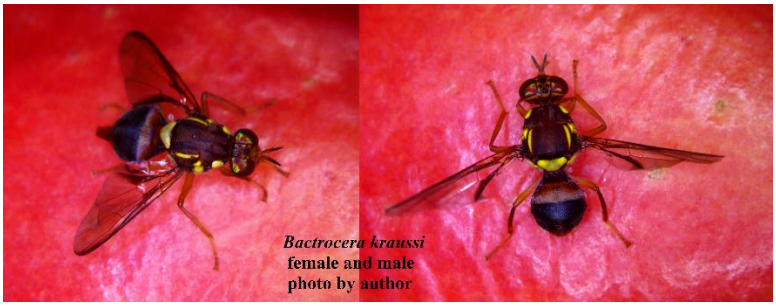


Oleic acid

Chapter Five

Behavioural and electrophysiological responses to rectal gland secretions and headspace volatiles emitted by *Bactrocera kraussi* (Hardy) (Tephritidae)

Prepared for submission to Journal of Chemoecology



Bactrocera kraussi
female and male
photo by author

Author contributions:
S. J. Park and J. Perez helped with Y-tube observations. V. Mendez and S. J. Park helped with GC-EAD experiments. All other experiments and data analysis were conducted by S. Noushini. The manuscript was drafted by S. Noushini. All authors read the manuscript and provided feedback.

At the begging of this chapter there is a short summery of phylogenetic situation of *Bactrocera kraussi* and species which are known as close relatives to this species.

At the end of this chapter, after the manuscript, figure 3 has been provided summarising structure of all identified compounds in male and female *Bactrocera kraussi*.

Preamble

Bactrocera kraussi (Hardy) is a highly polyphagous pest species belongs to order Diptera and family Tephritidae.[1,2] This species is very similar to *Bactrocera tryoni* (Froggatt). The differences between these two species is that *B. kraussi* has tint only in both costal cells while *B. tryoni has* tint and microtrichia in both costal cells. *Bactrocera kraussi* has longer less tapered vittae, lateral spots on the abdomen (instead of a wraparound T), narrow mesopleural stripe, broad basal band on the scutellum and dark apices of the femora.[1]

All molecular markers including COI, POP4, EIF3L, RPA2 and DDOSTS2 separate these two morphologically similar species.[1,3]

Pheromone profile of *B. tryoni* has been discussed in details in Chapter 6.

References:

1. Schutze, M. *et al. The Australian Handbook for the Identification of Fruit Flies. Version 3.1*. (Plant Health Australia, 2018). doi:10.1016/j.jasms.2007.01.008
2. Leblanc, L., Tora, E., Drew, R. A. I. & Allwood, A. J. Host plant records for fruit flies (Diptera: Tephritidae: Dacini) in the Pacific Islands. *Proc. Hawaiian Entomol. Soc.* **44**, 11–53 (2013).
3. Blacket, M. J., Semeraro, L. & Malipatil, M. B. Barcoding Queensland fruit flies (Bactrocera tryoni): impediments and improvements. *Mol. Ecol. Resour.* **12**, 428–436 (2012).

Behavioural and Electrophysiological Responses to Rectal Gland Secretions and Headspace Volatiles Emitted by *Bactrocera kraussi* (Hardy) (Tephritidae)

Saeedeh Noushini[1,3*], Soo Jean Park[2,3], Jeanneth Perez[2,3], Danielle Holgate[1], Vivian Mendez Alvarez[2,3], Ian Jamie[1,3], Joanne Jamie[1], Phillip Taylor[2,3]

[1] Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia.

[2] Applied BioSciences, Macquarie University, Sydney, NSW 2109, Australia.

[3] Australian Research Council Industrial Transformation Training Centre for Fruit Fly Biosecurity Innovation, Macquarie University, Sydney, NSW 2109, Australia.

Corresponding author:

Saeedeh Noushini, Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia

E-mail:

ORCID**:** 0000-0001-5558-1656

Abstract Tephritid fruit flies typically release volatile compounds, usually interpreted as sex pheromones, as an integral element of their sexual biology. Understanding the composition and function of released volatiles is an important aspect of understanding fruit fly sexual biology and can also provide valuable knowledge for the development of attractants that can be used in monitoring and in pest management. *Bactrocera kraussi* (Hardy) (Diptera: Tephritidae) is a pest fruit fly for which knowledge of released volatiles is incomplete, limiting both understanding of its mating system and the potential development of novel attractants. Here we (1) establish chemical profiles of rectal gland contents and volatile emissions at the time of mating activity by using gas chromatography-mass spectrometry (GC-MS) and (2) evaluate the detection and function of natural blends of both sexes by gas chromatography-electroantennogram detection (GC-EAD), gas chromatography-electropalpogram detection (GC-EPD) and Y-tube olfactometers. Spiroacetals were found to be the dominant compounds in male rectal gland extracts whereas saturated/unsaturated fatty acid esters were the main compounds in female rectal gland extracts. Analysis of rectal gland extracts by GC-EAD/EPD showed that five volatile compounds, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, methyl laurate, ethyl laurate, ethyl myristate and ethyl palmitate elicited antennal/palpal responses in males and two volatile compounds, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, elicited antennal/palpal responses in females. Interestingly, Y-tube olfactometer behavioural assays found that the natural blend of female rectal glands attracted males at dusk but did not attract females, and the natural blend of male rectal glands did not attract either males or females. The biological significance of male-produced volatiles may be in functions other than mate attraction, such as species recognition or signals of quality, or may function as attractants only when combined with other visual and acoustic cues associated with fruit fly mating behaviour.

Keywords Tephritidae, *Bactrocera kraussi*, Pheromone, Olfaction, Electrophysiology

Introduction

Chemical communication plays an important role in the mating system of many tephritid fruit flies (Witzgall et al. 2010; Benelli et al. 2014). In particular, volatile compounds stored in the rectal glands and emitted into the air during calling and courtship can attract members of the opposite sex (Nation 1972; Perkins 1990; Sivinski et al. 2000; Cruz-López et al. 2015). These emissions can also attract members of the same sex to mating aggregations (Sivinski and Calkins 1986; Hendrichs et al. 2002). Studies of volatile compounds in Dacine fruit flies have focused on chemical profiles of male fruit flies because males have typically been considered as the major sex pheromone producers (El-Sayed 2019). However, in some species females have been found to produce sex pheromones. In *Bactrocera oleae,* sex pheromones are produced mainly by female flies (Haniotakis 1974; Mazomenos and Haniotakis 1985) while males produce a compound that only acts as a close-range attractant for females (Carpita et al. 2012; Canale et al. 2013). Similarly, in *Zeugodacus cucurbitae* (Coquillett) and *B. dorsalis* (Hendel), both male and female volatile emissions have been reported to attract the opposite sex (Baker et al. 1982; Baker and Bacon 1985; Nishida et al. 1988a, b).

Bactrocera kraussi (Hardy) is endemic to Australia and is well established in the Torres Strait Islands and Northeast Queensland, as far south as Townsville (Schutze et al. 2018). It is a polyphagous and economically important pest species, infesting a wide range of both wild and commercial fruit and vegetable hosts including mango (*Mangifera indica*), banana (*Musa* spp.), guava (*Psidium guajava*), feijoa (*Acca sellowiana*), peach (*Prunus persica*), citrus and tamarind (*Tamarindus indica*) (Hancock et al. 2000; Schutze et al. 2018). Compounds extracted from rectal glands and collected from the headspace volatiles of *B. kraussi* males have been partially described by Fletcher et al. (1992), who reported (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5,5]undecane as a major compound, along with six other spiroacetals, and five other minor compounds. To date, however, chemical profiles of volatiles produced by female *B. kraussi* are unknown, and the biological significance of the volatile compounds produced by *B. kraussi* males and females has also not been investigated. The present study (1) establishes the chemical profiles of *B. kraussi* females and re-evaluates chemical profiles of *B. kraussi* males by using gas chromatography-mass spectrometry (GC-MS) of rectal gland exudates and headspace collections; (2) evaluates antennal and palpal sensitivity to each compound in natural

blends by use of gas chromatography-electroantennogram detection (GC-EAD) and gas chromatography-electropalpogram detection (GC-EPD); and (3) evaluates attraction of males and females to natural blends of both sexes of *B. kraussi* by use of Y-tube olfactometers.

Methods and materials

Insects

A laboratory-reared population of *B. kraussi* (G27), maintained using carrot-based larval diet, was obtained from the Queensland Department of Agriculture and Fisheries (Cairns). At Macquarie University, Sydney (Australia), approximately 500 pupae were placed in a 47.5 × 47.5 × 47.5 cm fine mesh cage (Megaview Bugdorm 4S4545, Taiwan) for emergence and kept in a controlled environment room at 25 ± 0.5 °C, 65 ± 5% relative humidity (RH) and 11.5:0.5:11.5:0.5 light/dusk/dark/dawn photoperiod. Adult flies were fed with sugar and yeast hydrolysate (MP Biomedicals LLC) provided separately and tap water through a soaked sponge. Flies were reared for one generation using a standard carrot diet (Steiner and Mitchell 1966) and following the methodology described by Pérez et al. (2018). Flies were separated by sex within 3 days after emergence and transferred to 12.5 L clear plastic cages (180 flies per cage). No mating was observed before separating the flies. All cages were kept with the same diet and environmental conditions described above. All experiments used 13- to 18-day old virgin flies.

Rectal Gland Extraction

Gland extracts were obtained from sexually mature males and females. Handling of the flies and the gland extractions followed the procedure of Kitching et al. (1989). Flies were chilled on dry ice to kill them. The abdomen was gently squeezed with tweezers such that the glands protruded slightly. The glands were then gently pulled out with tweezers, and the secretory sac separated. Glands were carefully placed in a tear-drop vial in dry ice. Once 20 glands were collected, the vials were removed from the dry ice and the contents were extracted into 200 μL of *n*-hexane (HPLC grade, Sigma-Aldrich) by saturating the glands with solvent and leaving them to stand at room temperature for 10 minutes. The extracts were then transferred to a new vial, labelled and stored at -20 °C until analysed.

Six replicates per sex were collected using 20 glands per replicate. Samples were stored at – 20 °C until analysed.

Headspace Collection

Headspace collections were conducted during the immediate pre-dust, dusk and immediate post-dusk light phases, in a controlled environment room under the same conditions that the flies were kept (*i.e.*, 25 ± 0.5 °C, 65 ± 5% RH). The time of the day was selected based on our observations of males calling and mating at dusk. Thirty sexually mature males and 30 sexually mature females of *B. kraussi* were separately placed into a glass chamber (150 mm long × 40 mm ID) 30 minutes before dusk and charcoal-filtered air at a flow rate of 1.0 L/min (air pulling system) was drawn over the flies for a period of 1.5 hours, beginning 30 minutes before dusk. Released volatiles were adsorbed onto 50 mg of Tenax adsorbent (Scientific Instrument Services, Inc, Tenax-GR Mesh 60/80) packed into glass cartridges (6 × 50 mm) and fitted with glass wool plugs. Volatiles were subsequently extracted from the Tenax into 1 mL of *n*-hexane (HPLC grade, Sigma-Aldrich). Samples were stored at -20 °C until analysis. Five replicates per sex were collected. To distinguish any possible contaminants an air control sample, comprising an empty glass chamber, was run and analysed along with every volatile collection. Tenax traps were conditioned at 200 °C for three hours under a nitrogen stream (75 mL/min) prior to each headspace collection. Glass chambers were washed with 5% Extran aqueous solution, rinsed with hot tap water, and heated at 200 °C for 18 hours. Activated charcoal filters were thermally conditioned by heating them at 200 °C for 18 hours prior to each headspace collection (El-Sayed et al. 2008).

Analysis of Rectal Gland Extracts and Headspace Collections

Mass spectra were recorded by gas chromatography-mass spectrometry (GC-MS) on a Shimadzu GCMS-QP2010 instrument using a capillary column with 35% diphenyl / 65% dimethyl polysiloxane as the stationary phase (30 m × 0.25 mm ID × 0.25 μm film thickness) and helium (99.999%) (ultra-high purity, BOC, Australia) as a carrier gas with a constant flow of 1 mL/min. The temperature program was 50 °C (4 min) to 250 °C (6 min) at a rate of 10 °C/min, with an injector temperature of 270 °C. Mass spectra were recorded in EI mode (70 keV), scanning from 40 to 620 *m/z*. The interface and ion source

temperatures were 200 °C and 250 °C, respectively. Impurities were identified through comparison with the air control samples.

All compounds including esters, amides and spiroacetals were identified through comparison with gas chromatography retention times and mass spectra of authentic samples. Of the 25 compounds detected in *B. kraussi,* 13 were commercially available and were purchased from Sigma-Aldrich (Castle Hill, Australia), Alfa-Aesar (United Kingdom), Nu-Chek-Prep, INC (Minnipolis, USA). This included 2-ethyl-1-hexanol (≥ 98%) (**1**), diethyl succinate (99%) (**8**), methyl laurate (≥ 98%) (**12**), ethyl laurate (≥ 98%) (**13**), ethyl tridecanoate (99%) (**14**), methyl myristate (≥ 98%) (**16**), ethyl myristate (99%) (**17**), ethyl myristoleate (97%) (**18**), isoamyl laurate (≥ 97%) (**19**), methyl palmitate (≥ 99%) (**20**), methyl palmitoleate (≥ 99%) (**21**), ethyl palmitate (≥ 99%) (**22**), ethyl oleate (98%) (**25**). (*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**2**), 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**3**), *N*-(2-methylbutyl)acetamide (**5**), *N*-(3-methylbutyl)acetamide (**6**), 6-oxononan-1-ol (**11**), propyl laurate (**15**), ethyl palmitoleate (**23**) and ethyl elaidate (**24**) were not available commercially, and were synthesised following literature procedures (see Electronic Supplementary Material for synthesis details). 2-Methyl-6-pentyl-3,4-dihydro-2*H*-pyran (**4**), (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**7**), (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**9**) and 2,8-dimethyl-1,7-dioxaspiro[5.5]undecan-3-ol (**10**) were tentatively identified based on the literature mass spectral fragmentation pattern (Perkins 1990; Fletcher et al. 1992; Booth et al. 2007). All compounds were found to have identical GC-MS profiles to samples in the extracts, confirming their presence in the headspace samples and/or rectal gland extracts. The relative percentage of each compound in the rectal gland blend or headspace was obtained by dividing its individual peak area by the total peak area and multiplying the result by 100.

Electrophysiology

The response of female and male antennae to the rectal gland extract of the opposite sex was evaluated using coupled gas chromatography-electroantennogram detection (GC-EAD) analysis. The system comprised of an Agilent 7890B gas chromatograph, using a capillary column with SH-Rtx-35 (30 m × 0.25 mm ID × 0.25 μm film thickness) fused silica capillary and hydrogen (99.999% pure) supplied by a generator (MGG-2500-220 Parker Balston, New York) with a constant flow of 2.5 mL/min as a carrier gas. The

temperature program was 50 °C (1 min) to 250 °C (3 min) at a rate of 10 °C/min, with an injector and detector temperature of 270 °C and 290 °C, respectively. The effluent of the column was mixed with 30 mL/min make-up nitrogen gas and split at 1:1.5 (*v/v*) ratio, with one part going to the internal FID and the other through a heated transfer line (TC-02, Syntech, Hilversum, The Netherlands), kept at constant temperature of 200 °C. Male rectal gland extracts and female rectal gland extracts were separately subjected to using heads from females and males *B. kraussi*, respectively, to detect active compounds.

The head of a male or female fly was mounted between two silver wires with capillary electrodes filled with an electrically conductive gel (Spectra 360). One electrode was placed at the tip of an antenna and the other electrode at the back of the head. The mounted heads were under charcoal filtered and humidified air flow (400 mL/min) controlled by a flow controller (Syntech Stimulus Controller CS-55, Syntech, Hilversum, The Netherlands). Signals were captured and processed with a data acquisition controller (IDAC-4, Syntech, Hilversum, The Netherlands) and analysed using GC-EAD 2014 software version 1.2.5. Before injection of a sample, the antenna was stimulated with 1-hexanol to check sensitivity, then 1 µL of the rectal gland extract from the opposite sex was injected. Nine GC-EAD recordings per sex were obtained. Responses were considered genuine if present in at least six of the nine replicates collected. The identity of the compounds eliciting electrophysiological response was confirmed by comparing retention times with that of GC-MS chromatograms.

Behavioural assays

The responses of sexually mature (13-18 days old) *B. kraussi* males and females toward the rectal glands content of the same and opposite sex were evaluated using Y-tube olfactometers. The system comprised of a clear acrylic Y shaped tube with one central arm (6.5 cm × 4.5 cm × 5 cm) in which the release chamber (5cm × 5cm × 5cm) was located, and two upwind lateral arms (12.5 cm × 4.5 cm × 5 cm), each of them connected to a rectangular chamber (7.5 cm × 5 cm × 5 cm) (see Electronic Supplementary Material). The Y-tube olfactometer was positioned horizontally on a white table and a humidified and charcoal-filtered air stream was passed through the Y-tube at a flow rate of 140 ± 5 mL/min. The stimulus cartridge was prepared by crushing 15 rectal glands of *B. kraussi* (males or females) on a 1.0 cm^2 filter paper (Advantec, Japan) inserted in a glass Pasteur pipette (145 mm long). The control cartridge was prepared using 1 cm^2 filter paper inserted

in the same type of glass Pasteur pipette. One cartridge of each type was fitted to one of the Y-tube upwind arms using Tygon tubing (Tygon® formula E-3603, Sigma-Aldrich). An individual fly was placed in the release chamber to acclimatize 30 minutes before experiments started at dusk in a controlled environment room, under the same conditions the flies were kept. Every trial lasted 30 minutes. Once the two cartridges (stimulus and control) were connected to the upwind arms, the system was allowed to equilibrate for two minutes and then the barriers of the two upwind arms and the release chamber were removed. A choice was recorded when the fly reached one of the two upwind arms and stayed there for at least one minute. Those flies that did not make any choice, that is, remained in the release chamber, did not reach one of the two upwind arms or did not stay in one arm for one minute, were not counted. For each treatment, at least 42 replicates from responsive flies were carried out. The position (left or right) of the stimulus and the control was alternated every trial to counter potential positional effects. Each fly was tested only once and fresh rectal glands were used each day. Before each replicate, the Y-tube olfactometer was washed with 5% Extran aqueous solution, rinsed with hot tap water and air-dried. To compare the number of flies choosing the stimulus over the control, a binomial test with the probability level of $P < 0.05$ was used.

Results

Analysis of Rectal Gland Extracts and Headspace Collections

The composition of *B. kraussi* rectal gland extracts and volatile emissions from sexually mature males and females is presented in Table 1. There were distinct differences in the volatile profiles of females and males. Spiroacetals were the dominant class of compounds in male rectal gland extracts whereas saturated/unsaturated esters were the main class of compounds in female rectal gland extracts.

For *B. kraussi* males, the identified compounds included five spiroacetals (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**2**), 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**3**), (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**7**), (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**9**), 2,8-dimethyl-1,7-dioxaspiro[5.5]undecan-3-ol (**10**), two amides *N*-(2-methylbutyl)acetamide (**5**) and *N*-(3-methylbutyl)acetamide (**6**), and 2-ethyl-1-hexanol (**1**), 2-methyl-6-pentyl-3,4-dihydro-2*H*-pyran (**4**), diethyl succinate **(8)** and 6-

oxononan-1-ol (**11**). In the headspace collections for males, only 2-ethyl-1-hexanol (**1**), (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**2**), *N*-(2-methylbutyl)acetamide (**5**), *N*-(3-methylbutyl)acetamide (**6**) and (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**7**) were detected. The most abundant compound in both male rectal gland extracts and headspace collections was (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**2**), representing about 86% and 45% of the blends, respectively. Male volatile emissions included *N*-(3-methylbutyl)acetamide (**6**) at a similar ratio as the main compound (~45%).

For *B. kraussi* females, a total of eighteen compounds were identified in rectal gland extracts (Table 1), including three spiroacetals (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**2**), 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**3**), (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**7**), one amide *N*-(3-methylbutyl)acetamide (**6**), 14 esters methyl laurate (**12**), ethyl laurate (**13**), ethyl tridecanoate (**14**), propyl laurate (**15**), methyl myristate (**16**), ethyl myristate (**17**), ethyl myristoleate (**18**), isoamyl laurate (**19**), methyl palmitate (**20**), methyl palmitoleate (**21**), ethyl palmitate (**22**), ethyl palmitoleate (**23**), ethyl elaidate (**24**) and ethyl oleate (**25**). Of these, thirteen compounds, **2**, **6**, **7**, **12**-**19**, **22** and **23,** were also detected in headspace samples. The main compound present in female gland extracts and headspace samples was the most volatile ethyl ester, ethyl laurate (**13**), although it was found in higher proportions in the headspace samples (70% *vs* 39%). The second major compound, ethyl myristate (**17**), had similar relative abundance in rectal gland extracts and headspace samples (26% and 24%, respectively).

Electrophysiology

Figure 1 illustrates the electroantennographic and electropalpographic response of male and female *B. kraussi* to the rectal gland extract of conspecific males and females. The three most abundant esters emitted by *B. kraussi* females, ethyl laurate (**13**), ethyl myristate (**17**) and ethyl palmitate (**22**), and spiroacetal (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**2**), elicited antennal responses from male *B. kraussi*. Among male rectal gland components, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**2**) elicited antennal and palpal responses from males. Male palps also responded to (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**2**), methyl laurate (**12**) and ethyl laurate (**13**) from female rectal gland extracts. Female maxillary palps and antenna shared the detection of two spiroacetals, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**2**) and (*E*,*E*)-2-ethyl-8-

methyl-1,7-dioxaspiro[5.5]undecane (**7**). No other compounds elicited palpal or antennal responses in females.

Behavioural assays

Sexually mature *B. kraussi* males significantly preferred the upwind arm containing female rectal glands over the control upwind arm ($P = 0.0233$) (Fig. 2). In contrast, females did not show any preference for male rectal glands over the control ($P = 0.117$) and neither females nor males showed any preference when the rectal glands content of the same conspecific sex was presented ($P = 0.104$ and $P = 0.108$, respectively) (Fig. 2). The percentages of responsive flies were 81% and 73% when males were presented to female and male natural blends, respectively, and 82% and 79% when females were presented to male and female natural blends, respectively.

Discussion

The present study is the first to identify the chemical profiles of female *B. kraussi* and is also the first report of electrophysiological detection and behavioural evaluation of volatiles produced by *B. kraussi*. The present study also confirms and expands upon compounds identified in previous studies of male chemical profiles (Fletcher et al. 1992). This combined analysis of both rectal gland extracts and headspace (volatile) collections with GC-EAD/EPD and behavioural assays provides a valuable starting point for understanding chemical communication of *B. kraussi*.

Compounds **2**, **3**, **4**, **6** and **8**-**11**, found in sexually mature male *B. kraussi*, have been previously reported in *B. kraussi* males, with the spiroacetal **2** reported as the major component of both rectal gland extracts and headspace volatile emissions (Fletcher et al. 1992). While 2-hydroxyundecan-6-one and 3-methylbutan-1-ol were not detected in our headspace collections, we detected *N*-(2-methylbutyl)acetamide (**5**) and (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**7**), which have not been previously reported. The amide, *N*-(2-methylbutyl)acetamide found in this study has been previously reported in rectal glands of other fruit fly species (Bellas and Fletcher 1979; Baker et al. 1980). The spiroacetal (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane has been previously reported in male-produced volatiles of *B. nigrotibialis* (Perkins), *B. halfordiae* (Tryon), *B.*

dorsalis (Hendel), *B. kirki* (Froggatt), *B. latifrons* (Hendel) and *B. occipitalis* (Bezzi) as well as female-produced volatiles of *B. tryoni* (Froggatt) and *B. musae* (Tryon) (Perkins et al. 1990; Symonds et al. 2009; Benelli et al. 2014; El-Sayed et al. 2019; Noushini et al. 2020). Despite the similar sensitivity of the detection system employed in the present study to that of the previous study (Fletcher et al. 1992), two previously reported minor compounds, 3-methylbutan-1-ol and 2-hydroxyundecan-6-one, were not detected. The previous study identified compounds *via* GC-MS on a Hewlett- Packard 5970 Series GC-MS system using a non-polar column and a Finnigan Mat 1020 GC-MS. In the present study, compounds were identified by GC-MS on a Shimadzu GCMS-QP2010, using a mid-polarity phase column. Given that 2-hydroxyundecan-6-one is the open chain hydrated form of 2-methyl-6-pentyl-3,4-dihydro-2*H*-pyran (**4**), which is related biosynthetically to spiroacetals (Fletcher et al. 1992; Fletcher and Kitching 1995), perhaps this compound was not present in their rectal gland at the time of the day that samples were collected.

Sexually mature female *B. kraussi* released a more complex blend with lower volatility than males and with a very different dominance of compounds. As seen in both the rectal gland extracts and headspace collections (Table 1), the major compounds identified were ethyl laurate (**13**) and ethyl myristate (**17**). These have been also reported as major constituents of rectal glands and airborne volatiles of *B. musae* females (Noushini et al. 2020). The spiroacetals (*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**2**), 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**3**) and (*E,E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**7**) were present in females, but in minor amounts. Compounds **12** to **25** were female specific, with methyl laurate, ethyl laurate, ethyl myristate and ethyl palmitate EAD/EPD active. The saturated/unsaturated esters, methyl laurate (**12**), ethyl laurate (**13**), ethyl tridecanoate (**14**), methyl myristate (**16**), ethyl myristate (**17**), ethyl myristoleate (**18**), methyl palmitate (**20**), ethyl palmitate (**22**), ethyl palmitoleate (**23**), ethyl elaidate (**24**) and ethyl oleate (**25**) have also been reported in other *Bactrocera* species but in different proportions in each species (Fletcher and Kitching 1995; Canale et al. 2015; El-Sayed et al. 2019; Levi-zada et al. 2020; Noushini et al. 2020). Methyl laurate, ethyl laurate, ethyl myristate and ethyl palmitate have been also reported as EAD active compounds for males and females of *B. oleae*, in which ethyl laurate and methyl palmitate attracted conspecific females and males, respectively (Canale et al. 2015). Similarly (*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and ethyl laurate have been found to be EAD active for *B. musae* (Noushini et al. 2020). Our EAD/EPD and Y-tube olfactometer

behavioural assay results suggest *B. kraussi* uses some of the esters as mate-attracting sex pheromone, as well as for species identification.

Male and female antennae elicited EAD responses to four (**2**, **13**, **17** and **22**) and two (**2** and **7**) components, respectively, of natural blends of rectal gland extracts (Fig. 1). Although female maxillary palps detected the same compounds as female antenna, male palps and antenna detected different compounds. Differences in olfactory function between antennae and maxillary palps are known in other *Bactrocera* species, including *B. tryoni* (Verschut et al. 2018) and *B. depressa* (Shiraki) (Oh et al. 2019). Electropalpographic responses of males of these species to cuelure, a male specific lure, were higher than electroantennographic responses, suggesting that palps might serve in detection of some long-range odorants (Verschut et al. 2018; Oh et al. 2019). A functional study of antennae and palps of *B. kraussi* would help to clarify the roles of these organs in the detection of volatiles produced by conspecifics.

The electrophysiological results indicate that either or both sexes may respond behaviourally to the natural blends, with attraction being a key response to consider. Y-Tube olfactometry is an appropriate method to test attraction (Canale et al. 2015). Interestingly, only male attraction to female rectal gland volatiles was significant, suggesting that female rectal gland excretions may serve a role as mate-attracting sex pheromones in this species (Fig. 2). As mentioned above, female-produced sex pheromones have been reported in other Dacine fruit flies (Benelli et al. 2014): in *B. oleae* 1,7-dioxaspiro[5,5]undecane produced by females attracts males (Baker and Herbert 1987; Benelli et al. 2014), in *Z. cucurbitae* isomers of 2,8-dialkyl-1,7-dioxaspiro[5,5]undecanes and *N*-(3-methylbutyl)acetamide produced by females attract males (Baker and Bacon 1985), and in *B. dorsalis* spiroacetals including (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, (*E*,*E*)-8-ethyl-2-methyl-1,7-dioxaspiro[5.5]undecane and (*E*,*E*)-8-methyl-2-propyl-1,7-dioxaspiro[5.5]undecane produced by females attract males (Baker and Bacon 1985). While our bioassay results suggest that rectal gland exudates may not be key for aggregation of males or females, or for mate attraction by males, there are other potential functions of these products, such as species or sex identification and quality assessment, that cannot be excluded and warrant investigation. It may also be that male-produced emissions lose some aspects of function when isolated from the visual cues and sounds produced by the rapid wing fanning that is characteristic of calling behaviour.

Acknowledgments

We thank the Queensland Department of Agriculture and Fishers (QDAF) for providing fruit flies and pupae. We thank Daintree Rainforest Observatory (James Cook University) for providing space for the 2016 sample collection. We also acknowledge Stefano De Faveri, Peter Leach and Sybilla Oczkowicz for their help in sample collections and providing laboratory space. This research was funded through the Australian Research Council Industrial Transformation Training Centre (ITTC) for Fruit Fly Biosecurity Innovation (Project IC50100026), funded by the Australian Government.

References

Baker R, Bacon AJ (1985) The identification of spiroacetals in the volatile secretions of two species of fruit fly (*Dacus dorsalis, Dacus curcurbitae*). Experientia 41:1484–1485. doi: 10.1007/BF01950049

Baker R, Herbert R, Howse PE, et al (1980) Identification and synthesis of the major sex pheromone of the olive fly (*Dacus oleae*). J Chem Soc Chem Commun 52. doi: 10.1039/c39800000052

Baker R, Herbert RH (1987) Isolation and synthesis of 1,7-dioxaspiro[5.5]undecane and 1,7-dioxaspiro[5.5]undecan-3-and -4-ols from the olive fly (*Dacus oleae*). J Chem Soc Perkin Trans 1 1123. doi: 10.1039/p19870001123

Baker R, Herbert RH, Lomer RA (1982) Chemical components of the rectal gland secretions of male *Dacus cucurbitae*, the melon fly. Experientia 38:232–233. doi: 10.1007/BF01945082

Bellas TE, Fletcher BS (1979) Identification of the major components in the secretion from the rectal pheromone glands of the Queensland fruit flies *Dacus tryoni* and *Dacus neohumeralis* (Diptera: Tephritidae). J Chem Ecol 5:795–803. doi: 10.1007/BF00986564

Benelli G, Daane KM, Canale A, et al (2014) Sexual communication and related behaviours in Tephritidae: current knowledge and potential applications for integrated pest management. J Pest Sci 87:385–405. doi: 10.1007/s10340-014-0577-3

Booth YK, Hayes PY, Moore CJ, et al (2007) Synthesis and absolute configuration of a constitutionally-new [5.6] spiroacetal from *B. tryoni* (Queensland fruit fly). Org

Biomol Chem 5:1111–1117. doi: 10.1039/B701833A

Booth YK, Schwartz BD, Fletcher MT, et al (2006) A diverse suite of spiroacetals, including a novel branched representative, is released by female Bactrocera tryoni (Queensland fruit fly). Chem Commun 3975–3977. doi: 10.1039/B611953K

Canale A, Benelli G, Germinara GS, et al (2015) Behavioural and electrophysiological responses to overlooked female pheromone components in the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae). Chemoecology 25:147–157. doi: 10.1007/s00049-014-0183-0

Canale A, Germinara SG, Carpita A, et al (2013) Behavioural and electrophysiological responses of the olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), to male- and female-borne sex attractants. Chemoecology 23:155–164. doi: 10.1007/s00049-013-0131-4

Carpita A, Canale A, Raffaelli A, et al (2012) (*Z*)-9-tricosene identified in rectal gland extracts of *Bactrocera oleae* males: first evidence of a male-produced female attractant in olive fruit fly. Naturwissenschaften 99:77–81. doi: 10.1007/s00114-011-0868-y

Cruz-López L, Malo EA, Rojas JC (2015) Sex pheromone of *Anastrepha striata*. J Chem Ecol 41:458–464. doi: 10.1007/s10886-015-0581-y

El-Sayed AM (2019) The Pherobase: Database of pheromones and semiochemicals. http://www.pherobase.com/. Accessed 17 Mar 2019

El-Sayed AM, Byers JA, Manning LM, et al (2008) Floral scent of Canada thistle and its potential as a generic insect attractant. J Econ Entomol 101:720–727. doi: 10.1093/jee/101.3.720

El-Sayed AM, Venkatesham U, Unelius CR, et al (2019) Chemical composition of the rectal gland and volatiles released by female Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae). Environ Entomol. doi: 10.1093/ee/nvz061

Fletcher MT, Kitching W (1995) Chemistry of fruit flies. Chem Rev 95:789–828. doi: 10.1021/cr00036a001

Fletcher MT, Wells JA, Jacobs MF, et al (1992) Chemistry of fruit-flies. Spiroacetal-rich secretions in several *Bactrocera* species from the South-West Pacific region. J Chem Soc Perkin Trans 1 2827–2831. doi: 10.1039/P19920002827

Hancock DL, Hamacek EL, Lloyd AC, Elson-Harris MM (2000) The distribution and host plants of fruit flies (Diptera: Tephritidae) in Australia. Queensland Department of Primary Industries, Brisbane, Australia

Haniotakis GE (1974) Sexual attraction in the olive fruit fly, *Dacus oleae* (Gmelin).

444 Environ Entomol 3:82–86. doi: 10.1093/ee/3.1.82
445 Hendrichs J, Robinson AS, Cayol JP, Enkerlin W (2002) Medfly areawide sterile insect
446 technique programmes for prevention, suppression or eradication: the importance of
447 mating behavior studies. Fla Entomol 85:1–14. doi: 10.1653/0015-
448 4040(2002)085[0001:MASITP]2.0.CO;2
449 Kitching W, Lewis JA, Perkins M V, et al (1989) Chemistry of fruit flies. Composition of
450 the rectal gland secretion of (male) *Dacus cucumis* (cucumber fly) and *Dacus*
451 *halfordiae*. Characterization of (*Z,Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. J
452 Org Chem 54:3893–3902. doi: 10.1021/jo00277a028
453 Levi-zada A, Levy A, Rempoulakis P, et al (2020) Diel rhythm of volatile emissions of
454 males and females of the peach fruit fly *Bactrocera zonata*. J Insect Physiol
455 120:103970. doi: 10.1016/j.jinsphys.2019.103970
456 Mazomenos BE, Haniotakis GE (1985) Male olive fruit fly attraction to synthetic sex
457 pheromone components in laboratory and field tests. J Chem Ecol 11:397–405. doi:
458 10.1007/BF01411425
459 Nation JL (1972) Courtship behavior and evidence for a sex attractant in the male
460 Caribbean fruit fly, *Anastrepha suspensa*. Ann Entomol Soc Am 65:1364–1367. doi:
461 10.1093/aesa/65.6.1364
462 Nishida R, Tan KH, Fukami H (1988a) *Cis*-3,4-dimethoxycinnamyl alcohol from the rectal
463 glands of male oriental fruit fly, *Dacus dorsalis*. Chem Express 3:207–210
464 Nishida R, Tan KH, Serit M, et al (1988b) Accumulation of phenylpropanoids in the rectal
465 glands of males of the Oriental fruit fly, *Dacus dorsalis*. Experientia 44:534–536. doi:
466 10.1007/BF01958941
467 Noushini S, Perez J, Park SJ, et al (2020) Rectal gland chemistry, volatile emissions, and
468 antennal responses of male and female banana fruit fly, Bactrocera musae. Insects
469 11:32. doi: 10.3390/INSECTS11010032
470 Oh H, Jeong SA, Kim J, Park KC (2019) Morphological and functional heterogeneity in
471 olfactory perception between antennae and maxillary palps in the pumpkin fruit fly,
472 *Bactrocera depressa*. Arch Insect Biochem Physiol 101:. doi: 10.1002/arch.21560
473 Pérez J, Park SJ, Taylor PW (2018) Domestication modifies the volatile emissions
474 produced by male Queensland fruit flies during sexual advertisement. Sci Rep 8:1–10.
475 doi: 10.1038/s41598-018-34569-3
476 Perkins MV (1990) Characterisation and synthesis of Bactrocera fruit fly pheromones. PhD
477 Thesis, Department of Chemistry.The University of Queensland, Australia
478 Perkins MV, Kitching W, Drew RAI, et al (1990) Chemistry of fruit flies: composition of

the male rectal gland secretions of some species of South-East Asian Dacinae. Re-examination of *Dacus cucurbitae* (melon fly). J Chem Soc Perkin Trans 1 1111–1117. doi: 10.1039/P19900001111

Schutze M, McMahon J, Krosch M, et al (2018) The Australian Handbook for the Identification of Fruit Flies. Version 3.1. Plant Health Australia, Canberra, ACT

Sivinski J, Aluja M, Dodson GN, et al (2000) Topics in the evolution of sexual behavior in the Tephritidae. In: Aluja M, Norrbom AL (eds) Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior. CRC Press, pp 751–792

Sivinski JM, Calkins C (1986) Use of pheromones in tropical crops: pheromones and parapheromones in the control of tephritids. Florida Entomol 69:157–168. doi: 10.2307/3494757

Steiner LF, Mitchell S (1966) Tephritid fruit flies. In: Smith CN (ed) Insect colonization and mass production. Academic Press, pp 555–583

Symonds MRE, Moussalli A, Elgar MA (2009) The evolution of sex pheromones in an ecologically diverse genus of flies. Biol J Linn Soc 97:594–603

Verschut TA, Farnier K, Cunningham JP, Carlsson MA (2018) Behavioral and physiological evidence for palp detection of the male-specific attractant cuelure in the Queensland fruit fly (*Bactrocera tryoni*). Front Physiol 9:. doi: 10.3389/fphys.2018.00990

Witzgall P, Kirsch P, Cork A (2010) Sex pheromones and their impact on pest management. J Chem Ecol 36:80–100. doi: 10.1007/s10886-009-9737-y

Table 1 Chemical profiles of *B. kraussi* adults. RI = retention index, MW = molecular weight, HS = headspace, RG = rectal gland, ND = not detected

RI	Name	MW	Characteristic EI ions *m/z* (%)	Females		Males	
				HS (%)	RG (%)	HS (%)	RG (%)
1027	2-Ethyl-1-hexanol (**1**)	130.2	112 (M – H_2O, 2.1), 99 (β-cleavage product, 1.1), 98 (6.9), 83 (25.8), 70 (25.9), 69 (10.1), 57 (100), 56 ($CH_3CH_2CH{=}CH_2$, 25.3)	ND	ND	3.4	2.3
1140	(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**2**)	184.1	184 (M^+, 8.6), 169 (M – CH_3, 1.6), 140 (14.1), 125 (9.7), 115 ($CH_3(C_5H_7O){=}OH$, 92.2), 112 ($CH_3(C_5H_7O){=}CH_2$, 100), 97 (73.0), 69 (50.9), 55 (67.0)	<1	7.6	45.1	85.6
1162	2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**3**)	184.1	184 (M^+, 3.1), 155 (M – C_2H_5, 23.1), 140 (7.1), 115 (M – $C_5H_9^-$, 100), 112 (M – C_4H_8O, 60.2), 97 (69.4), 85 (60.5), 69 (48.7), 55 (68.5)	ND	<1	ND	<1
1174	2-Methyl-6-pentyl-3,4-dihydro-2*H*-pyran (**4**)	168.3	168 (12.4), 125 (M – C_3H_7, 22.8), 112 ($C_7H_{12}O$, 76.6), 97 (22.9), 84 (21.3), 83 (33.0), 70 (23.5), 55 (100), 43 (76.2)	ND	ND	ND	<1
1212	*N*-(2-Methylbutyl)acetamide (**5**)	129.1	129 (M^+, 9.7), 100 (M – C_2H_5, 34.7), 73 (β-cleavage product, 43.2), 72 (β-cleavage product, 95.6), 60 ($CH_3C(OH)NH^+$, 61.3), 43 (100)	ND	ND	5.1	<1
1219	*N*-(3-methylbutyl)acetamide (**6**)	129.1	129 (M^+, 4.5), 114 (M – CH_3, 9.7), 86 (M – C_3H_7, 25.2), 73 (β-cleavage product, 85.7), 72 (β-cleavage product, 72.2), 60 ($CH_3C(OH)NH^+$, 36.5), 43 (100)	<1	<1	44.3	2.2
1227	(*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**7**)	198.2	198 (M^+, 9.3), 169 (M - C_2H_5, 11.2), 140 (12.9), 129 ($CH_3CH_2(C_5H_7O){=}OH^+$, 40.2), 126 ($CH_3CH_2(C_5H_7O){=}CH_2$, 30.0), 115 $CH_3(C_5H_7O){=}OH$, 87.7), 112 ($CH_3(C_5H_7O){=}CH_2$, 83.0), 97 (58.4), 83 (55.5), 69 (67.7), 55 (100)	<1	<1	2.1	2.7
1246	Diethyl succinate (**8**)	174.2	174 (M^+, 0.7), 129 (M – OC_2H_5, 53.5), 128 (14.1), 101 (M – $COOC_2H_5$, 100), 73 (26.0), 74 (13.4), 55(32.6), 45 (18.5), 43 (10.1)	ND	ND	ND	<1
1321	(*Z*,*Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**9**)	184.1	184 (M^+, 4.3), 169 (M – CH_3, 2.8), 140 (3.9), 125 (6.5), 115 ($CH_3(C_5H_7O){=}OH$, 100), 112 ($CH_3(C_5H_7O){=}CH_2$, 34.4), 97 (71.7), 69 (71.0), 55 (60.0)	ND	ND	ND	<1
1421	2,8-Dimethyl-1,7-dioxaspiro[5.5]undecan-3-ol (**10**)	200.3	200 (M^+, 3.2), 156 (34.2), 128 (5.9), 112 ($CH_3(C_5H_7O){=}CH_2$, 100), 97 (28.6), 83 (31.2), 55 (23.9)	ND	ND	ND	<1

1431	6-Oxononan-1-ol (**11**)	158.2	158 (M^+, 0.6), 140 (M – H_2O, 1.4), 115 (9.4), 112 (2.9), 99 (3.6), 97 (20.2), 86 (33.1), 79 (9.6), 73 (12.7), 71 (67.1), 69 (64.0), 58 (53.2), 55 (29.9), 43 (100), 41 (66.8)	ND	ND	ND	4.9
1531	Methyl laurate (**12**)	214.2	214 (M^+, 2.6), 183 (M – OCH_3, 3.7), 171 (5.0), 143 (7.0), 129 (4.7), 87 (55.9), 74 (McLafferty rearrangement product, 100), 59 ($COOCH_3$, 10.8), 55 (29.1)	<1	<1	ND	ND
1593	Ethyl laurate (**13**)	228.4	228 (M^+, 2.8), 199 (M – C_2H_5, 1.9), 183 (M – OC_2H_5, 5.6), 157 (7.6), 101 (35.9), 88 (McLafferty rearrangement product, 100), 73 ($COOC_2H_5$, 20.7), 70 (21.8), 61 (14.9), 60 (13.7), 55 (27.0)	70.2	39.1	ND	ND
1661	Ethyl tridecanoate (**14**)	242.2	242 (M^+, 3.4), 213 (M – C_2H_5, 5.1), 197 (M – OC_2H_5, 2.1), 199 (12.4), 157 (13.7), 101 (59.3), 88 (McLafferty rearrangement product, 100), 73 ($COOC_2H_5$, 26.5), 57 (50.2), 55 (44.7)	<1	<1	ND	ND
1686	Propyl laurate (**15**)	242.2	242 (M^+, 1.5), 201 (21.9), 199 (M – C_3H_7, 3.4), 183 (M – OC_3H_7, 25.8), 157 (6.3), 129 (9.3), 115 (16.9), 102 (McLafferty rearrangement product, 29.3), 87 ($COOC_3H_7$, 9.8), 61 (100), 59 (6.1), 55 (33.5)	<1	<1	ND	ND
1727	Methyl myristate (**16**)	242.2	242 (M^+, 2.9), 211 (M – OCH_3, 1.2), 199 (5.8), 143 (7.5), 125 (7.3), 111 (19.2), 101 (5.0), 97 (32.3), 87 (47.2), 74 (McLafferty rearrangement product, 100), 59 ($COOCH_3$, 8.6), 55 (66.7)	<1	4.1	ND	ND
1789	Ethyl myristate (**17**)	256.4	256 (M^+, 4.4), 211 (M – OC_2H_5, 6.0), 213 (5.8), 157 (10.1), 101 (46.2), 88 (McLafferty rearrangement product, 100), 73 ($COOC_2H_5$, 21.8), 70 (22.7), 55 (32.2)	23.7	25.6	ND	ND
1797	Ethyl myristoleate (**18**)	254.2	254 (M^+, 2.5), 209 (M – OC_2H_5, 6.0), 208 (M – C_2H_5OH, 7.1), 166 (8.4), 124 (10.3), 88 (McLafferty rearrangement product, 32.8), 73 ($COOC_2H_5$, 14.6), 69 (45.4), 55 (100)	<1	<1	ND	ND
1833	Isoamyl laurate (**19**)	270.5	270 (M^+, 1.0), 201 (1.5), 183 (M – OC_5H_{11}, 4.3), 115 ($COOC_5H_{11}$, 2.0), 70 (100), 71(34.9), 55 (18.9), 43 (46.4)	<1	<1	ND	ND
1920	Methyl palmitate (**20**)	270.3	270 (M^+, 1.7), 227 (1.7), 143 (9.8), 87 (36.9), 74 (McLafferty rearrangement product, 100), 59 ($COOCH_3$, 9.3), 55 (81.1)	ND	<1	ND	ND

1932	Methyl palmitoleate (**21**)	268.4	268 (M^+, 3.5), 237 (M – OCH_3, 9.1), 236 (M – CH_3OH, 12.8), 194 (12.2), 152 (11.0), 96 (33.3), 74 (McLafferty rearrangement product, 53.7), 59 ($COOCH_3$, 19.9), 55 (100)	ND	<1	ND	ND
1984	Ethyl palmitate (**22**)	284.3	284 (M^+, 4.2), 241 (4.8), 157 (9.5), 101 (50.9), 255 (M – C_2H_5, 1.1), 239 (M – OC_2H_5, 3.9), 88 (McLafferty rearrangement product, 100), 73 ($COOC_2H_5$, 21.3), 55 (41.3), 43 (54.4)	<1	8.9	ND	ND
1994	Ethyl palmitoleate (**23**)	282.3	282 (M^+, 1.9), 237 (M – OC_2H_5, 10.1), 236 (M – C_2H_5OH, 10.7), 194 (10.0), 152 (8.8), 88 (McLafferty rearrangement product, 39.0), 73 ($COOC_2H_5$, 29.9), 69 (67.0), 55 (100)	<1	8.7	ND	ND
2176	Ethyl elaidate (**24**)	310.3	310 (M^+, 0.6), 265 (M – OC_2H_5, 6.8), 264 (M – C_2H_5OH, 10.0), 222 (7.3), 180 (7.1), 123 (9.5), 110 (16.7), 97 (38.5), 88 (McLafferty rearrangement product, 33.2), 83 (44.4), 73 ($COOC_2H_5$, 13.6), 69 (65.4), 55 (100), 43 (60.7), 41 (84.3)	ND	3.1	ND	ND
2182	Ethyl oleate (**25**)	310.3	310 (M^+, 1.9), 265 (M – OC_2H_5, 5.4), 264 (M – C_2H_5OH, 8.3), 222 (7.1), 180 (7.2), 123 (7.9), 110 (12.5), 97 (37.4), 88 (McLafferty rearrangement product, 25.6), 83 (42.3), 73 ($COOC_2H_5$, 11.2), 69 (71.6), 55 (100), 43 (50.2), 41 (71.5)	ND	2.5	ND	ND

Figure legends

Fig. 1 Simultaneous response of flame ionisation detector (FID) and electroantennographic detection (EAD)/electropalpographic detection (EPD) using *Bactrocera kraussi* male and female antenna and maxillary palps to rectal gland extract from conspecifics. Numbered peaks indicate electrophysiologically active compounds: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**2**) and (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (**7**), methyl laurate (**12**), ethyl laurate (**13**), ethyl myristate (**17**), ethyl palmitate (**22**)

Fig. 2 Response of sexually mature virgin *Bactrocera kraussi* males and females to rectal gland volatiles of the same and opposite sex, *vs* control (clean filter paper), in Y-tube olfactometer behavioural assays. * = significantly different at 0.02 level, ns = not significantly different, *n* = number of responding flies

Fig. 1

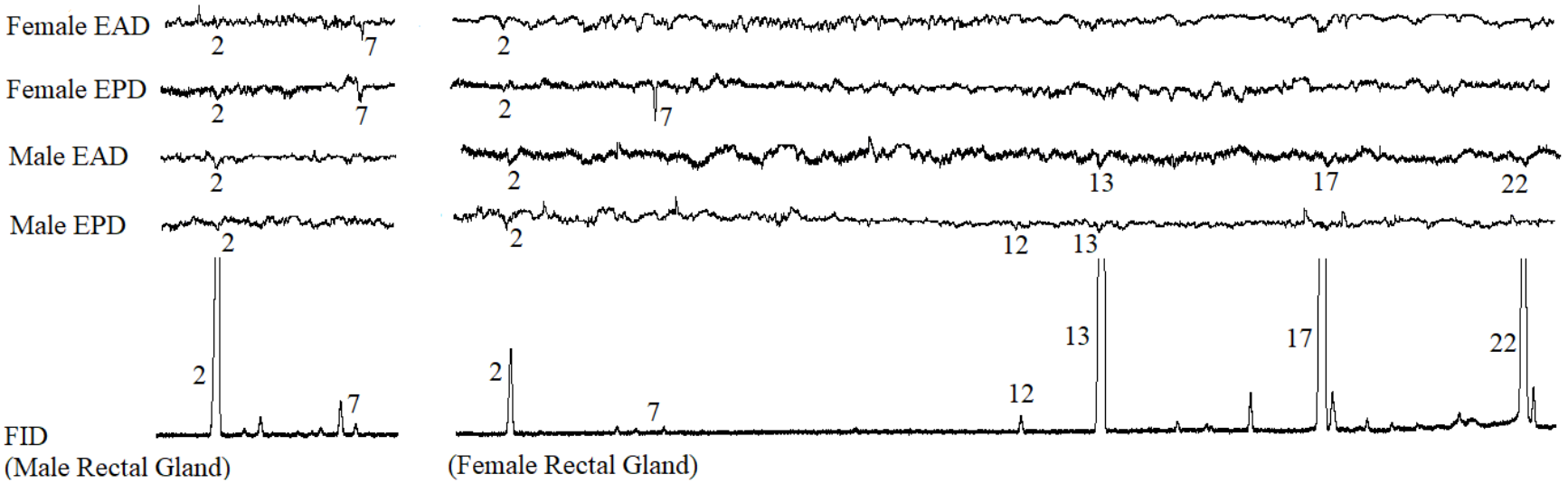

Female EAD
Female EPD
Male EAD
Male EPD
FID
(Male Rectal Gland)
(Female Rectal Gland)
2
7
12
13
17
22

Fig. 2

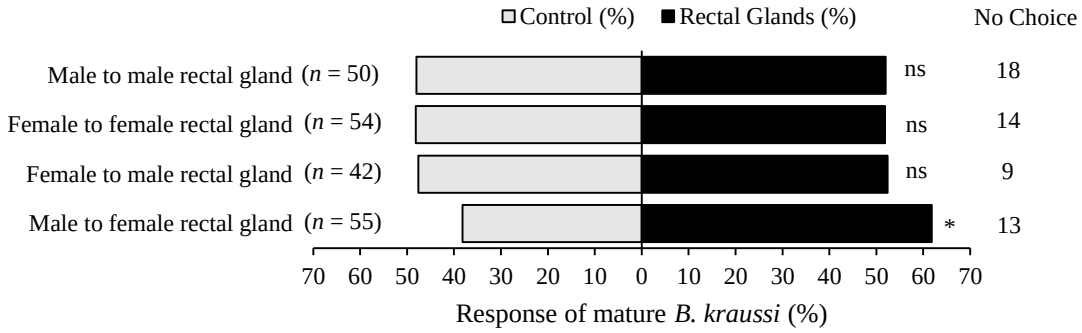
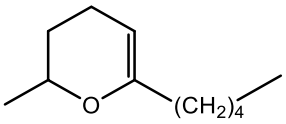
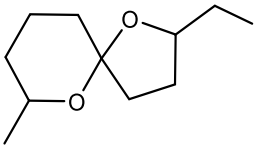

Control (%)
Rectal Glands (%)
No Choice
Male to male rectal gland (n = 50)
Female to female rectal gland (n = 54)
Female to male rectal gland (n = 42)
Male to female rectal gland (n = 55)
ns
ns
ns
*
18
14
9
13
70 60 50 40 30 20 10 0 10 20 30 40 50 60 70
Response of mature B. kraussi (%)

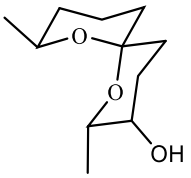
Figure 3. Structure of compounds identified in *Bactrocera kraussi* chemical profile.



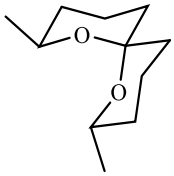
2-Methyl-6-pentyl-3,4-dihydro-2*H*-pyran



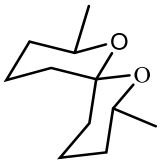
2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane



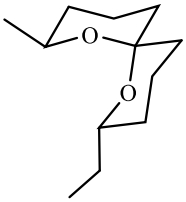
2,8-Dimethyl-1,7-dioxaspiro[5.5]undecan-3-ol



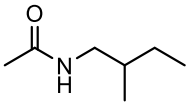
(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane



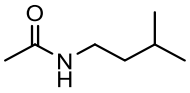
(*Z*,*Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane



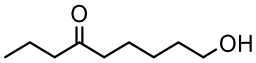
(*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane



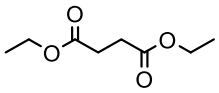
N-(2-Methylbutyl)acetamide



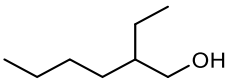
N-(3-methylbutyl)acetamide



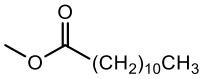
6-Oxononan-1-ol



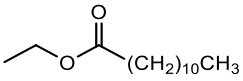
Diethyl succinate



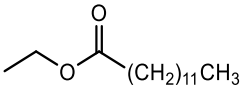
2-Ethyl-1-hexanol



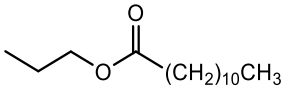
Methyl laurate



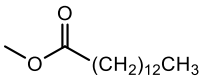
Ethyl laurate



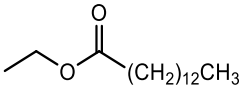
Ethyl tridecanoate



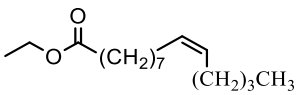
Propyl laurate



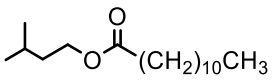
Methyl myristate



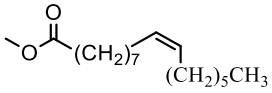
Ethyl myristate



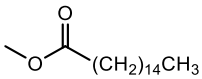
Ethyl myristoleate



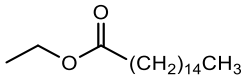
Isoamyl laurate



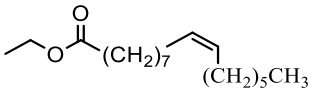
Methyl palmitoleate



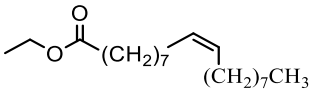
Methyl palmitate



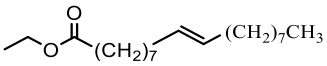
Ethyl palmitate



Ethyl palmitoleate



Ethyl oleate



Ethyl elaidate

Chapter Six

Sampling technique biases in the analysis of fruit fly volatiles: A case study of Queensland fruit fly

Published in Scientific Reports

Author contributions:
All experiments were conducted by S. Noushini. Data analysis was done by S. Noushini and P. Taylor. The manuscript drafted by S. Noushini. All authors read the manuscript and provided critical feedback.

At the end of this chapter, after the manuscript, figure 5 has been provided summarising structure of all identified compounds in male and female *Bactrocera tryoni*.

Noushini, S., Park, S. J., Jamie, I., Jamie, J., & Taylor, P. (2020). Sampling technique biases in the analysis of fruit fly volatiles: a case study of Queensland fruit fly. *Scientific Reports*, *10*, 19799.

DOI: https://doi.org/10.1038/s41598-020-76622-0

scientific reports

Check for updates

OPEN

Sampling technique biases in the analysis of fruit fly volatiles: a case study of Queensland fruit fly

Saeedeh Noushini[1,3]✉, Soo Jean Park[2,3], Ian Jamie[1,3], Joanne Jamie[1] & Phillip Taylor[2,3]

Diverse methods have been used to sample insect semiochemicals. Sampling methods can differ in efficiency and affinity and this can introduce significant biases when interpreting biological patterns. We compare common methods used to sample tephritid fruit fly rectal gland volatiles ('pheromones'), focusing on Queensland fruit fly, *Bactrocera tryoni*. Solvents of different polarity, *n*-hexane, dichloromethane and ethanol, were compared using intact and crushed glands. Polydimethylsiloxane, polydimethylsiloxane/divinylbenzene and polyacrylate were compared as adsorbents for solid phase microextraction. Tenax-GR and Porapak Q were compared as adsorbents for dynamic headspace sampling. Along with compounds previously reported for *B. tryoni*, we detected five previously unreported compounds in males, and three in females. Dichloromethane extracted more amides while there was no significant difference between the three solvents in extraction of spiroacetals except for (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane for which *n*-hexane extracted higher amount than both dichloromethane and ethanol. Ethanol failed to contain many of the more volatile compounds. Crushed rectal gland samples provided higher concentrations of extracted compounds than intact rectal gland samples, but no compounds were missed in intact samples. Of solid phase microextraction fibers, polyacrylate had low affinity for spiroacetals, ethyl isobutyrate and ethyl-2-methylbutanoate. Polydimethylsiloxane was more efficient for spiroacetals while type of fiber did not affect the amounts of amides and esters. In dynamic headspace sampling, Porapak was more efficient for ethyl isobutyrate and spiroacetals, while Tenax was more efficient for other esters and amides, and sampling time was a critical factor. Biases that can be introduced by sampling methods are important considerations when collecting and interpreting insect semiochemical profiles.

Semiochemicals, including pheromones, are of central importance in the biology of many insects, including tephritid fruit flies. Because semiochemicals are commonly produced and released at low concentrations, efficient sampling methods are needed for collection and subsequent identification and quantification[1–5]. Tephritid fruit flies typically store pheromones in rectal glands and release them into the air during sexual activity[4,6–13]. Diverse sampling methods have been used to sample fruit fly pheromones and, in addition to genuine biological differences, some variation in pheromones reported for different fruit flies may actually arise from differences in the chemical collection efficiencies of the sampling methods used. The most common method entails immersion of rectal glands in organic solvents. Common solvents that have been used for fruit fly rectal gland extraction vary in polarity and include *n*-pentane, *n*-hexane, acetone, dichloromethane and ethanol[12,14–17]. In some studies the glands have been intact while in others the glands have been crushed[18–20].

Rather than focusing on compounds stored in the rectal glands, some studies have instead focused on collecting the emitted volatiles. While this approach does not identify the glandular source of the emissions, it has the advantage of being a whole-animal method, thus detecting volatiles that might be produced and emitted by glands other than the rectal glands, and hence more fully represents the array of compounds, and blends, that might be encountered by receivers. In most studies, emitted volatiles are trapped onto an adsorbent material using either dynamic or static sampling techniques[21]. Dynamic headspace sampling techniques involve passing an airflow to purge and trap volatiles onto an adsorbent material such as Porapak (ethylvinylbenzene-divinylbenzene copolymer), activated charcoal, or Tenax (porous polymer based on 2,6-diphenyl-*p*-phenylene oxide). Tenax[15,22,23] and Porapak Q[24,25] have been widely used in sampling of fruit fly pheromones. Static headspace sampling techniques

[1]Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia. [2]Applied BioSciences, Macquarie University, Sydney, NSW 2109, Australia. [3]Australian Research Council Industrial Transformation Training Centre for Fruit Fly Biosecurity Innovation, Macquarie University, Sydney, NSW 2109, Australia. ✉email: sally.noushini@mq.edu.au

involve use of adsorbent materials without airflow. The most commonly used static sampling method utilizes SPME (solid phase microextraction) adsorbent fibers, such as polydimethylsiloxane (PDMS), carboxen (CAR), divinylbenzene (DVB), polyacrylate (PA), or a mixed-phase coating, which vary in efficiency depending on the polarity of targeted compounds. PDMS fibers are widely used for collection of non-polar compounds[6,26–28]. Polyacrylate (PA) has a high affinity to more polar compounds and hence has been used for polar semiochemicals[29,30]. PDMS/divinylbenzene (DVB), a mixed-phase coating that covers a broader spectrum due to their distinct polarity, has also been used for collection of semiochemicals[19,20,29,31–33]. Because adsorbent materials differ in affinity for particular groups of semiochemicals, a poor choice of material can result in substantial under-sampling, or even failure to even detect some compounds.

Bactrocera tryoni is the most economically important pest fruit fly in Australia[34,35], being highly polyphagous and attacking most fruit crops[36]. Volatile profiles of male and female *B. tryoni* have been described previously[19,37–39]. Similar to many other fruit flies, *B. tryoni* stores secreted volatiles in the rectal glands[7,8,40,41]. *Bactrocera tryoni* mating is limited to a period of about 30 min at dusk[37,41,42]. During calling and courtship, males release the sweet-smelling volatile blend containing six aliphatic amides that have been generally interpreted as sex pheromones[7,37,41–45]. Although the functions of the individual components of the male *B. tryoni* sex pheromone blend have not been studied, virgin mature females are attracted to volatiles from crushed male glands or calling males[43,44]. The secretions reported for *B. tryoni* females have differed somewhat between studies, and this may reflect differences in sampling methods[19,38]. Booth et al.[19] reported a diverse suite of spiroacetals as predominant compounds from *n*-pentane extracts of the whole crushed abdomen, while El-Sayed et al.[38] found saturated/unsaturated esters as predominant compounds from *n*-hexane extracts of intact rectal glands. The contrast between these studies may indicate that while spiroacetals are not present in the rectal glands they may be produced in other glands elsewhere in the abdomen[38].

The present study considers the effects of sampling methods on the detection and quantification of fruit fly volatiles, using *B. tryoni* as a model species. The main purpose of this study was to highlight advantages and disadvantages of the different methods adopted for rectal gland extractions and headspace collection. We investigated the effect of (1) solvent polarity, (2) crushing of sampled glands in solvent, (3) adsorbent types in both dynamic and static sampling techniques, and (4) volume of air sampled in dynamic sampling techniques. In addition, we identified six previously unreported compounds in male *B. tryoni* rectal gland contents/emissions and three in females. These compounds resolve a long-standing discord between the perceptible odor and known blend composition in *B. tryoni*.

Materials and methods

Insects. All experiments were conducted using *B. tryoni* from a laboratory culture at Macquarie University, Sydney, Australia (originating from central coastal New South Wales, G27). Adults were provided sugar and yeast hydrolysate (MP Biomedicals LLC) as food, and tap water through a soaked sponge. Virgin male and female flies were segregated within 4 days after eclosion, transferred to 12.5 L clear plastic cages (180 flies per cage) and maintained in controlled environment rooms (25 ± 0.5 °C, 65 ± 5% relative humidity (RH) and 11.5: 0.5: 11.5: 0.5 light/dusk/dark/dawn photoperiod) until they were used in experiments. No calling or mating was observed prior to separating the sexes. Flies used for all experiments were 13—18 days old (sexually mature) virgins (see Perez-Staples et al.[46]).

Chemicals. *n*-Hexane, dichloromethane, ethanol and the following chemicals were purchased from Sigma-Aldrich (St Louis, MO, US), Alfa-Aesar (Ward Hill, MA, US), Chem-Supply (Bedford St, Gillman, SA) and Nu-Chek-Prep and INC (Elysian, MN, US), with the purities noted in parentheses, and were used without further purification: hexadecane (99%), ethyl propanoate (99%), ethyl isobutyrate (≥ 98%), ethyl 2-methylbutanoate (99%), propyl isobutyrate (≥ 97%), ethyl 2-methylpentanoate (≥ 98%), diethyl succinate (99%), methyl laurate (≥ 98%), ethyl laurate (≥ 98%), methyl myristate (≥ 98%), ethyl myristate (99%), ethyl myristoleate (97%), methyl palmitoleate (≥ 99%), ethyl palmitate (≥ 99%) and ethyl oleate (98%). Propyl laurate, ethyl palmitoleate, ethyl elaidate, *N*-(2-methylbutyl)acetamide, *N*-(3-methylbutyl)acetamide, *N*-(2-methylbutyl)propanamide, *N*-(3-methylbutyl)propanamide, *N*-(2-methylbutyl)isobutyrate and *N*-(3-methylbutyl)isobutyrate and (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane were synthesized (see Supplementary Information for synthesis details). 2,7-Dimethyl-1,6-dioxaspiro[4.5]decane[47], (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane[18], (*E*,*Z*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane[32], (*E*,*E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane[19] and (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane[32], were tentatively identified based on literature mass spectral fragmentation patterns (Table 1).

GC–MS analysis. Gas Chromatography-Mass Spectrometry (GC–MS) analyses were performed using a Shimadzu GCMS-QP2010 or GCMS-TQ8040 instrument, which was equipped with a capillary column with 5% diphenyl/95% dimethyl polysiloxane as the stationary phase (30 m × 0.25 mm I.D. × 0.25 µm film thickness). Helium (99.999%, BOC, North Ryde, NSW, Australia) at a flow rate of 1.0 mL/min was used as a carrier gas. The oven temperature was held at 50 °C for 4 min or 40 °C for 1 min then programmed at 10 °C/min to 250 °C, with splitless injection mode at 270 °C. The temperatures of interface and ion source were 290 and 200 °C, respectively. Mass detection was performed in EI mode at a voltage of 70 eV. The spectra were obtained over a mass range of 45 to 500 m/*z*. The condition used for determining the Kovats retention index was the same as above, with the oven initial temperature at 40 °C for 1 min.

Rectal gland samples. *n*-Hexane, dichloromethane (DCM) and ethanol (EtOH) were used for separate rectal gland extractions. For each solvent 10 replicates containing 10 glands were collected for each sex. Flies

No	Name	Characteristic EI ions *m/z* (%)	I	KI
1	Ethyl propanoate*	102 (M^+, 10), 74 (14.5), 57 (100)	AS	672
2	Ethyl isobutyrate*	116 (M^+, 24.8), 88 (43.9), 71 (100)	AS	727
3	Ethyl 2-methylbutanoate*	130 (M^+, 1.23), 115 (8.0), 102 (60.8), 85 (37.9), 74 (25.6), 57 (100)	AS	844
4	Propyl isobutyrate*	130 (M^+, 0.5), 102 (8.2), 101 (5.9), 89 (83.7), 71 (100)	AS	850
5	Ethyl 2-methylpentanoate*	144 (M^+, 2.9), 115 (M – C_2H_5, 9.9), 102 (67.5), 99 (M – OC_2H_5, 18.1), 74 (41.3), 55 (23.5), 45 (100)	AS	933
6	*N*-(2-Methylbutyl)acetamide*#	129 (M^+, 6.0), 114 (M – CH_3, 12.2), 100 (M – C_2H_5, 52.3), 73 (β-cleavage/H rearrangement, 57.4), 72 (M – C_4H_9,100), 60 ($CH_3C(OH)NH^+$, 60.3), 58 (27.9), 55 (16.1)	AS	1123
7	*N*-(3-Methylbutyl)acetamide*#	129 (M^+, 5.3), 114 (M – CH_3, 16.0), 86 (M – C_3H_7, 29.2), 73 (β-cleavage/H rearrangement, 100), 72 (M – C_4H_9, 74.7), 60 ($CH_3C(OH)NH^+$, 30.1), 55 (17.3)	AS	1129
8	Diethyl succinate*	174 (M^+, 0.4), 129 (M – OC_2H_5, 57.6), 128 (20.1), 101 (M – $COOC_2H_5$, 100), 73 (21.1), 74 (11.2), 55(15.6)	AS	1172
9	*N*-(2-Methylbutyl)propanamide*#	143 (M^+, 8.0), 114 (M – C_2H_5, 15.4), 87 (β-cleavage/H rearrangement, 50.6), 86 (M – C_4H_9, 100), 74 ($CH_3CH_2C(OH)NH^+$, 83.1), 58 (39.7), 57(95.0)	AS	1198
10	*N*-(3-Methylbutyl)propanamide*#	143 (M^+, 4.8), 128 (M – CH_3, 10.4), 114 (M – C_2H_5, 16.3), 100 (15.2), 87 (β-cleavage/H rearrangement, 100), 86 (M – C_4H_9, 62.9), 74 ($CH_3CH_2C(OH)NH^+$, 28.1), 57 (66.6)	AS	1204
11	*N*-(2-Methylbutyl)isobutyrate*	157 (M^+, 9.0), 128 (M – C_2H_5, 15.3), 114 (M – C_3H_7, 10.2), 101 (β-cleavage/H rearrangement, 15.2), 100 (M – C_4H_9, 16.2) 88 ($CH_3CHCH_3C(OH)NH^+$, 77.2), 71 (100)	AS	1226
12	*N*-(3-Methylbutyl)isobutyrate*#	157 (M^+, 9.5), 142 (M – CH_3, 17.2), 114 (M – C_3H_7, 23.6), 101 (β-cleavage/H rearrangement, 75.6), 100 (M – C_4H_9, 20.1), 88 ($CH_3CHCH_3C(OH)NH^+$, 27.0), 71 (100)	AS	1230
13	2,7-Dimethyl-1,6-dioxaspiro[4.5]decane#	170 (M^+, 0.3), 155 (M – CH_3, 0.8), 126 (17.4), 115 (12.8), 111 (4.2), 101 ($CH_3(C_4H_5O)=OH^+$, 100), 98 ($CH_3(C_4H_5O)=CH_2$, 88.7), 83 (43.8), 69 (29.4), 55 (52.7)	L[47]	1076
14	(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane#	184 (M^+, 9.4), 169 (M – CH_3, 1.8), 140 (12.7), 125 (8.1), 115 ($CH_3(C_5H_7O)=OH^+$, 86.5), 112 ($CH_3(C_5H_7O)=CH_2$, 100), 97 (61.2), 69 (46.6), 55 (43.1)	AS	1148
15	(*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane#	198 (M^+, 10.9), 169 (M—C_2H_5, 11.6), 140 (15.2), 129 ($CH_3CH_2(C_5H_7O)=OH^+$, 47.7), 126 ($CH_3CH_2(C_5H_7O)=CH_2$, 43.6), 115 ($CH_3(C_5H_7O)=OH^+$, 100), 112 ($CH_3(C_5H_7O)=CH_2$, 90.7), 97 (59.2), 83 (51.6), 69 (71.3), 55 (70.8)	L[18]	1239
16	(*E*,*Z*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane#	184 (M^+, 1.1), 168 (0.8), 155 (M – C_2H_5, 13.2), 140 (1.5), 115 ($CH_3(C_5H_7O)=OH^+$ and $CH_3CH_2(C_4H_5O)=OH^+$, 100), 112 ($CH_3CH_2(C_5H_7O)=CH_2$ and $CH_3CH_2(C_4H_5O)=CH_2$, 41.8), 97 (91.7), 85 (9.1), 83 (8.3), 69 (85.8), 55 (54.9)	L[32]	1275
17	(*E*,*E*)-2-Ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane#	212 (M^+, 2.6), 183 (M – C_2H_5, 12.8), 143 ($CH_3(C_5H_6O)=OHCH_2CH_3^+$, 57.2), 140 ($CH_3(C_5H_6O)=CH_2CH_2CH_3$, 11.2), 125 ($CH_2CH(C_5H_7O)CH_3$,100), 115 ($CH_3(C_5H_7O)=OH^+$, 46.3),112 ($CH_3(C_5H_7O)=CH_2$, 70.7), 97 (57.6), 82 (55.6), 83 (60.6), 55 (75.1)	L[19]	1318
18	(*E*,*E*)-2-Propyl-8-methyl-1,7-dioxaspiro[5.5]undecane#	212 (M^+, 7.3), 169 (M – C_3H_7, 13.2), 143 ($CH_3CH_2CH_2(C_5H_6O)=OH^+$, 31.5), 140 ($CH_3CH_2CH_2(C_5H_6O)=CH_2CH_2CH_3$, 36.5), 125 (44.5), 115 ($CH_3(C_5H_7O)=OH^+$, 100),112 ($CH_3(C_5H_7O)=CH_2$, 73.4), 97 (72.1), 82 (26.2), 83 (34.6), 69 (46.8), 55 (69.2)	L[32]	1324
19	Methyl laurate#	214 (M^+, 3.68), 183 (M – OCH_3, 8.0), 171 (14.6), 143 (18.2), 129 (9.5), 87 (60.1), 74 (McLafferty rearrangement product, 100), 59 ($COOCH_3$, 8.5), 55 (22.8)	AS	1524
20	Ethyl laurate#	228 (M^+, 4.3), 199 (M – C_2H_5, 4.7), 183 (M – OC_2H_5, 5.6), 157 (7.6), 101 (35.9), 88 (McLafferty rearrangement product, 100), 73 ($COOC_2H_5$, 20.7), 70 (21.8), 61 (14.9), 60 (13.7), 55 (27.0)	AS	1595
21	Propyl laurate#	242 (M^+, 1.6), 201 (40.4), 199 (M – C_3H_7, 1.0), 183 (M – OC_3H_7, 36.5), 157 (9.2), 129 (15.2), 115 (26.7), 102 (McLafferty rearrangement product, 29.7), 87 ($COOC_3H_7$, 11.2), 61 (100), 59 (4.1), 57 (30), 55 (26.0)	AS	1691
22	Methy myristate#	242 (M^+, 6.6), 211 (M – OCH_3, 6.3), 199 (16.2), 143 (25.6), 125 (1.1), 111 (2.6), 101 (8.8), 97 (6.3), 87 (64.4), 74 (McLafferty rearrangement product, 100), 59 ($COOCH_3$, 7.8), 55 (23.4)	AS	1727
23	Ethyl myristate#	256 (M^+, 7.1), 213 (13.8), 211 (M – OC_2H_5, 8.1), 157 (22.0), 101 (53.8), 88 (McLafferty rearrangement product, 100), 73 ($COOC_2H_5$, 17.8), 70 (22.1), 55 (20.1)	AS	1795
24	Ethyl myristoleate#	254 (M^+, 4.1), 209 (M – OC_2H_5, 13.9), 208 (M – C_2H_5OH, 15.0), 155 (9.3), 166 (28.9), 124 (23.7), 88 (McLafferty rearrangement product, 46.4), 73 ($COOC_2H_5$, 16.6), 69 (25.2), 55 (100)	AS	1785
25	Methyl palmitoleate#	268 (M^+, 5.1), 237 (M – OCH_3, 14.2), 236 (M – CH_3OH, 18.6), 194 (18.0), 152 (24.1), 97 (51.6), 96 (51.4), 74 (McLafferty rearrangement product, 52.3), 69 (63.6), 59 ($COOCH_3$, 17.1), 55 (100)	AS	1909
26	Ethyl palmitoleate#	282 (M^+, 6.7), 237 (M – OC_2H_5, 14.0), 236 (M – C_2H_5OH, 21.3), 194 (23.2), 152 (28.6), 88 (McLafferty rearrangement product, 57.3), 73 ($COOC_2H_5$, 16.8), 69 (68.7), 55 (100)	AS	1977
27	Ethyl palmitate#	284 (M^+, 11.3), 255 (M – C_2H_5, 4.1), 241 (13.2), 239 (M – OC_2H_5, 7.5), 157 (21.3), 115 (8.4), 101 (57.5), 88 (McLafferty rearrangement product, 100), 73 ($COOC_2H_5$, 16.2), 55 (21.1)	AS	1995
28	Ethyl oleate#	310 (M^+, 1.2), 265 (M – OC_2H_5, 3.8), 264 (M – C_2H_5OH, 8.170), 222 (5.4), 180 (5.0), 125 (13.6), 123 (13.6), 111 (18.5), 97 (39.8), 88 (McLafferty rearrangement product, 35.4), 83 (50.1), 73 ($COOC_2H_5$, 15.2), 69 (77.1), 55 (100)	AS	2144
29	Ethyl elaidate#	310 (M^+, 0.5), 265 (M – OC_2H_5, 4.6), 264 (M – C_2H_5OH, 9.3), 222 (5.8), 180 (5.4), 123 (14.1), 110 (18.7), 97 (38.2), 88 (McLafferty rearrangement product, 33.2), 83 (44.4), 73 ($COOC_2H_5$, 13.6), 69 (70.1), 55 (100)	AS	2172

Table 1. Compounds produced by adults of *Bactrocera tryoni*. * Compounds identified in males, # Compounds identified in females, No = Number, I = Identification, AS = authentic sample, L = literature, KI = Kovats index.

were first killed by chilling them on dry ice 3 – 5 h before the onset of dusk. Rectal glands were extracted by gently pressing the abdomen and pulling the gland out with fine forceps. Glands were carefully placed in a 1.1 mL tear-drop vial in dry ice. Once 10 glands were collected, the vials were removed from the dry ice and 100 µL of solvent was added. Glands were saturated with solvent at room temperature for 10 min The extracts were then transferred to a new vial and stored at -20 °C until analyzed. Hexadecane was used as an internal standard, with 2 µL of 1.35 mg/mL stock solution being added to each extract. To assess the effects of crushing the glands, an additional 10 samples were assessed using *n*-hexane as a solvent. For these samples, when 100 µL of *n*-hexane was added, the 10 rectal glands were crushed using a capillary glass tube. Other steps were as for the intact gland samples. Gloves (Ni-Tek) were used when collecting and handling samples to minimize risk of contamination.

Headspace samples. *Dynamic method.* Tenax-GR Mesh 60/80, 50 mg (Scientific Instrument Services, Inc) and Porapak Q 80–100 mesh, 50 mg (Waters, USA) were packed into 6 × 50 mm glass cartridges and held in place with glass wool plugs (Table S1). Tenax and Porapak were separately conditioned under nitrogen (75 mL/min) at 200 °C and 180 °C respectively for three hours before each sample collection. For each collection, 30 males or 30 females were placed into a glass chamber (150 mm long and 40 mm ID) 30 min before dusk to acclimatize. Dusk in the controlled environment room was simulated for 30 min. While the period of active calling is well known from observations of wing fanning behaviour[37,41,42] it is possible that emissions continue beyond this time. Charcoal-filtrated air (0.5 L/min, air pulling system) was passed over the flies for 10 min and 20 min, starting from the end of dusk phase, 40 min, 60 min and 90 min, starting from beginning of dusk, to cover all likely release times. Volatiles were subsequently eluted from Tenax or Porapak using 1 mL *n*-hexane, and concentrated to 200 µL under a gentle nitrogen stream. Six replicates per sex per sampling period were collected for each sorbent. Samples were stored at -20 °C until analyzed. To distinguish between volatile compounds released by the flies and possible contaminants, an air control sample comprising an empty glass chamber was run and analyzed along with each headspace collection. Hexadecane was then used as an internal standard, with 2 µL of 2.7 mg/mL stock solution being incorporated to each concentrated samples. 1 µL of each sample was injected for GC–MS analysis.

Static method. A manual holder (Supelco, Bellefonte, PA, US) was used with three different fibers; 100 µm film thickness PDMS, 65 µm PDMS–DVB and 85 µm PA (Table S1). Fibers were thermally conditioned in the GC injection port for 30 min at 270 °C. Six replicates per sex were carried out for each fiber. For each replicate, 5 males or females were placed in a 40 mL clear glass vial 30 min before dusk to acclimatize. When dusk was finished, the fiber was exposed for 10 min, which was sufficient time for adsorption of volatiles without saturating the fiber. The loaded SPME fiber was then injected into the GC–MS.

Prior to each headspace collection, glass chambers and vials were washed with 5% Extran aqueous solution, rinsed with hot tap water, and heated at 200 °C for 18 h. Activated charcoal filters were thermally conditioned by heating them at 200 °C for 18 h prior to each headspace collection[48]. To distinguish any possible contaminants, an air control sample comprising an empty glass vial was run and analyzed along with each headspace collection.

Data analysis

Normalized GC peak areas were used to compare the proportions of each compound. Normalized peak areas were obtained by dividing the peak area of interested compounds by the peak area of the internal standard. The data sets were not normally distributed and were transformed to log (x + 1) for statistical analysis. All graphics were generated using normalized GC peak areas without log-transformation, except for SPME, for which the actual peak areas were used. The effects of sampling method were analyzed by ANOVA, followed by a Tukey post hoc test ($\alpha = 0.05$) for multiple comparisons using SPSS (IBM Corp. released 2012 IBM SPSS Statistics for Windows, v. 21.0. Armonk, NY, IBM Corp.). When comparing the extraction efficiency of different solvents of rectal gland contents, solvent and compound were fixed factors. When comparing extractions from intact and crushed rectal glands in *n*-hexane, crushing treatment and compound were fixed factors. When comparing SPME fibers for static headspace sampling, fiber and compound were fixed factors. When comparing sorbents and collection time for active headspace sampling, sorbent, compound and time were fixed factors. Due to low concentrations, ethyl propanoate, propyl isobutyrate and ethyl 2-methylpentanoate were excluded from statistical analysis of male rectal glands and headspace while propyl laurate and (*E*,*Z*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5] decane were excluded from statistical analysis of female rectal glands and headspace. For the analysis of amides, the peak area from male samples were used for statistical analysis as females produced the same amides as males but in lower concentration.

Results

A full listing of the compounds identified in rectal gland extracts and emissions of *B. tryoni* is provided in Table 1. Twenty two compounds were detected in rectal gland extracts and headspace collections of sexually mature virgin female *B. tryoni*, including five amides (**6**, **7**, **9**, **10** and **12**), six spiroacetals (**13**–**18**) and eleven esters (**19**–**29**). Of these, 19 compounds were detected in the headspace. Ethyl oleate (**28**), ethyl elaidate (**29**) and *N*-(2-methylbutyl)acetamide (**6**) were not detected in the headspace. Of the 22 compounds detected in our study, three minor compounds, *N*-(2-methylbutyl)acetamide (**6**), propyl laurate (**21**) and methyl myristate (**22**) have not been previously reported in *B. tryoni*.

Twelve compounds were detected in rectal gland extracts and headspace collections of male *B. tryoni*, including the seven previously reported compounds, *N*-(2-methylbutyl)acetamide, *N*-(3-methylbutyl)acetamide, *N*-(2-methylbutyl)propanamide, *N*-(3-methylbutyl)propanamide, *N*-(2-methylbutyl)isobutyrate, *N*-(3-methylbutyl)isobutyrate and ethyl isobutyrate and five additional compounds that have not been previously reported

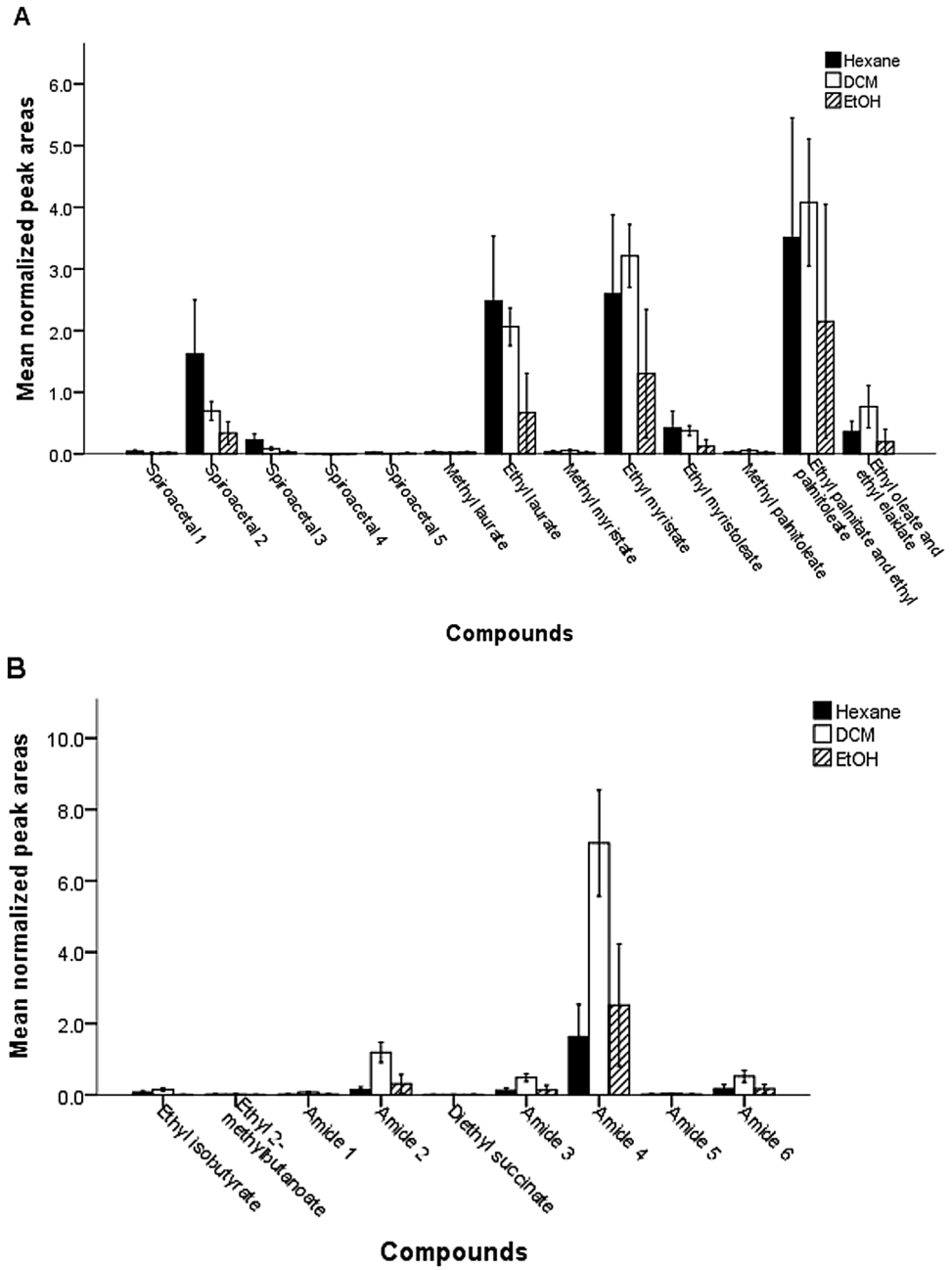


Figure 1. Graphical display of mean normalized peak areas ($n = 10$) obtained for rectal glands extract of female (**A**) and male (**B**) *Bactrocera tryoni* using three different solvents, *n*-hexane, dichloromethane (DCM) and ethanol (EtOH). Error bars represent the confidence interval for the mean at 95% confidence level. Amide 1: *N*-(2-methylbutyl)acetamide, Amide 2: *N*-(3-methylbutyl)acetamide, Amide 3: *N*-(2-methylbutyl) propanamide, Amide 4: *N*-(3-methylbutyl)propanamide, Amide 5: *N*-(2-methylbutyl)isobutyrate, Amide 6: *N*-(3-methylbutyl)isobutyrate, Spiroacetal 1: 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, Spiroacetal 2: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 3: (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 4: (*E*,*E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 5: (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane.

(Table 1); ethyl propanoate, ethyl 2-methylbutanoate, propyl isobutyrate, ethyl 2-methylpentanoate and diethyl succinate.

Rectal gland extractions. *Effect of solvent.* GC–MS analysis of rectal glands showed that solvent was a significant factor for the amounts of compounds (solvent: $F_{2, 594} = 66.653$, $P < 0.001$, compound: $F_{21, 594} = 118.087$, $P < 0.001$, solvent × compound: $F_{41, 594} = 4.576$, $P < 0.001$). DCM and *n*-hexane generally extracted similar amounts of esters, and both generally extracted greater amounts than ethanol (Fig. 1A, Table S2). The overall

patterns of solvents are similar for the compounds although the lower efficiency of ethanol was less evident for compounds that were less abundant overall in all solvents, notably methyl laurate, methyl myristate, methyl palmitoleate and diethyl succinate (Table S2, Figure S1). Ethanol extracts typically did not contain ethyl isobutyrate and ethyl 2-methylbutanoate, which were detected in the DCM and *n*-hexane extracts.

The DCM extracts consistently contained greater amounts of amides than either *n*-hexane or ethanol (Fig. 1B, Table S2). There was no difference between *n*-hexane and ethanol in amounts of amides (Table S2). In case of spiroacetals, there was no significant difference between *n*-hexane, DCM and ethanol except for the most abundant spiroacetal, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane for which *n*-hexane extracted greater amounts than DCM ($P < 0.001$) or ethanol ($P < 0.001$), while DCM extracted greater amount than ethanol ($P = 0.001$).

Effect of crushing glands. Crushing of rectal glands significantly increased extraction of compounds (crushing: $F_{1, 396} = 65.862$, $P < 0.001$, compound: $F_{21, 396} = 159.600$, $P < 0.001$, crushing × compound: $F_{21, 396} = 6.171$, $P < 0.001$). The overall patterns of crushing are similar for the compounds (Figure S2) although the higher efficiency of crushing glands was less evident for compounds that were less abundant overall in male and female rectal glands, notably ethyl isobutyrate, ethyl 2-methylbutanoate, diethyl succinate, *N*-(2-methylbutyl)acetamide, *N*-(2-methylbutyl)propanamide, *N*-(2-methylbutyl)isobutyrate, methyl laurate, methyl myristate, methyl palmitoleate, ethyl myristoleate and ethyl oleate and ethyl elaidate (Fig. 2, Table S3).

Effect of SPME fibers in static headspace sampling

The type of SPME fiber affected the amounts of compounds trapped from female and male headspace samples (fiber: $F_{2, 315} = 7.636$, $P = 0.001$, compound: $F_{20, 315} = 11.041$, $P < 0.001$, fiber × compound: $F_{40, 315} = 2.622$, $P < 0.001$) (Fig. 3 A and B). PDMS trapped more spiroacetals than PA (Figure S3, Table S4). There was no significant difference between PDMS/DVB and PA or PDMS (Table S4). The type of fiber did not affect the amounts of amides and esters except for the two lighter esters, ethyl isobutyrate and ethyl-2-methylbutanoate. PDMS/DVB trapped more ethyl isobutyrate and ethyl-2-methylbutanoate than PDMS ($P = 0.002$ and $P = 0.005$, respectively), and both PDMS/DVB and PDMS trapped more than PA (Table S4).

Effect of sorbent material and time in dynamic headspace sampling. Analysis of headspace samples revealed that the type of sorbent material and sampling duration significantly affected the amounts of compounds trapped (sorbent: $F_{1, 950} = 11.727$, $P = 0.001$, time: $F_{4, 950} = 170.902$, $P < 0.001$, compound: $F_{18, 950} = 945.192$, $P < 0.001$, sorbent × time: $F_{4, 950} = 8.605$, $P < 0.001$, sorbent × compound: $F_{18, 950} = 64.468$, $P < 0.001$, time × compound: $F_{72, 950} = 20.828$, $P < 0.001$, sorbent × time × compound: $F_{72, 950} = 4.144$, $P < 0.001$).

For spiroacetals, while Porapak trapped more 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane (spiroacetals 1, 2 and 3) than Tenax, this was not the case for (*E*,*E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane (spiroacetals 4 and 5) (Fig. 4, Table S5 and S6). Generally, the amounts of spiroacetals increased with sampling time for both Tenax and Porapak until 60 min (Fig. 4). However, there were some subtle differences among compounds in the relationship between time and amount (Table S5).

While Porapak and Tenax trapped very similar amounts of most esters, Tenax trapped significantly more ethyl laurate, ethyl myristate, ethyl myristoleate and ethylpalmitate/palmitoleate than Porapak at 90 min (Fig. 4, Table S6). Generally, the amounts of long chain esters increased with sampling time for Tenax, while they increased until 60 min for Porapak (Fig. 4). However, there were some subtle differences among compounds in the relationship between time and amount (Table S5 and S6).

Tenax generally collected higher amounts of amides than Porapak (Fig. 4). The amounts of amides increased with sampling time until 60 min (Fig. 4). The overall patterns are similar for the different amides, with only subtle differences (Fig. 4, Table S6). Both Tenax and Porapak trapped trace amounts of diethyl succinate and ethyl 2-methylbutanoate, but the amounts of these compounds were not able to be statistically analyzed.

Discussion

Rectal gland volatiles of Queensland fruit fly. Adding to the six aliphatic amides that have been reported previously in the rectal glands of male *B. tryoni*[37], we here describe an additional six previously unreported compounds in rectal glands and emissions of male *B. tryoni*. While ethyl isobutyrate has been tentatively identified in the volatiles of male *B. tryoni* by Kumaran et al.[43] it was not reported as a rectal gland component. Kumaran et al.[43] also tentatively identified only 3 amides as components of rectal glands including, *N*-(3-methylbutyl)acetamide, *N*-hexylpropanamide and *N*-propylbutyramide, two of which are different from those reported by Bellas and Fletcher[37] and the present study. Of these, *N*-(3-methylbutyl)acetamide) and *N*-hexylpropanamide, as well as 2-hydroxypropanamide and two propanoic acid derivatives, 2-methyl propanoic acid and 2-methylundecyl propenoate, were also reported in the volatiles released by male *B. tryoni* by Kumaran et al.[43] Although pheromone composition may be affected qualitatively and quantitatively by larval diets[49], the additional compounds found in the present study have most likely been overlooked previously. The ethyl and propyl esters are highly volatile and may have been lost through volatilization during extraction or trapping[50]. The four short chain esters—ethyl propanoate, ethyl 2-methylbutanoate, propyl isobutyrate and ethyl 2-methylpentanoate – are here identified for the first time in fruit flies. Some of these compounds have been reported previously in fruits. For example, ethyl propanoate, which has a pineapple-like odor[51], is a common volatile in many ripe fruits that attract *B. tryoni* females including mango and apple[52]. Ethyl propanoate has also been considered as an attractant for other frugivorous pest insects. For instance, ethyl propanoate increases attraction of the African palm weevil *Rhynchophorus phoenicis* to aggregation pheromone[53]. Ethyl isobutyrate has been reported as an important contributor to the sweet aroma of fresh pineapple[54] and other fruits includ-

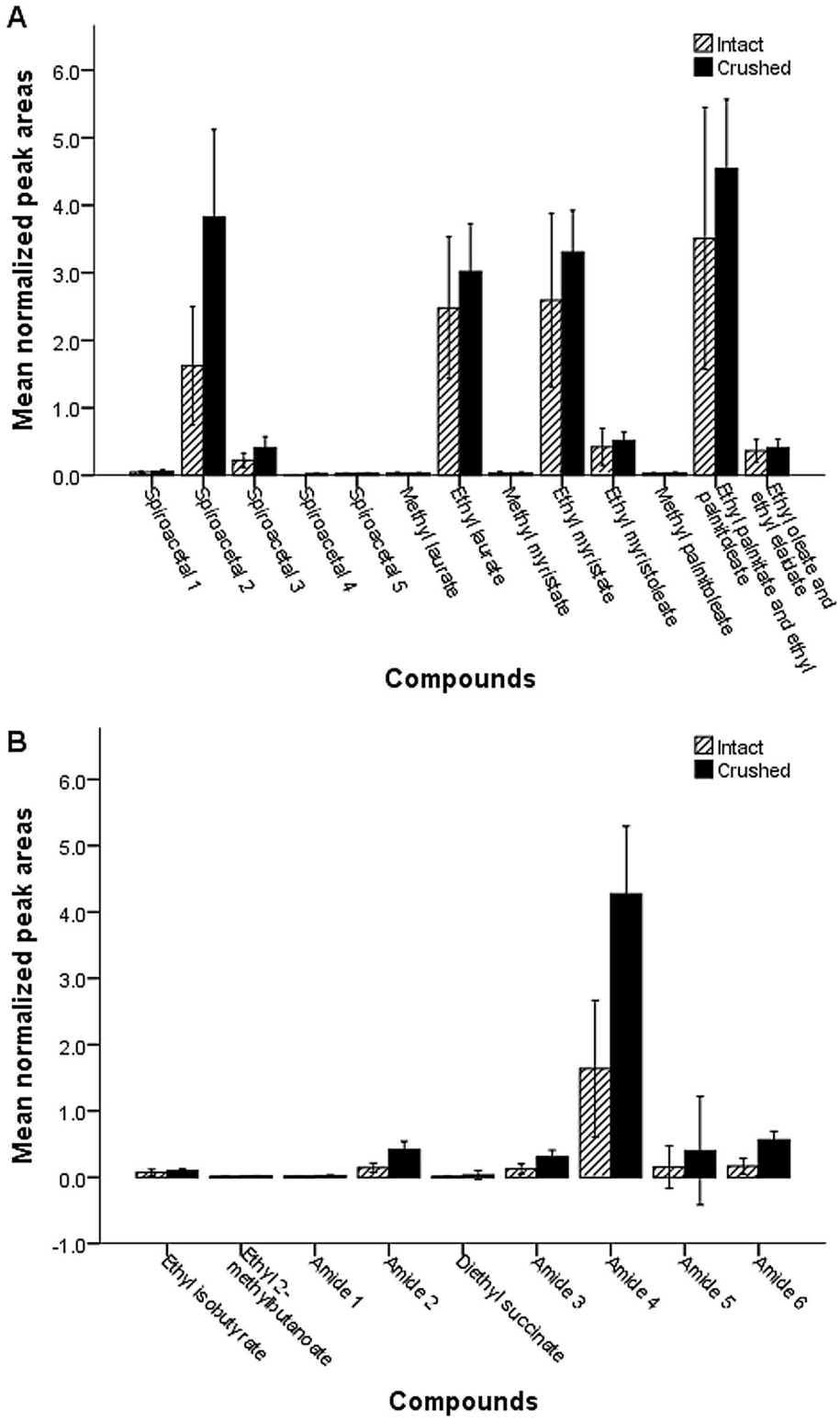


Figure 2. Graphical display of mean normalized peak areas ($n = 10$) obtained for rectal glands extract of female (**A**) and male (**B**) *Bactrocera tryoni* using crushed rectal glands and non-crushed rectal glands. Error bars represent the confidence interval for the mean at 95% confidence level. Amide 1: *N*-(2-methylbutyl)acetamide, Amide 2: *N*-(3-methylbutyl)acetamide, Amide 3: *N*-(2-methylbutyl)propanamide, Amide 4: *N*-(3-methylbutyl) propanamide, Amide 5: *N*-(2-methylbutyl)isobutyrate, Amide 6: *N*-(3-methylbutyl)isobutyrate, Spiroacetal 1: 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, Spiroacetal 2: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 3: (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 4: (*E*,*E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 5: (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane.

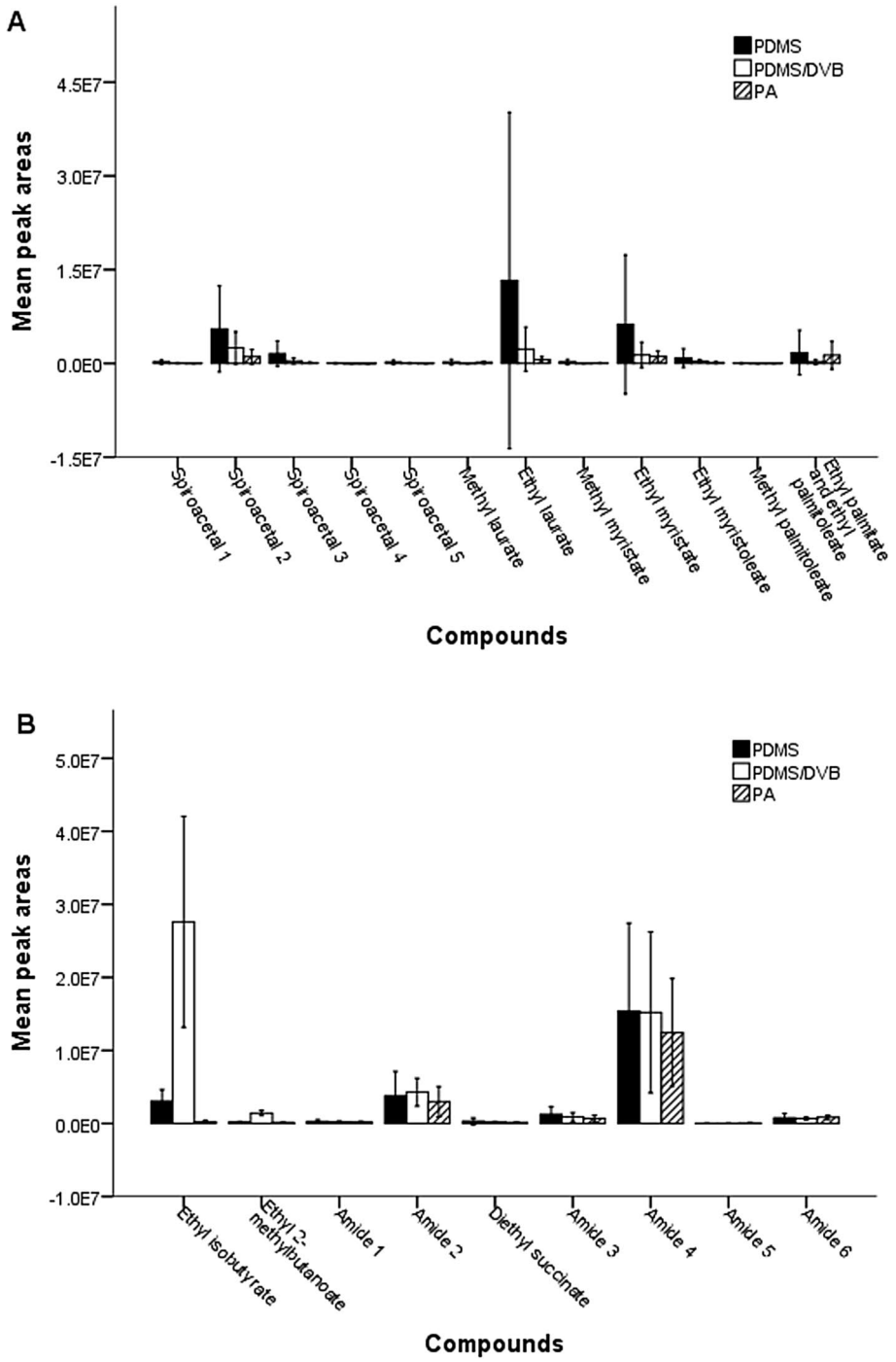


Figure 3. Graphical display of mean peak areas ($n = 6$) obtained for headspace collection of female (A) and male (B) *Bactrocera tryoni* using three SPME fibers, polydimethylsiloxane (PDMS), poly(dimethylsiloxane)–divinylbenzene (PDMS–DVB) and polyacrylate (PA). Error bars represent the confidence interval for the mean at 95% confidence level. Amide 1: *N*-(2-methylbutyl)acetamide, Amide 2: *N*-(3-methylbutyl)acetamide, Amide 3: *N*-(2-methylbutyl)propanamide, Amide 4: *N*-(3-methylbutyl)propanamide, Amide 5: *N*-(2-methylbutyl) isobutyrate, Amide 6: *N*-(3-methylbutyl)isobutyrate, Spiroacetal 1: 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, Spiroacetal 2: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 3: (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 4: (*E*,*E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 5: (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane.

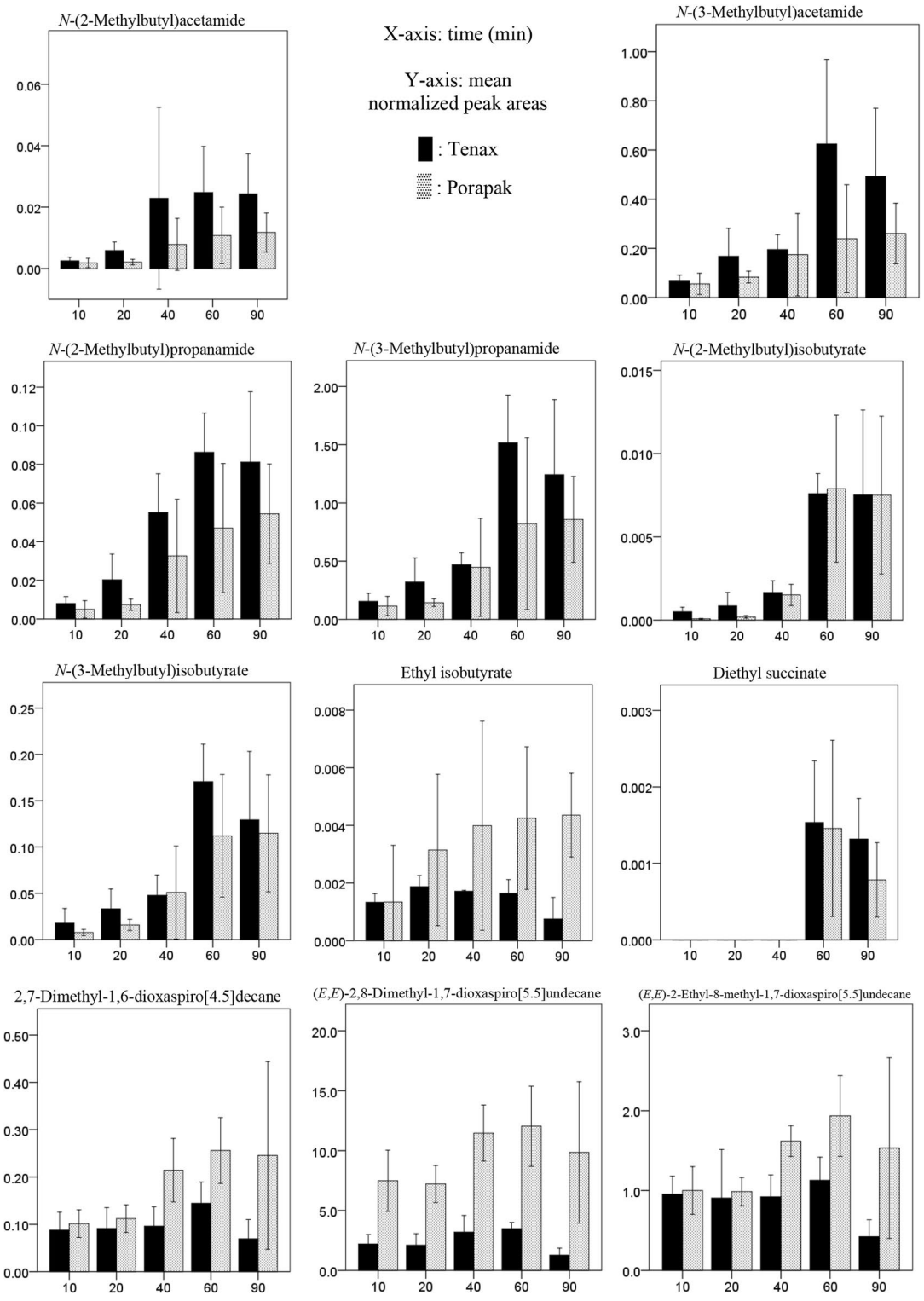


Figure 4. Graphical display of estimated mean normalized peak area ($n = 6$) obtained for headspace collection of male and female *Bactrocera tryoni* using two polymer sorbents, Tenax-GR and Porapak Q. Compounds obtained from males include: *N*-(2-methylbutyl)acetamide, *N*-(3-methylbutyl)acetamide, *N*-(2-methylbutyl)propanamide, *N*-(3-methylbutyl)propanamide, *N*-(2-methylbutyl)isobutyrate and *N*-(3-methylbutyl)isobutyrate, ethyl isobutyrate and diethyl succinate. Compounds obtained from females include: 2,7-dimethyl-1,6-dioxaspiro[4.5] decane, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane , (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, (*E*,*E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane, methyl laurate, ethyl laurate, methyl myristate, ethyl myristate, ethyl myristoleate, methyl palmitoleate, ethyl palmitate and ethyl palmitoleate. Error bars represent the confidence interval for the mean at 95% confidence level.

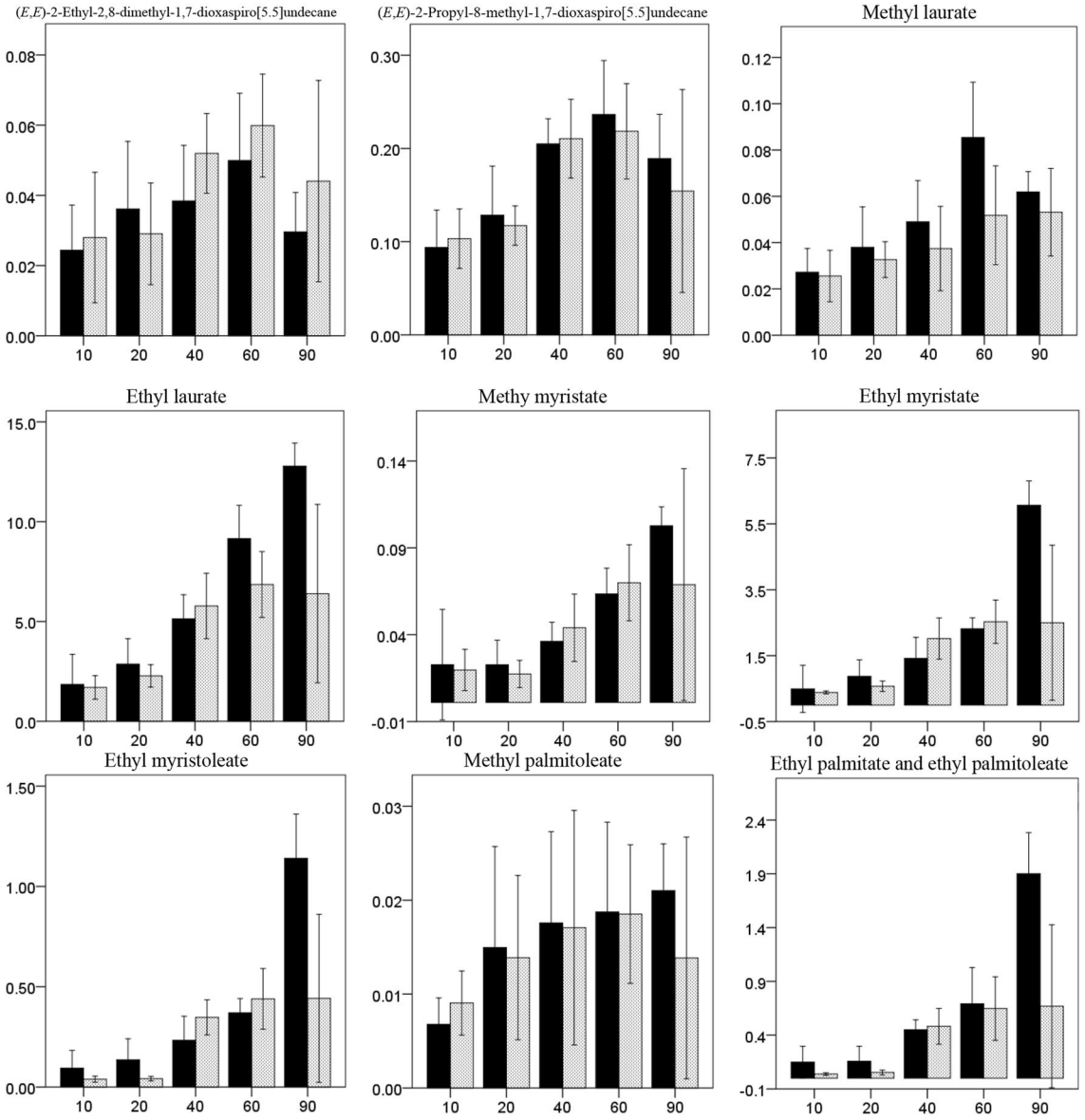


Figure 4. (continued)

ing apple[55], orange[56] and berries[57,58]. Ethyl isobutyrate has been also reported as a strong electrophysiologically active compound for the female blueberry fruit fly, *Rhagoletis mendax*[57]. Ethyl 2-methylbutanoate is found in apples[55], pineapples[59,60], oranges[61], and berries[57,58]. Diethyl succinate has been found in rectal glands of male *B. halfordiae*[15] and *B. kraussi*[62]. This compound is known as an attractant for the spotted wing Drosophila, *Drosophila suzukii*[63]. The strong sweet smell of volatiles released by *B. tryoni* males during sexual activity does not resemble that of the amides described previously by Bellas and Fletcher[37] but does resemble that of the esters reported in the present study.

In female *B. tryoni*, we found three compounds that have not been reported previously[19,38]. While ethyl (9,12)-octadecadienoate was not detected in our rectal gland extracts, despite being reported in a previous study[38] , *N*-(2-methylbutyl)acetamide, propyl laurate and methyl palmitoleate were detected for the first time. These are all present at low concentrations and this likely explains why they were not detected previously. It is possible that differences between studies in the reporting of these compounds is a result of differences in rearing conditions and especially larval diet[49].

Rectal glands. Although relying only on rectal gland extraction can mean that volatiles released from elsewhere on the flies are missed, this is a practical, rapid and selective way to collect compounds that fruit flies emit from these glands[7,8] and is important for confirming the source of compounds collected in headspace samples. We assessed the effectiveness of three different solvents for rectal gland extractions, including the non-polar *n*-hexane, medium-polarity DCM and polar ethanol. In general, under the conditions used in this study, GC–MS of ethanol extracts showed very incomplete mass profiles for ethyl isobutyrate and ethyl 2-methylbutanoate.

It was difficult to identify these compounds without the assistance of the *n*-hexane and DCM extracts. Since ethanol is the most polar solvent employed, it is possible that ethanol did not absorb the volatile esters during the extraction time used in this study. DCM and *n*-hexane extracted similar concentrations of lower volatility long chain fatty acid esters but there were differences in the extraction of more volatile compounds. DCM was the most effective solvent for extraction of amides, whereas there was no significant difference between the three solvents for the extraction of spiroacetals except for (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane that *n*-hexane was more effective. Extracts from crushed rectal glands contained the same compounds as those from intact glands, but at larger amounts. This shows that there is a benefit to crushing glands, and importantly also demonstrates that studies where extracts were taken from intact rectal glands are at least qualitatively comparable to those extracts obtained from crushed glands as no compounds were missed in intact gland samples.

Headspace. The selection of fiber is a critical aspect of using SPME. The three SMPE fibers used in this study exhibited different performances. In general, the most polar fiber, PA, was found to be inefficient at collecting spiroacetals, ethyl isobutyrate and ethyl-2-methylbutanoate. PDMS was found to have better or at least the same performance as PMDS/DVB for collection of the more polar and volatile compounds except for ethyl isobutyrate and ethyl-2-methylbutanoate . The different concentration of analyte on the fiber may result from several factors including the chemical properties of the analyte, the equilibrium time[64,65], the experimental conditions (temperature and humidity)[66], and storage conditions[67].Of these factors, only the properties of the analytes would have affected the outcomes in this study because the experiments were conducted in controlled environment rooms (25 ± 0.5 °C, 65 ± 5% RH), and used the same equilibrium time and storage conditions. The short chain esters are volatile but have polar surfaces that would require sorbent affinity with volatile and slightly polar properties. PDMS/DVB would be suitable for such applications, and this is consistent with our results. Because of the substantial differences amongst headspace compounds in collection efficiency on different SPME fibers, there is a particular need for care in both the selection of fibers and in the interpretation of analyzed samples.

In dynamic headspace sampling, Porapak was found to be more effective for spiroacetals, while Tenax was more effective for esters and amides. Based on sorbent properties these results were anticipated. Sampling period and flow rate are also important factors. Tenax started to lose the six amides, diethyl succinate and all spiroacetals once air was passed through for 60 min (Fig. 4). Porapak showed similar capacity for *N*-(2-methylbutyl)isobutyramide, all spiroacetals and esters (except methyl laurate, ethyl myristoleate and ethyl palmitate/palmitoleate). Porapak showed greater capacity to retain the other five amides, methyl laurate, ethyl myristoleate and ethyl palmitate/palmitoleate after 60 min, whereas Tenax showed greater capacity to retain all the long chain esters after 60 min (Fig. 4).

Active and passive headspace sampling techniques each have advantages and disadvantages. Advantages of SPME include higher sensitivity, ease of handling, shorter adsorption time, no solvent peak in GC and easy sequential sampling. However, quantitation of analytes in many types of SPME matrices is a major challenge[67–69]. The other disadvantage is that the sample can be used only once[67]. While dynamic headspace sampling can have lower sensitivity, quantitation of analytes can be conveniently achieved as the calibration of a compound using an internal standard is easily achieved in liquid samples. Liquid samples can also be re-used, in their original form or after concentration or dilution if needed, and can also be used later for other assays (*e.g.* electrophysiological assays or bioassays)[70]. A disadvantage is that if samples contain highly volatile compounds, the solvent peak may mask these compounds in GC. Overall, dynamic headspace methods are more effective for quantitation, while SPME has some advantages for qualitative analysis.

In brief, each sampling technique may bias the interpretation of rectal gland contents or released volatile compositions. Therefore, it is important to consider differences that can be introduced by sampling techniques when interpreting volatiles of tephritid fruit flies, and especially when comparing studies that have used different techniques. There are advantages and disadvantages for each method, and it may often be useful to employ more than one method to ensure comprehensive sample collection and analysis.

Received: 19 February 2020; Accepted: 26 October 2020
Published online: 13 November 2020

References

1. Nojima, S., Classen, A., Groot, A. T. & Schal, C. Qualitative and quantitative analysis of chemicals emitted from the pheromone gland of individual *Heliothis subflexa* females. *PLoS ONE* **13**, (2018).
2. Golub, M. A. & Weatherston, I. Techniques for extracting and collecting sex pheromones from live insects and from artificial sources. in *Techniques in Pheromone Research* (eds. Hummel, H. E. & Miller, T. A.) 223–285 (Springer, New York, 1984). doi:https://doi.org/10.1007/978-1-4612-5220-7_10
3. Al-Khshema, H., Agarwal, M. & Ren, Y. Optimization and validation for determination of volatile organic compounds from Mediterranean fruit fly (Medfly) *Ceratitis capitata* (Diptera: Tephritidae) by using HS-SPME-GC-FID/MS. *J. Biol. Sci.* **17**, 347–352 (2017).
4. Piccardi, P. Insect sex-communication and prospects for pheromones in pest management. *Bolletino di Zool.* **47**, 397–408 (1980).
5. Baker, T. C., Gaston, L. K., Pope, M. M., Kuenen, L. P. S. & Vetter, R. S. A high-efficiency collection device for quantifying sex pheromone volatilized from female glands and synthetic sources. *J. Chem. Ecol.* **7**, 961–968 (1981).
6. Levi-Zada, A. *et al.* Analyzing diurnal and age-related pheromone emission of the olive fruit fly, *Bactrocera oleae* by sequential SPME-GCMS analysis. *J. Chem. Ecol.* **38**, 1036–1041 (2012).
7. Fletcher, B. S. The structure and function of the sex pheromone glands of the male Queensland fruit fly, *Dacus tryoni*. *J. Insect Physiol.* **15**, 1309–1322 (1969).
8. Fletcher, B. S. Storage and release of sex pheromone by the Queensland fruit fly, *Dacus tryoni* (Diptera:Trypetidae). *Nature* **219**, 631–632 (1968).

9. Ponce, W. P., Nation, J. L., Emmel, T. C., Smittle, B. J. & Teal, P. E. A. Quantitative analysis of pheromone production in irradiated Caribbean fruit fly males, *Anastrepha suspensa* (Loew). *J. Chem. Ecol.* **19**, 3045–3056 (1993).
10. Perkins, M. V., Kitching, W., Drew, R. A. I., Moore, C. J. & Konig, W. A. Chemistry of fruit flies: composition of the male rectal gland secretions of some species of South-East Asian Dacinae. Re-examination of *Dacus cucurbitae* (melon fly). *J. Chem. Soc. Perkin Trans. 1* 1111–1117 (1990). doi:https://doi.org/10.1039/P19900001111
11. Liedo, P. *et al.* Effect of post-teneral diets on the performance of sterile *Anastrepha ludens* and *Anastrepha obliqua* fruit flies. *J. Appl. Entomol.* **137**, 49–60 (2013).
12. Tokushima, I., Orankanok, W., Tan, K. H., Ono, H. & Nishida, R. Accumulation of phenylpropanoid and sesquiterpenoid volatiles in male rectal pheromonal glands of the guava fruit fly, *Bactrocera correcta*. *J. Chem. Ecol.* **36**, 1327–1334 (2010).
13. Wee, S. L. & Tan, K. H. Female sexual response to male rectal volatile constituents in the fruit fly, *Bactrocera carambolae* (Diptera: Tephritidae). *Appl. Entomol. Zool.* **40**, 365–372 (2005).
14. Kitching, W. *et al.* Spiroacetals in rectal gland secretions of Australasian fruit fly species. *J. Chem. Soc. Chem. Commun.* **11**, 853 (1986).
15. Kitching, W. *et al.* Chemistry of fruit flies. Composition of the rectal gland secretion of (male) *Dacus cucumis* (cucumber fly) and *Dacus halfordiae*. Characterization of (*Z,Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. *J. Org. Chem.* **54**, 3893–3902 (1989).
16. Zhang, X. *et al.* Chemical compounds from female and male rectal pheromone glands of the guava fruit fly, *Bactrocera correcta*. *Insects* **10**, (2019).
17. Canale, A. *et al.* Behavioural and electrophysiological responses to overlooked female pheromone components in the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae). *Chemoecology* **25**, 147–157 (2015).
18. Perkins, M. V. Characterisation and synthesis of Bactrocera fruit fly pheromones. (PhD Thesis, Department of Chemistry. The University of Queensland, Australia, 1990). https://doi.org/https://doi.org/10.14264/uql.2015.222
19. Booth, Y. K. *et al.* A diverse suite of spiroacetals, including a novel branched representative, is released by female *Bactrocera tryoni* (Queensland fruit fly). *Chem. Commun* https://doi.org/10.1039/B611953K (2006).
20. Schwartz, B. D., Booth, Y. K., Fletcher, M. T., Kitching, W. & Voss, J. J. De. Spiroacetal biosynthesis in fruit flies is complex : distinguishable origins of the same major spiroacetal released by different *Bactrocera* spp. 1526–1528 (2010). doi:https://doi.org/10.1039/b917977a
21. Barbosa-Cornelio, R., Cantor, F., Coy-Barrera, E. & Rodríguez, D. Tools in the investigation of volatile semiochemicals on insects: from sampling to statistical analysis. *Insects* **10**, 241 (2019).
22. Nation, J. L. Biology of pheromone release by male Caribbean fruit flies, *Anastrepha suspensa* (Diptera: Tephritidae). *J. Chem. Ecol.* **16**, 553–572 (1990).
23. Pérez, J., Park, S. J. & Taylor, P. W. Domestication modifies the volatile emissions produced by male Queensland fruit flies during sexual advertisement. *Sci. Rep.* **8**, 1–10 (2018).
24. Heath, R. R., Landolt, P. J., Robacker, D. C., Dueben, B. D. & Epsky, N. D. Sexual pheromones of tephritid flies: clues to unravel phylogeny and behavior. in *Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior* (eds. Aluja, M. & Norrbom, A. L.) 793–809 (CRC Press, Boca Raton, 2000).
25. Ohinata, K. *et al.* Oriental fruit fly and melon fly: biological and chemical studies of smoke produced by males. *J. Environ. Sci. Heal. Part A Environ. Sci. Eng.* **17**, 197–216 (1982).
26. Booth, Y. K., Kitching, W. & De Voss, J. J. Biosynthesis of insect spiroacetals. *Nat. Prod. Rep.* **26**, 490–525 (2009).
27. Ohmura, W. *et al.* Chemical composition of the defensive secretion of the longhorned beetle, *Chloridolum loochooanum*. *J. Chem. Ecol.* **35**, 250–255 (2009).
28. Robacker, D. C., Aluja, M., Bartelt, R. J. & Patt, J. Identification of chemicals emitted by calling males of the sapote fruit fly, *Anastrepha serpentina*. *J. Chem. Ecol.* **35**, 601–609 (2009).
29. Cerkowniak, M., Boguś, M. I., Włóka, E., Stepnowski, P. & Gołębiowski, M. Application of headspace solid-phase microextraction followed by gas chromatography coupled with mass spectrometry to determine esters of carboxylic acids and other volatile compounds in *Dermestes maculatus* and *Dermestes ater* lipids. *Biomed. Chromatogr.* **32**, (2018).
30. Levi-Zada, A. *et al.* Circadian release of male-specific components of the greater date moth, *Aphomia (Arenipses) Sabella*, using sequential SPME/GC/MS analysis. *J. Chem. Ecol.* **40**, 236–243 (2014).
31. Cruz-López, L., Malo, E. A. & Rojas, J. C. Sex pheromone of *Anastrepha striata*. *J. Chem. Ecol.* **41**, 458–464 (2015).
32. Schwartz, B. D. *et al.* Spiroacetal biosynthesis in insects from Diptera to Hymenoptera: The giant ichneumon wasp *Megarhyssa nortoni nortoni* Cresson. *J. Am. Chem. Soc.* **130**, 14853–14860 (2008).
33. Gilley, D. C., DeGrandi-Hoffman, G. & Hooper, J. E. Volatile compounds emitted by live European honey bee (*Apis mellifera* L.) queens. *J. Insect Physiol.* **52**, 520–527 (2006).
34. Hancock, D. L., Hamacek, E. L., Lloyd, A. C. & Elson-Harris, M. M. *The distribution and host plants of fruit flies (Diptera: Tephritidae) in Australia*. (Queensland Department of Primary Industries, 2000).
35. Clarke, A. R., Powell, K. S., Weldon, C. W. & Taylor, P. W. The ecology of *Bactrocera tryoni* (Diptera: Tephritidae): what do we know to assist pest management?. *Ann. Appl. Biol.* **158**, 26–54 (2011).
36. Vargas, R. I. *et al.* An overview of pest species of *Bactrocera* fruit flies (diptera: tephritidae) and the integration of biopesticides with other biological approaches for aheir management with a focus on the pacific region. *Insects* **6**, 297–318 (2015).
37. Bellas, T. E. & Fletcher, B. S. Identification of the major components in the secretion from the rectal pheromone glands of the Queensland fruit flies *Dacus tryoni* and *Dacus neohumeralis* (Diptera: Tephritidae). *J. Chem. Ecol.* **5**, 795–803 (1979).
38. El-Sayed, A. M. *et al.* Chemical composition of the rectal gland and volatiles released by female Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae). *Environ. Entomol.* https://doi.org/10.1093/ee/nvz061 (2019).
39. Fletcher, M. T. & Kitching, W. Chemistry of fruit flies. *Chem. Rev.* **95**, 789–828 (1995).
40. Tychsen, P. H. & Fletcher, B. S. Studies on the rhythm of mating in the Queensland fruit fly, *Dacus tryoni*. *J. Insect Physiol.* **17**, 2139–2156 (1971).
41. Tychsen, P. Mating behaviour of the Queensland fruit fly, *Dacus tryoni* (Diptera: Tephritidae), in field cages. *J. Aust. Entomol. Soc* **16**, 459–465 (1977).
42. Pike, N. & Meats, A. Tendency for upwind movement in the sibling fruit fly species, *Bactrocera tryoni* and *B. neohumeralis* and their hybrids (Diptera: Tephritidae): influence of time of day, sex and airborne pheromone. *Bull. Entomol. Res.* **93**, 173–178 (2003).
43. Kumaran, N., Hayes, R. A. & Clarke, A. R. Cuelure but not zingerone make the sex pheromone of male *Bactrocera tryoni* (Tephritidae: Diptera) more attractive to females. *J. Insect Physiol.* **68**, 36–43 (2014).
44. Weldon, C. W. Influence of male aggregation size on female visitation in *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae). *Aust. J. Entomol.* **46**, 29–34 (2007).
45. Tan, H. K. & Nishida, R. Incorporation of raspberry ketone in the rectal glands of males of the Queensland fruit fly, *Bactrocera tryoni* (Froggat) (Diptera: Tephritidae). *Appl. Entomol. Zool.* **30**, 494–497 (1995).
46. Perez-Staples, D., Prabhu, V. & Taylor, P. W. Post-teneral protein feeding enhances sexual performance of Queensland fruit flies. *Physiol. Entomol.* **32**, 225–232 (2007).
47. Mitchell, R. F. *et al.* Evidence that cerambycid beetles mimic vespid wasps in odor as well as appearance. *J. Chem. Ecol.* **43**, 75–83 (2017).
48. El-Sayed, A. M. *et al.* Floral scent of Canada thistle and its potential as a generic insect attractant. *J. Econ. Entomol.* **101**, 720–727 (2008).

49. Merli, D. *et al.* Larval diet affects male pheromone blend in a laboratory strain of the medfly, *Ceratitis capitata* (Diptera: Tephritidae). *J. Chem. Ecol.* **44**, 339–353 (2018).
50. Ulberth, F., Gabernig, R. G. & Schrammel, F. Flame-ionization detector response to methyl, ethyl, propyl, and butyl esters of fatty acids . *JAOCS J. Am. Oil Chem. Soc.* **76**, 263–266 (1999).
51. Cossé, A. A., Todd, J. L., Millar, J. G., Martínez, L. A. & Baker, T. C. Electroantennographic and coupled gas chromatographic-electroantennographic responses of the mediterranean fruit fly, *Ceratitis capitata*, to male-produced volatiles and mango odor. *J. Chem. Ecol.* **21**, 1823–1836 (1995).
52. Cunningham, J. P., Carlsson, M. A., Villa, T. F., Dekker, T. & Clarke, A. R. Do fruit ripening volatiles enable resource specialism in polyphagous fruit flies?. *J. Chem. Ecol.* **42**, 931–940 (2016).
53. Gries, G. *et al.* Ethyl propionate: Synergistic kairomone for african palm weevil, *Rhynchophorus phoenicis* L. (Coleoptera: Curculionidae). *J. Chem. Ecol.* **20**, 889–897 (1994).
54. Takeoka, G. *et al.* Volatile constituents of pineapple (*Ananas Comosus* [L.] Merr.). in *Flavor Chemistry: Trends and Developments* (eds. Teranishi, R., Buttery, R. G. & Shahidi, F.) 223–237 (American Chemical Society, 1989). doi:https://doi.org/10.1021/bk-1989-0388.ch018
55. Matich, A. J., Rowan, D. D. & Banks, N. H. Solid phase microextraction for quantitative headspace sampling of apple volatiles. *Anal. Chem.* **68**, 4114–4118 (1996).
56. Hinterholzer, A. & Schieberle, P. Identification of the most odour-active volatiles in fresh, hand-extracted juice of Valencia late oranges by odour dilution techniques. *Flavour Fragr. J.* **13**, 49–55 (1998).
57. Lugemwa, F. N., Lwande, W., Bentley, M. D., Mendel, M. J. & Alford, A. R. Volatiles of wild blueberry, *Vaccinium angustifolium*: possible attractants for the blueberry maggot fruit fly, *Rhagoletis mendax*. *J. Agric. Food Chem.* **37**, 232–233 (1989).
58. Schieberle, P. & Hofmann, T. Evaluation of the character impact odorants in fresh strawberry juice by quantitative measurements and sensory studies on model mixtures. *J. Agric. Food Chem.* **45**, 227–232 (1997).
59. Zheng, L.-Y. *et al.* Aroma volatile compounds from two fresh pineapple varieties in China. *Int. J. Mol. Sci* **13**, 7383–7392 (2012).
60. Wei, C.-B. *et al.* Characteristic aroma compounds from different pineapple parts. *Molecules* **16**, 5104–5112 (2011).
61. Buettner, A. & Schieberle, P. Evaluation of aroma differences between hand-squeezed juices from Valencia late and Navel oranges by quantitation of key odorants and flavor reconstitution experiments. *J. Agric. Food Chem.* **49**, 2387–2394 (2001).
62. Fletcher, M. T. *et al.* Chemistry of fruit-flies. Spiroacetal-rich secretions in several *Bactrocera* species from the South-West Pacific region. *J. Chem. Soc. Perkin Trans.* **1**, 2827–2831. https://doi.org/10.1039/P19920002827 (1992).
63. Cha, D. H., Adams, T., Rogg, H. & Landolt, P. J. Identification and field evaluation of fermentation volatiles from wine and vinegar that mediate attraction of spotted wing Drosophila, *Drosophila suzukii*. *J. Chem. Ecol.* **38**, 1419–1431 (2012).
64. Ai, J. Solid phase microextraction for quantitative analysis in nonequilibrium situations. *Anal. Chem.* **69**, 1230–1236 (1997).
65. Martos, P. A. & Pawliszyn, J. Calibration of solid phase microextraction for air analyses based on physical chemical properties of the coating. *Anal. Chem.* **69**, 206–215 (1997).
66. Chai, M. & Pawliszyn, J. Analysis of environmental air samples by solid-phase microextraction and gas chromatography/ion trap mass spectrometry. *Environ. Sci. Technol.* **29**, 693–701 (1995).
67. Fäldt, J., Eriksson, M., Valterová, I. & Borg-Karlson, A. K. Comparison of headspace techniques for sampling volatile natural products in a dynamic system. *Z. Naturforsch. C.* **55**, (2000).
68. Yang, X. & Peppard, T. Solid-phase microextraction for flavor analysis. *J. Agric. Food Chem.* **42**, 1925–1930 (1994).
69. Merkle, S., Kleeberg, K. & Fritsche, J. Recent developments and applications of solid phase microextraction (SPME) in food and environmental analysis—A review. *Chromatography* **2**, 293–381 (2015).
70. Agelopoulos, N. G. & Pickett, J. A. Headspace analysis in chemical ecology: effects of different sampling methods on ratios of volatile compounds present in headspace samples. *J. Chem. Ecol.* **24**, 1161–1172 (1998).

Acknowledgments

This research was supported by the Australian Research Council Industrial Transformation Training Centre (ITTC) for Fruit Fly Biosecurity Innovation (Project IC50100026), funded by the Australian Government. This research received additional support from the SITplus collaborative fruit fly program. Project *Raising Q-fly Sterile Insect Technique to World Standard* (HG14033) is funded by the Hort Frontiers Fruit Fly Fund, part of the Hort Frontiers strategic partnership initiative developed by Hort Innovation, with co-investment from Macquarie University and contributions from the Australian Government.

Author contributions

S.N., S.J.P., P.T., J.J. and I.J. designed the experiments. S.N. performed the experiments. S.N. and P.T. analysed the data. S.N. wrote the manuscript. All authors revised the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

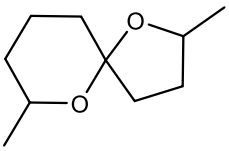
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-76622-0.

Correspondence and requests for materials should be addressed to S.N.

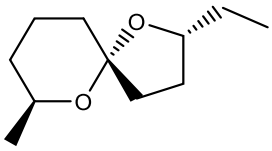
Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

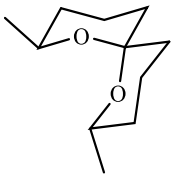
Figure 5. Structure of compounds identified in *Bactrocera tryoni* adults.



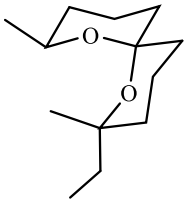
2,7-Dimethyl-1,6-dioxaspiro[4.5]decane



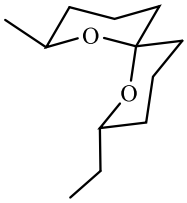
(*E*,*Z*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane



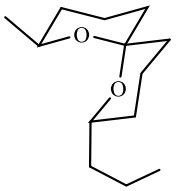
(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane



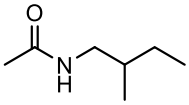
(*E*,*E*)-2-Ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane



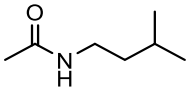
(*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane



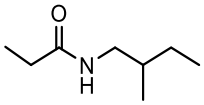
(*E*,*E*)-2-Propyl-8-methyl-1,7-dioxaspiro[5.5]undecane



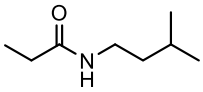
N-(2-Methylbutyl)acetamide



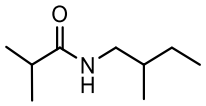
N-(3-methylbutyl)acetamide



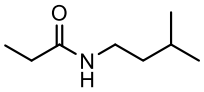
N-(2-Methylbutyl)propanamide



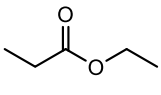
N-(3-Methylbutyl)propanamide



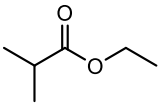
N-(2-Methylbutyl)isobutyrate



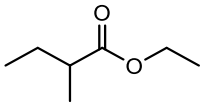
N-(3-Methylbutyl)isobutyrate



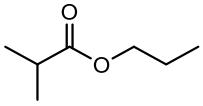
Ethyl propanoate



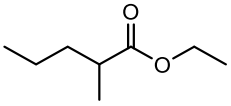
Ethyl isobutyrate



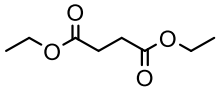
Ethyl 2-methylbutanoate



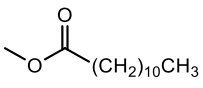
Propyl isobutyrate



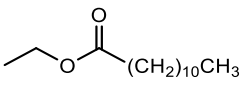
Ethyl 2-methylpentanoate



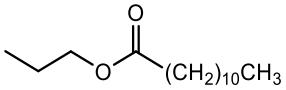
Diethyl succinate



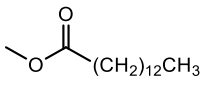
Methyl laurate



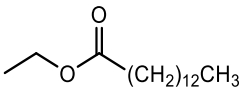
Ethyl laurate



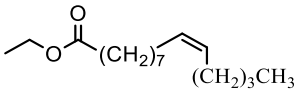
Propyl laurate



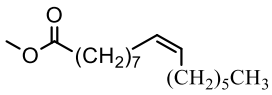
Methyl myristate



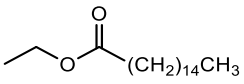
Ethyl myristate



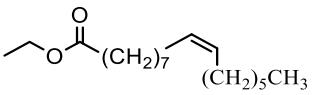
Ethyl myristoleate



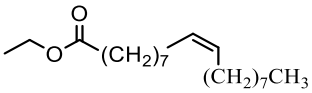
Methyl palmitoleate



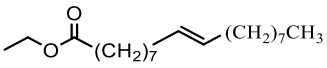
Ethyl palmitate



Ethyl palmitoleate



Ethyl oleate



Ethyl elaidate

Chapter Seven

Conclusion

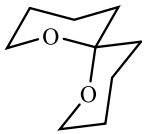
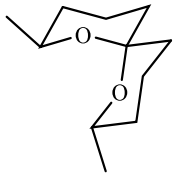
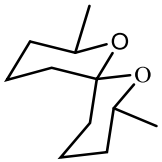
Conclusion

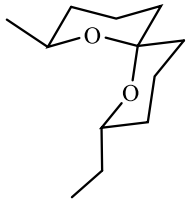
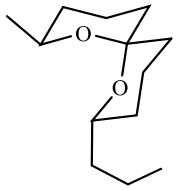
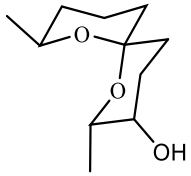
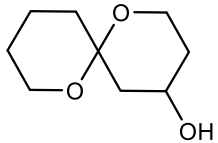
Tephritid fruit flies are significant horticultural pests, with some species belonging to the genus *Bactrocera* amongst the most damaging of all insect pests globally. Due to increasing restrictions on the use of organophosphate insecticides, alternative fruit fly control methods are necessary to protect fruits and vegetables from fruit fly damage. 'Lure and kill' is one technique that is promising as an alternative control method. The effectiveness of this technique depends on the use of efficient chemical lures. Sex pheromones of *Bactrocera* fruit flies are well known as short- and long-range attractants of the opposite sex, and are therefore of interest as chemical lures.

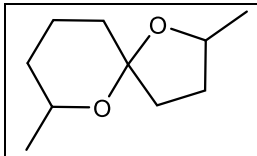
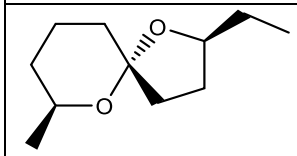
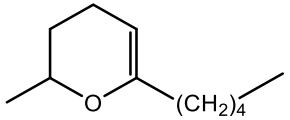
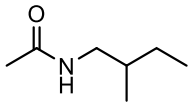
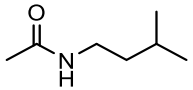
In this study, as described in Chapters 2-5, rectal gland and headspace volatile profiles of males and females of four Australian pest fruit fly species, *B. musae*, *B. frauenfeldi*, *B. bryoniae* and *B. kraussi*, were investigated as potential fruit fly attractants. Identification of volatile compounds were conducted using gas chromatography-mass spectrometry (GC-MS). Compounds were identified through comparison with retention times and mass spectra of authentic samples, where available commercially or through synthesis, or NIST library and mass spectra published in the literature, where authentic samples were not available.

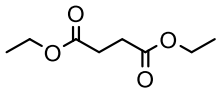
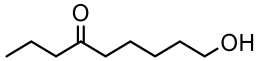
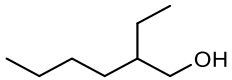
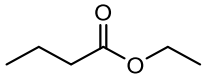
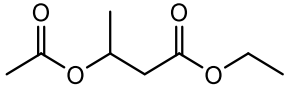
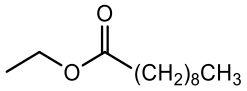
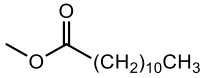
Overall, a total of 38 volatile compounds from four major chemical groups were identified across male and female rectal glands of *B. musae, B. frauenfeldi*, *B. bryoniae* and *B. kraussi*. These included spiroacetals, acetamides, fatty acids and fatty acid esters. The simple acids and esters were formally identified following comparison with commercial samples, and the amides and long-chain esters following comparison with authentic synthetic samples. Identification of the spiroacetals was more challenging, with only (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and (*E*,*E*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane formally identified following synthesis. The remaining spiroacetals (2,7-dimethyl-1,6-dioxaspiro[4.5]decane, (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane, 2,8-dimethyl-1,7-dioxaspiro[5.5]undecan-3-ol and 4-hydroxy-1,7-dioxaspiro[5.5]undecane) were tentatively identified from their characteristic mass spectral fragmentation patterns, which are well described in the literature.

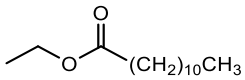
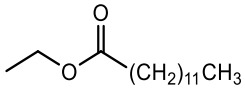
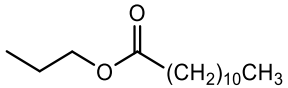
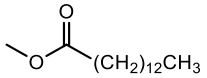
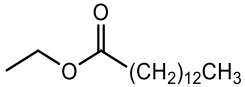
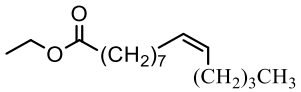
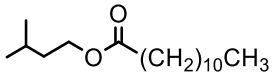
Table 1. Chemical Compound produsedby adults of *B. musae*, *B. frauenfeldi*, *B. bryoniae*, *B. kraussi*. M = male, F = female, + = detected in GCMS, - = not detected in GCMS, NA = not active

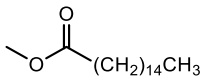
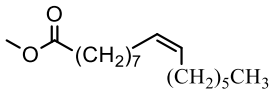
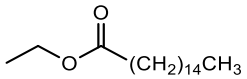
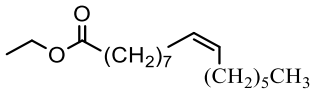
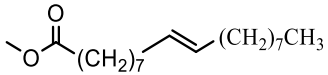
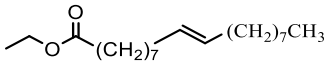
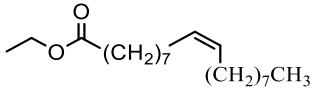
Compounds		Rectal gland extract				Released volatile				GC-EAD				GC-EPD		
		B. musae	*B. frauenfeldi*	*B. bryoniae*	*B. kraussi*	*B. musae*	*B. frauenfeldi*	*B. bryoniae*	*B. kraussi*	*B. musae*	*B. frauenfeldi*	*B. bryoniae*	*B. kraussi*	*B. frauenfeldi*	*B. bryoniae*	*B. kraussi*
 1,7-Dioxaspiro[5,5]undecane	M	-	-	-	-	-	-	-	-	NA	NA	Active	NA	NA	Active	NA
	F	-	-	+		-	-	+	-	NA	NA	Active	NA	NA	Active	NA
 (*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane	M	-	+	+	+	-	+	-	+	Active	Active	Active	Active	Active	NA	Active
	F	+	+	+	+	+	+	+	+	NA	Active	NA	Active	Active	Active	Active
 (*Z*,*Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane	M	-	-	-	+	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	-	+	-	-	-	+	-	NA	NA	NA	NA	NA	Active	NA

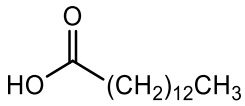
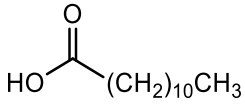
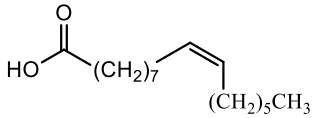
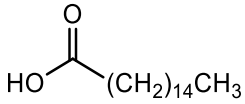
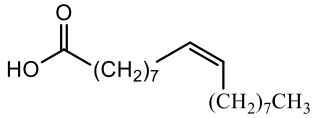
 (*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane	M	-	+	-	+	-	+	-	+	NA	NA	NA	NA	Active	NA	NA
	F	+	+	+	+	+	+	+	+	NA	Active	NA	Active	Active	Active	Active
 (*E*,*E*)-2-Propyl-8-methyl-1,7-dioxaspiro[5.5]undecane	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	-	+	-	-	-	+	-	NA	NA	NA	NA	NA	Active	NA
 2,8-Dimethyl-1,7-dioxaspiro[5.5]undecan-3-ol	M	-	-	-	+	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
 4-Hydroxy-1,7-dioxaspiro[5.5]undecane	M	-	-	+	-	-	-	+	-	NA	NA	Active	NA	NA	NA	NA
	F	-	-	-		-	-	-		NA	NA	Active	NA	NA	Active	NA

 2,7-Dimethyl-1,6-dioxaspiro[4.5]decane	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	-	+	-	-	-	-	-	NA	NA	NA	NA	NA	Active	NA
 (*E*,*E*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane	M	-	-	-	+	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	-	+	+	-	-	+	-	NA	NA	NA	NA	NA	Active	NA
 2-Methyl-6-pentyl-3,4-dihydro-2*H*-pyran	M	-	-	-	+	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
 *N*-(2-Methylbutyl)acetamide	M	-	-	+	+	-	-	+	+	NA	NA	NA	NA	NA	NA	NA
	F	-	+	+	-	-	+	-	-	NA	NA	NA	NA	NA	NA	NA
 *N*-(3-methylbutyl)acetamide	M	+	-	+	+	+	-	+	+	NA	NA	Active	NA	NA	NA	NA
	F	-	+	+	+	-	+	+	+	NA	NA	NA	NA	NA	NA	NA
	M	-	-	-	+	-	-	-	-	NA	NA	NA	NA	NA	NA	NA

 Diethyl succinate	F	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
 6-Oxononan-1-ol	M	-	-	-	+	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
 2-Ethyl-1-hexanol	M	-	-	-	+	-	-	-	+	NA	NA	NA	NA	NA	NA	NA
	F	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
 Ethyl butanoate	M	+	-	-	-	+	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	-	-	-	-	-	-	-	Active	NA	NA	NA	NA	NA	NA
 Ethyl 3-acetoxybutanoate	M	-	-	+	-	-	-	+	-	NA	NA	NA	NA	NA	Active	NA
	F	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	Active	NA
 Ethyl caprate	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	+	+	+	-	+	-	+	-	NA	Active	NA	NA	NA	Active	NA
 Methyl laurate	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	Active
	F	+	+	+	+	+	+	+	+	NA	NA	NA	NA	Active	NA	NA
	M	+	-	-	-	-	-	-	-	Active	NA	Active	Active	NA	NA	Active

 Ethyl laurate	F	+	+	+	+	+	+	+	+	NA	NA	Active	NA	NA	NA	NA
 Ethyl tridecanoate	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	+	+	+	+	-	-	+	+	NA	NA	NA	NA	NA	NA	NA
 Propyl laurate	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	+	+	+	+	-	-	+	+	NA	NA	NA	NA	NA	NA	NA
 Methyl myristate	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	+	+	+	+	+	+	+	+	NA	NA	NA	NA	NA	NA	NA
 Ethyl myristate	M	+	-	-	-	-	-	-	-	NA	NA	Active	Active	NA	NA	NA
	F	+	+	+	+	+	+	+	+	NA	NA	Active	NA	NA	NA	NA
 Ethyl myristoleate	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	+	+	-	+	+	+	-	+	NA	NA	NA	NA	NA	NA	NA
 Isoamyl laurate	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	-	-	+	-	-	-	+	NA	NA	NA	NA	NA	NA	NA

 Methyl palmitate	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	+	+	+	+	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
 Methyl palmitoleate	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	+	-	+	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
 Ethyl palmitate	M	-	-	-	-	-	-	-	-	NA	NA	Active	Active	NA	NA	NA
	F	+	+	+	+	+	-	+	+	NA	NA	Active	NA	NA	NA	NA
 Ethyl palmitoleate	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	+	+	+	+	+	+	+	+	NA	NA	NA	NA	NA	NA	NA
 Methyl elaidate	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	+	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
 Ethyl elaidate	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	+	+	-	+	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
 Ethyl oleate	M	-	+	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	+	+	+	+	-	-	-	-	NA	NA	NA	NA	NA	NA	NA

 Myristic acid	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	+	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
 Lauric acid	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	-	+	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
 Palmitoleic acid	M	-	+	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	+	+	-	-	-	+	-	NA	NA	NA	NA	NA	NA	NA
 Palmitic acid	M	-	+	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	+	+	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
 Oleic acid	M	-	-	-	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA
	F	-	-	+	-	-	-	-	-	NA	NA	NA	NA	NA	NA	NA

For each species, headspace analyses showed similar composition to the rectal gland extracts, except that fatty acids were not found in the headspace volatile collections. Males typically produced and released only one major component, usually a spiroacetal, along with additional minor components. The only exception was with males of *B. musae*, which were found to produce and release a short-chain ester as a major component. In contrast, females produced and released a more complex blend than males, including spiroacetals, acetamides, fatty acids (rectal gland only) and fatty acid esters, with the latter being the major components. The similar composition of compounds in the rectal gland contents and headspace volatiles, suggests that in *B. musae, B. frauenfeldi, B. bryoniae* and *B. kraussi* the rectal glands are the main source of headspace volatiles.

To begin to ascertain whether any of the identified volatile compounds would be useful as an attractant, gas chromatography-electroantennography (GC-EAG) and gas chromatography-electropalpography (GC-EPG) studies were conducted. While EAG/EPG alone cannot show if a compound would be an attractant, they can indicate whether a compound can be detected by the antennae/maxillary palps of fruit flies. The EAG and EPG responses of male and female *B. frauenfeldi, B. bryoniae* and *B. kraussi* to the rectal gland of the same and opposite conspecifics were recorded. For *B. musae,* only the EAG responses of males and females to the rectal gland of opposite sex were recorded. EPG was not conducted due to lack of continued availability of *B. musae* colonies. Differences in the detection of compounds between antennae and maxillary palps were observed. This is also consistent with what is seen with other *Bactrocera* species. For all the species tested, male and female antenna and maxillary palps responded to two classes of compounds, spiroacetals and esters. Functional studies on antennae and maxillary palps were beyond the scope of this study, but would be a useful future research avenue.

Although males and females of each species detected specific compounds from their own volatile profile, it was found that males and female shared the detection of some compounds across all species. (*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane was detected by antenna and maxillary palps of female *B. frauenfeldi, B. bryoniae* and *B. kraussi* as well as antenna of male *B. musae*, *B. frauenfeldi*, *B. bryoniae* and *B. kraussi* and maxillary palps of male *B. frauenfeldi* and *B. kraussi*. (*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane was detected by maxillary palps of female *B. frauenfeldi*, *B. bryoniae*, *B. kraussi* and male *B. frauenfeldi* as well as antenna of female *B. frauenfeldi*. Other spiroacetals were also electrophysiologically active including 1,7-dioxaspiro[5,5]undecane, 4-hydroxy-1,7-

dioxaspiro[5.5]undecane, 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, (*E*,*E*)-2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane, (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane. These were detected by either antennae or maxillary palps of *B. bryoniae* males and/or females. Ethyl laurate, was detected by male and female *B. bryoniae* and males of *B. kraussi* and *B. musae*. Ethyl myristate and ethyl palmitate were also detected by male and female *B. bryoniae* and male *B. kraussi*. Methyl laurate was detected by *B. kraussi* males and *B. frauenfeldi* females. Ethyl caprate was detected by females of *B. frauenfeldi* and *B. bryoniae*. Two short chain esters, ethyl butanoate and ethyl 3-acetoxybutanoate, were detected by females of *B. musae* and *B. bryoniae*, respectively. The electrophysiological activity of these compounds suggests a possible biological role of these compounds in the mating system of each species.

Y-Tube olfaction bioassays were conducted to investigate the behavioural response of *B. frauenfeldi*, *B. bryoniae* and *B. kraussi* to the natural blend of rectal gland volatile compounds of the same and opposite conspecifics. Due to lack of continued availability of *B. musae* colonies, the olfaction bioassays were not investigated for this species. These bioassays showed that female *B. frauenfeldi* were attracted to the volatiles from conspecific male rectal glands but males were not. Neither males nor females were attracted to volatiles from female rectal glands. In combination with GC-EAG/GC-EPG, this suggests (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane and (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane act as sex pheromones in *B. frauenfeldi*. Interestingly, in *B. kraussi*, the natural blend of female rectal glands attracted conspecific males but did not attract females, and the natural blend of male rectal glands did not attract either males or females. These results in combination with GC-EAG/GC-EPG suggest (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, methyl laurate, ethyl laurate, ethyl myristate and ethyl palmitate are sex pheromones for *B. kraussi*. Unattractiveness of male-produced volatiles in this species suggests that male emissions may lose some aspects of function when isolated from visual and acoustic cues associated with mating behaviour. Not being attracted to the rectal gland exudates of same sex conspecifics in *B. frauenfeldi* and *B. kraussi* suggest that rectal gland exudates may not be key for aggregation of males or females of these species, however there may be other potential functions of these products, such as species or sex identification and quality assessment. For *B. bryoniae* rectal gland products from males were attractive to females and males, while the rectal gland products from females were only attractive to males. In combination with GC-EAG/GC-EPG, this suggests that ethyl 3-acetoxybutanoate, 1,7-dioxaspiro[5,5]undecane, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, 4-hydroxy-

1,7-dioxaspiro[5.5]undecane, ethyl laurate, ethyl myristate and ethyl palmitate may be components of *B. bryoniae* sex pheromone and ethyl 3-acetoxybutanoate, *N*-(3-methylbutyl)acetamide, 1,7-dioxaspiro[5,5]undecane and 4-hydroxy-1,7-dioxaspiro[5.5]undecane may be components of male aggregation pheromone. The results presented in this study contribute to the understanding of pheromone communication in *B. frauenfeldi*, *B. bryoniae*, *B. kraussi* and *B. musae* and pave the way for developing new monitoring and control methods.

Although in some species of Dacine fruit fly, females have been found to produce sex pheromones, previous studies of volatile compounds have mainly focused on chemical profiles of male fruit flies because males have typically been considered as the major sex pheromone producers. According to our findings in this study (*B. kraussi* and *B. bryoniae*), there is more evidence now that females specifically produce sex pheromones.

Among similar species to the four species investigated in this study, volatile profiles of *B. dorsalis, B. albistrigata* and *B. kirki* have been reported.[1–7] As discussed in Chapter 2, female *B. dorsalis*, which is a closely related species to *B. musae* and *B. bryoniae*, produce and release *N*-(3-methylbutyl)acetamide, (*E*,*E*)-2,8-dimethyl-l,7-dioxaspiro[5.5]undecane, (*E*,*E*)-8-ethyl-2-methyl-l,7-dioxaspiro[5.5]undecane, (*E*,*E*)-8-methyl-2-propyl-l,7-dioxaspiro[5.5]undecane as well as a series of fatty acid esters including ethyl laurate, ethyl myristate, ethyl myristoleate and ethyl palmitate. The main spiroacetal in this species, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, was also produced by female *B. musae* and *B. bryoniae*, and was electrophysiologically active for the opposite sex in both species. The fatty acid esters were also found in rectal glands of female *B. musae* and *B. bryoniae* of which ethyl laurate was electrophysiologically active for male of both species. Two other esters, ethyl myristate and ethyl palmitate as well as *N*-(3-methylbutyl)acetamide, were also detected by male *B. bryoniae* antenna. For males of *B. dorsalis*, long-chain fatty acids including acids of C_{12}, C_{14}, C_{16}, C_{18} and a fatty acid ester, ethyl laurate, were reported as the major components of rectal gland volatiles. *Bactrocera musae* also produced a fatty acid ester, ethyl butanoate, as the major component of their pheromonal gland composition, which is electrophysiologically active for conspecific females; while in *B. bryoniae* the main component was a spiroacetal, 1,7-dioxaspiro[5,5]undecane that along with two other compounds *N*-(3-methylbutyl)acetamide and 4-hydroxy-1,7-dioxaspiro[5.5]undecane were electrophysiologically active for females. The latter compounds, *N*-(3-methylbutyl)acetamide and 4-hydroxy-1,7-dioxaspiro[5.5]undecane have similar structures

to the minor compounds that have been reported in male *B. dorsalis*, *N*-(3-methylbutyl)acetamide and 3-hydroxy-2,8-dimethyl-l,7-dioxaspiro[5.5]undecane.

The male rectal gland secretions of the two similar species to *B. frauenfeldi*, *B. albistrigata* and *B. kirki*, were rich in spiroacetals. The major component of rectal glands of male *B. frauenfeldi* was (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, which was electrophysiologycally active for females. Another minor spiroacetal was also found in the male volatile profile of *B. frauenfeldi*, (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, which was also detected by female antenna and maxillary palps. Both these spiroacetals were also found in male rectal gland secretions of *B. albistrigata* or *B. kirki*. (*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecanes was the major component in male *B. kirki* and a minor compound in male *B. albistrigata*. (*E*,*E*)-2-Ethyl-8-methyl-l,7-dioxaspiro[5.5lundecane was a minor component of male *B. kirki* rectal gland extract.

Fruit flies typically produce and release chemicals at low concentration, therefore efficient methods for sample collection are needed. The use of different collection methods affects the extraction efficiency and traceability of the test compounds. In Chapter 6, common methods used for sampling of fruit fly rectal gland volatiles were compared using *B. tryoni* as a model species. The effect of solvent polarity, crushing of glands in solvent, adsorbent types in both dynamic and static (SPME) headspace sampling techniques, and volume of air sampled in dynamic headspace sampling techniques, were investigated. Among the three solvents (*n*-hexane, dichloromethane and ethanol) examined for rectal gland extractions, dichloromethane was the most effective solvent for extraction of amides. There was no significant difference between the three solvents in extraction of spiroacetals except for (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane for which *n*-hexane extracted higher amount than both dichloromethane and ethanol. Ethanol was an unsuitable solvent as it failed to contain many of the more volatile compounds. Although extractions from intact rectal glands yielded lower concentrations of compounds than extractions from crushed rectal glands, studies that used the two different methods for gland extractions are qualitatively comparable. Of SPME fibres, polydimethylsiloxane (PDMS) showed more affinity to spiroacetals than polyacrylate (PA). The type of fiber did not affect the amounts of amides and esters except for ethyl isobutyrate and ethyl-2-methylbutanoate. Polydimethylsiloxane/divinylbenzene (PDMS/DVB) trapped more ethyl isobutyrate and ethyl-2-methylbutanoate than PDMS, and both PDMS/DVB and PDMS trapped more than PA. Polyacrylate had comparatively low affinity for short chain esters and spiroacetals. In

dynamic headspace sampling methods, Porapak was found to be more effective for more volatile compounds, while Tenax was more effective for more polar compound, and sampling time was a critical factor to consider for the optimum results. The results presented in Chapter 6 contribute to the understanding of the differences among the various sampling methods and provide guidance for choosing the most appropriate method according to the nature of target compounds. In addition, this study identified six previously unreported compounds from male rectal glands in *B. tryoni* including ethyl propanoate, ethyl isobutyrate, ethyl 2-methylbutanoate, propyl isobutyrate, ethyl 2-methylpentanoate and diethyl succinate, as well as three additional unreported compounds from female rectal glands including *N*-(2-methylbutyl)acetamide, propyl laurate and methyl myristate. These findings resolve a long-standing discord between the perceptible odour and known blend composition.

In summary, this PhD study has achieved its goal of determining the putative pheromone profile of *B. frauenfeldi, B. bryoniae, B. kraussi* and *B. musae*. This study identified a total of 38 volatile compounds in the chemical profiles across males and females of these species. In addition to benefits for understanding of pheromonal communication in fruit flies, information about the pheromone chemistry of these species is an important foundation for the potential application of volatiles as attractants to improve the monitoring and control of these pest species. This PhD study has also achieved its second goal of comparing the effects of common sampling methods and materials on the detection and quantification of fruit fly volatiles and provided guidance for choosing the best method.

References:

1. Shen, J. *et al.* Allyl-2,6-dimethoxyphenol, a female-biased compound, is robustly attractive to conspecific males of *Bactrocera dorsalis* at close range. *Entomol. Exp. Appl.* **167**, 811–819 (2019).
2. Nishida, R. *et al.* Accumulation of phenylpropanoids in the rectal glands of males of the Oriental fruit fly, *Dacus dorsalis*. *Experientia* **44**, 534–536 (1988).
3. Baker, R. & Bacon, A. J. The identification of spiroacetals in the volatile secretions of two species of fruit fly (*Dacus dorsalis, Dacus curcurbitae*). *Experientia* **41**, 1484–1485 (1985).
4. Perkins, M. V., Fletcher, M. T., Kitching, W., Drew, R. A. I. & Moore, C. J. Chemical studies of rectal gland secretions of some species of *Bactrocera dorsalis*

complex of fruit flies (diptera: Tephritidae). *J. Chem. Ecol.* **16**, 2475–2487 (1990).

5. Ohinata, K. *et al.* Oriental fruit fly and melon fly; biological and chemical studies of smoke produced by males. *J. Environ. Sci. Heal. . Part A Environ. Sci. Eng.* **17**, 197–216 (1982).
6. Fletcher, M. T. *et al.* Chemistry of fruit-flies. Spiroacetal-rich secretions in several *Bactrocera* species from the South-West Pacific region. *J. Chem. Soc. Perkin Trans. 1* 2827–2831 (1992). doi:10.1039/P19920002827
7. Symonds, M. R. E., Moussalli, A. & Elgar, M. A. The evolution of sex pheromones in an ecologically diverse genus of flies. *Biol. J. Linn. Soc.* **97**, 594–603 (2009).

Appendix

Appendix I:

Supplementary material for

Rectal Gland Chemistry, Volatile Emissions, and Antennal Responses of Male and Female Banana Fruit Fly, *Bactrocera musae*

Saeedeh Noushini[1,3*], Jeanneth Perez[2,3], Soo Jean Park[2,3], Danielle Holgate[1], Ian Jamie[1,3], Joanne Jamie[1], Phillip Taylor[2,3]

[1] Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia.

[2] Applied BioSciences, Macquarie University, Sydney, NSW 2109, Australia.

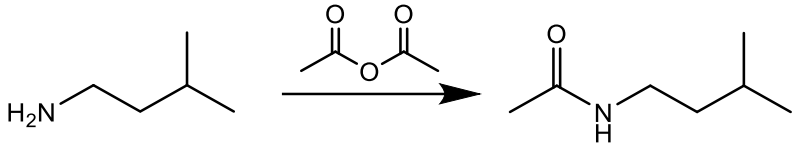
[3] Australian Research Council Industrial Transformation Training Centre for Fruit Fly Biosecurity Innovation, Macquarie University, Sydney, NSW 2109, Australia.

Corresponding author:
Saeedeh Noushini, Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia
E-mail:
ORCID: 0000-0001-5558-1656

Synthesis of compounds.

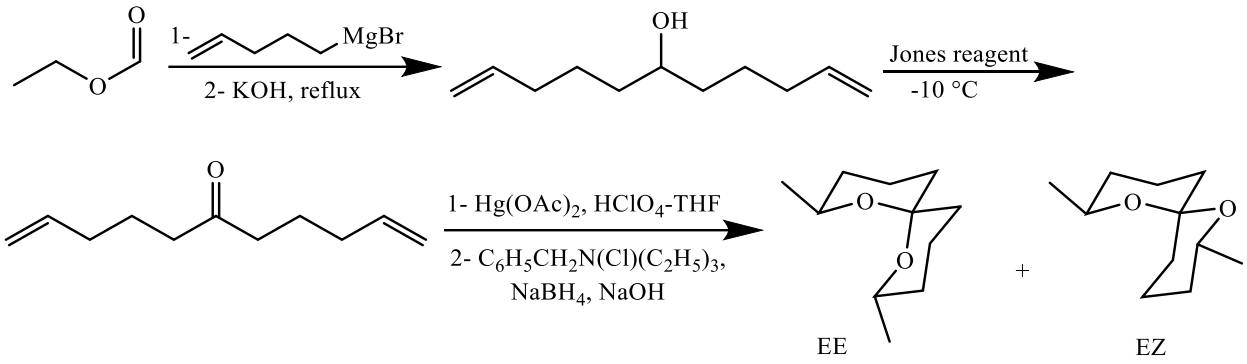
All reagents were purchased from Sigma-Aldrich and used without further purification. All solvents were anhydrous or analytical grade (Sigma-Aldrich) and used without further purification. The reaction progress was monitored by GC-MS (using the procedure given in the main text). Solvents were removed under reduced pressure using a Büchi Rotavapor R-200 and Büchi B-490 heating bath set to 40 °C. Mixtures were further dried under high vacuum using an Alcatel Pascal 2005SD vacuum pump. NMR spectra were recorded on a Bruker AVANCE-400 instrument (^{1}H NMR: 400 MHz, ^{13}C NMR: 101 MHz) or a Bruker AVANCE-600 instrument equipped with a cryoprobe (^{1}H NMR: 600 MHz, ^{13}C NMR: 150 MHz) using $CDCl_3$ and C_6D_6. The ^{1}H NMR chemical shifts were referenced to the residual protonated solvent peaks at δH 7.26 for chloroform-d and 7.15 for benzene-d_6. ^{13}C NMR chemical shifts were referenced to the central solvent peaks of bulk solvent at δC 77.16 for chloroform-d and 127.68 for benzene-d_6. *J* values are given in Hz.

Synthesis of *N*-(3-methylbutyl)acetamide (**2**).



The synthesis was conducted using the method of Naik et al.[1] To a mixture of 3-methylbutylamine (5.0 g, 57 mmol) in water (50 mL) was added acetic anhydride (8.7 g, 86 mmol). The clear reaction mixture was stirred at room temperature for 0.5 hour and the completion of the reaction at this time was determined by GC-MS. The clear reaction mixture was extracted with ethyl acetate (3 × 50 mL). The combined organic layer was washed with 5% aqueous sodium bicarbonate solution (150 mL), dried over sodium sulfate and concentrated under reduced pressure to give the crude product, which was purified by vacuum distillation (150 – 160 °C, 20 mm Hg) to afford *N*-(3-methylbutyl)acetamide (**2**) as a clear liquid (5.4 g, 73% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85 (6 H, d, *J* = 6.6, $CH(C\mathbf{H}_3)_2$), 1.33 (2 H, m, $C\mathbf{H}_2CH_3$), 1.56 (1 H, non, *J* = 6.7, C**H**), 1.92 (3 H, s, $C\mathbf{H}_3CO$), 3.18 (2 H, m, $NC\mathbf{H}_2$), 6.21 (1 H, bs, N**H**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 22.4, 23.1, 25.8, 38.0, 38.3, 170.4. GC-MS (EI) *m/z* (%) 129 (M^+, 5), 114 (M^+-CH_3, 12), 73 ($M^+-CH_2CH_2CH(CH_3)_2$, 100). MS data match with those in the literature.[2] NMR data are not available in the literature.

Synthesis of racemic 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**).



1,10-Undecadien-6-ol. Following the method of Kitching et al.,[3] Grignard reaction followed by hydrolysis was conducted to give 1,10-undecadien-6-ol. In brief, a flame-dried argon-flushed two-necked round bottom flask was charged with magnesium (1.8 g, 74 mmol), a single crystal of iodine, and a magnetic stirrer bar, and fitted with a condenser. Dry diethyl ether (60 mL) was added and the suspension was brought to reflux. 5-Bromopent-1-ene (10 g, 67 mmol) in diethyl ether (30 mL) was added dropwise and then the colourless suspension was stirred at reflux for 4 hours. The colourless suspension was cooled to 0 °C and ethyl formate (2.6 g, 34 mmol) was added. The suspension was warmed to room temperature, stirred for 1 hour, then quenched with saturated ammonium chloride and extracted with diethyl ether (3 × 15 mL). The combined organic layers were washed with saturated aqueous brine and dried over magnesium sulfate. After solvent removal by rotary evaporation, the yellow oil was refluxed in 15% aqueous potassium hydroxide solution for 3 hours. The solution was cooled to room temperature and extracted with diethyl ether (3 × 20 mL). Solvent was removed under reduced pressure to give the crude product as a yellow oil, which was purified by distillation (110 – 115 °C, 10 mm Hg) to afford 1,10-undecadien-6-ol as a colourless oil (3.7 g, 60% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.81 (2 H, ddt, *J* = 17, 10.3, 6.7 Hz, C**H**=), 5.01 (2 H, dq, *J* = 17.1, 1.7 Hz, C**H**$_2$=), 4.91 – 5.01 (2 H, m, C**H**$_2$=), 3.61 (1 H, bs, C**H**OH), 2.00 – 2.13 (4 H, m, C**H**$_2$CH=CH$_2$), 1.26 – 1.61 (9 H, m, including OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.7 (CH=), 114.6 (CH$_2$=), 71.7 (CHOH), 36.9 (CH$_2$), 33.7 (CH$_2$), 24.9 (CH$_2$). GC-MS (EI) *m/z* (%) 84 (12.3), 81 (100), 80 (10), 79 (19.5), 69 (9.2), 68 (9.3), 67 (20.6), 58 (9.5), 57 (30.2), 55 (72.3), 54 (27.7), 53 (9.4), 43 (32.1), 42 (9), 41 (43.8). Spectral data were consistent with the literature.[3]

Undeca-1,10-dien-6-one. To a solution of 1,10-undecadien-6-ol (3.99 g, 23.7 mmol) in acetone (10 mL) at -10 °C, freshly prepared Jones reagent (2.7 g of chromium trioxide in 4

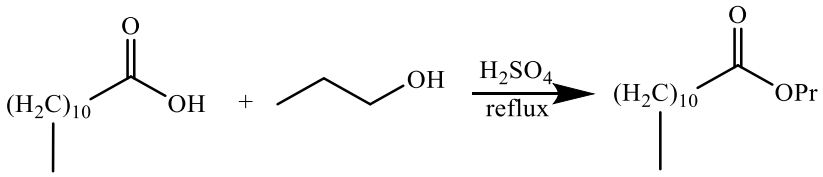
mL of sulfuric acid and 12 mL of distilled water) was added dropwise and the reaction monitored by GC-MS. After completion of the reaction (2 hours), the green suspension was filtered through a pad of Celite. The filtrate was washed with saturated aqueous sodium bicarbonate (17 mL), extracted with diethyl ether (4 × 50 mL) and washed with water (50 mL) and saturated aqueous brine (50 mL), then dried over magnesium sulfate. Concentration by rotary evaporation yielded undeca-1,10-dien-6-one as a colourless oil (2.9 g, 75% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.80 (2 H, ddt, *J* = 17.2, 10.3, 6.7 Hz, C**H**=), 4.87 – 4.97 (4 H, m, C**H**$_2$=), 2.33 (4 H, t, *J* = 7.5 Hz, C**H**$_2$CO), 1.98 (4 H, m, C**H**$_2$CH=CH_2), 1.60 (4 H, quin, *J* = 7.3 Hz, CH_2C**H**$_2$$CH_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 210.9 (CO), 138.0 (CH=), 115.2 (CH_2=), 41.9 (CH_2), 33.1 (CH_2), 22.8 (CH_2). GC-MS (EI) *m/z* (%) 112 (14.6), 97 (30), 84 (27.8), 83 (10.6), 70 (14.1), 69 (59.5), 58 (48.7), 55 (49.6), 43 (24.5), 41 (100). Spectral data were consistent with the literature.[3]

2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane. $Hg(OAc)_2$ (1.9 g, 6.1 mmol) was added to a stirred solution of undeca-1,10-dien-6-one (0.5 g, 3 mmol) in 1% aqueous perchloric acid: tetrahydrofuran (15 mL:15 mL) and the solution was stirred for 15 hours. Benzyltriethylammonium chloride (2.4 g, 10.5 mmol) in 10% aqueous sodium hydroxide (15 mL) and dichloromethane (5 mL) was added followed by sodium borohydride (0.09 g, 2.3 mmol) in 10% aqueous sodium hydroxide (5 mL). The gray suspension was stirred and monitored by GC-MS. After completion of the reaction (20 minutes), the gray suspension was filtered through a pad of Celite, which was then washed with 30 mL of diethyl ether. The aqueous phase was then extracted with diethyl ether (3 × 30 mL) and the combined organic layer (from Celite wash and extraction) were washed with saturated aqueous brine (50 mL) and dried over magnesium sulfate. After solvent removal by rotary evaporation the product was purified by Kugelrohr distillation (bp 110 °C; 30 mm Hg). According to the literature [3] under this condition a mixture of *E,E* diastereomer with some *E,Z* and no *Z,Z* isomer is obtained. These configurational isomers produced different MS fragmentation patterns that were matched with those in the literature.[3]

*(*E,E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane* (**3**). ^{13}C NMR (101 MHz, C_6D_6) δ 95.75 (CO_2), 64.8 (CO), 35.33 (CH_2), 32.90 (CH_2), 21.92 (CH_3), 19.03 (CH_2). GC-MS (EI) *m/z* (%) 184 (M^+, 5.6), 169 (M^+−CH_3, 1.9), 140 (M^+−CH_3CHO, 11.6), 125 (8.2), 115 (M^+−$CH_3CH_2CH_2CHCH^-$, 92.4), 114 (43.2), 113 (8.6), 112 (M^+−$CH_2CHCH(OH)CH_3$, 100), 97 (68.4), 84 (15.7), 83 (23.8), 73 (24.8), 71 (18.5), 70 (15.8), 69 (54.9), 58 (18.2), 55 (52.1), 43 (69.4), 42 (35.9), 41 (56.1).

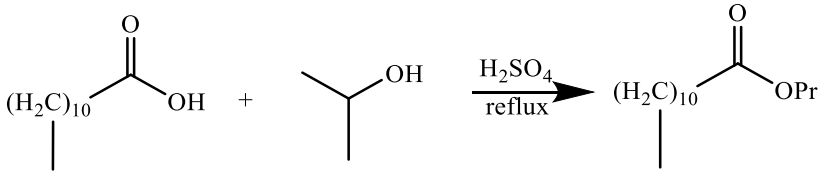
*(*E,Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane*. GC-MS (EI) *m/z* (%) 184 (M^+, 8.1), 115 (M^+−$CH_3CH_2CH_2CHCH^-$, 100), 114 (37), 112 (M^+−$CH_2CHCH(OH)CH_3$, 39.5), 97 (73.1), 83 (11.8), 73 (27.5), 71 (12.4), 69 (59.9), 55 (41.8), 43 (38.6), 42 (24.6), 41 (38.4).

Synthesis of propyl laurate (**9**).



A mixture of lauric acid (1.0 g, 5 mmol), 1-propanol (10 mL) and concentrated sulfuric acid (2 drops) was heated to reflux for 1.5 hours. After cooling, diethyl ether (10 mL) and 5% *w/v* aqueous sodium bicarbonate (10 mL) were added to the reaction mixture. The organic layer was separated and washed with 5% *w/v* aqueous sodium bicarbonate (3 × 10 mL) and dried over sodium sulfate. The solvent was removed under reduced pressure, yielding the crude product, which was purified by distillation to give ethyl palmitoleate as a colourless liquid (0.44 g, 38% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.87 (3 H, t, *J* = 7.0 Hz, $CH_2C\mathbf{H}_3$), 0.93 (3 H, t, *J* = 7.4 Hz, $OCH_2CH_2C\mathbf{H}_3$), 1.25-1.29 (16 H, m, $C\mathbf{H}_2$), 1.57-1.66 (4 H, m, $C\mathbf{H}_2CH_2COOPr$, $CH_3C\mathbf{H}_2CH_2OCO$), 2.29 (2 H, t, *J* = 7.5 Hz, $C\mathbf{H}_2COOPr$), 4.02 (2 H, t, *J* = 6.7 Hz, $C\mathbf{H}_2OCO$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 10.5 (CH_3), 14.2 (CH_3), 22.1 (CH_2), 22.8 (CH_2), 25.1 (CH_2), 29.3 (CH_2), 29.40 (CH_2), 29.47 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 32.0 (CH_2), 34.5 (CH_2), 65.9 (OCH_2), 174.1 (C=O). GC-MS (EI) *m/z* (%) 242 (M^+, 4.3), 213 (1.4), 201 (M^+−$CH_2CH_2CH_3$, 27.5), 183 (M^+−$OCH_2CH_2CH_3$, 25.7), 171 (6.6), 157 (6.8), 143 (3.3), 129 (8.7), 115 (21.8), 102 (McLafferty rearrangement product, 32.5), 97 (7.7), 85 (12.2), 73 (39.3), 61 (100), 57 (30.9), 43 (80.2).

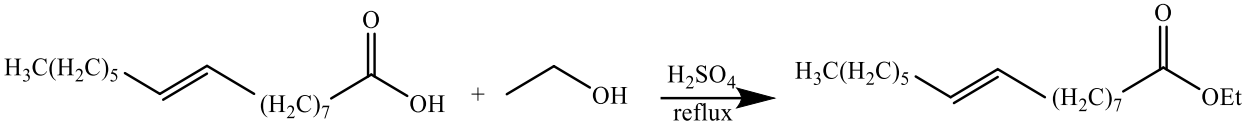
Synthesis of isopropyl laurate.



Using similar conditions to above, lauric acid (1.0 g, 5 mmol), was esterified with 2-propanol (10 mL) in the presence of concentrated sulfuric acid (2 drops), quenched with sodium bicarbonate, extracted with diethyl ether and purified by distillation to afford isopropyl laurate as a white waxy solid, mp 175-185 °C (0.34 g, 29% yield). ^{1}H NMR (400

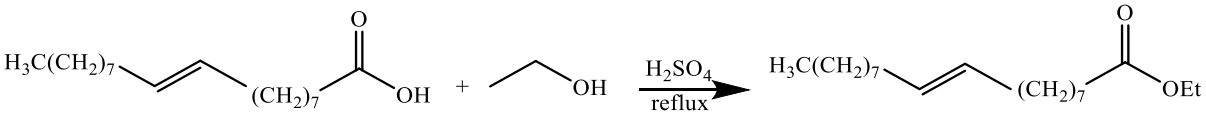
MHz, $CDCl_3$) δ 0.87 (3H, t, *J* = 6.8 Hz, $CH_2C\mathbf{H}_3$), 1.22 (6H, d, *J* = 6.2 Hz, $OCH(C\mathbf{H}_3)_2$), 1.25 – 1.28 (16H, m, $C\mathbf{H}_2$), 1.58 – 1.62 (2H, m, $C\mathbf{H}_2CH_2COO$*i*Pr), 2.25 (2H, t, *J* = 7.6 Hz, $C\mathbf{H}_2COO$*i*Pr), 4.97 – 5.03 (1H, m, $OC\mathbf{H}(CH_3)_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.5 (CH_3), 22.2 (CH_2), 23.1 (CH_2), 25.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 29.8 (CH_2), 29.9 (CH_2), 32.3 (CH_2), 35.1 (CH_2), 67.7 (OCH), 173.8 (C=O). GC-MS (EI) *m/z* (%) 242 (M^+, 0.9), 200 ($M^+-CH(CH_3)_2$, 26.8), 183 ($M^+-OCH(CH_3)_2$, 17.8), 171 (3.1), 157 (8.4), 143 (3.6), 129 (10.1), 115 (7.0), 102 (51.0), 97 (10.3), 85 (16.8), 73 (28.1), 60 (70.0), 57 (43.3), 43 (100). Experimental spectra were consistent with literature data.[4,5]

Synthesis of ethyl palmitoleate (**15**).



Using similar conditions to above, palmitoleic acid (0.50 g, 1.9 mmol), was esterified with ethanol (10 mL) in the presence of concentrated sulfuric acid (2 drops), quenched with sodium bicarbonate and diethyl ether and purified by distillation to afford ethyl palmitoleate as a colourless oil (0.11 g, 19% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.32 – 5.35 (2 H, m, C**H**=C**H**), 4.11 (2 H, q, *J* = 7.2 Hz, $OC\mathbf{H}_2CH_3$), 2.28 (2 H, t, *J* = 7.5 Hz, $C\mathbf{H}_2COOEt$), 1.98 – 2.01 (4 H, m, $C\mathbf{H}_2CH{=}CHC\mathbf{H}_2$), 1.59 – 1.63 (2 H, m, $C\mathbf{H}_2CH_2COOEt$), 1.23 – 1.30 (19 H, m, $C\mathbf{H}_2$), 0.88 (3 H, t, *J* = 6.9 Hz, $CH_2C\mathbf{H}_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.0 (C=O), 130.1 (CH), 129.9 (CH), 60.2 (OCH_2), 34.5 (CH_2), 31.9 (CH_2), 29.87 (CH_2), 29.82 (CH_2), 29.3 (CH_2), 29.26 (CH_2), 29.23 (CH_2), 29.1 (CH_2), 27.36 (CH_2), 27.30 (CH_2), 25.1 (CH_2), 22.8 (CH_2), 14.4 (CH_3), 14.2 (CH_3). GC-MS (EI) *m/z* (%) 282 (M^+, 3.8), 236 ($M^+-OCH_2CH_3$, 14.3), 218 (1.4), 207 (1.4), 194 ($M^+-CH_2COOCH_2CH_3$, 15.0), 179 (1.65), 165 (2.8), 152 ($M^+-(CH_2)_4COOCH_2CH_3$, 14.9), 138 (6.9), 123 (11.9), 101 (31.8), 88 (50.6), 83 (44.9), 69 (64.1), 55 (100), 41 (81.8). Spectral data were not available in the literature.

Synthesis of ethyl elaidate (**17**).



Using similar conditions to above, elaidic acid (0.45 g, 1.6 mmol), was esterified with ethanol (10 mL) in the presence of concentrated sulfuric acid (2 drops), quenched with sodium bicarbonate and diethyl ether and purified by distillation to afford ethyl elaidate as a colourless oil (113 mg, 23% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 – 5.28 (2 H, m, C**H**=C**H**), 4.11 (2 H, q, *J* = 7.1 Hz, OC**H**$_2$$CH_3$), 2.27 (2 H, t, *J* = 7.6 Hz, C**H**$_2$COOEt), 1.95 – 1.96 (4 H, m, C**H**$_2$CH=CHC**H**$_2$), 1.57 – 1.60 (2 H, m, C**H**$_2$$CH_2$COOEt), 1.23 – 1.28 (23 H, m, CH_2), 0.87 (3 H, t, *J* = 6.7 Hz, CH_2CH_2C**H**$_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.0 (C=O), 130.6 (CH), 130.4 (CH), 60.3 (OCH_2), 34.5 (CH_2), 32.78 (CH_2), 32.73 (CH_2), 32.0 (CH_2), 29.8 (CH_2), 29.7 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.36 (CH_2), 29.30 (CH_2), 29.1 (CH_2), 25.1 (CH_2), 22.8 (CH_2), 14.4 (CH_3), 14.2 (CH_3). GC-MS (EI) *m/z* (%) 310 (M^+, 3.5), 281 ($M^+-CH_2CH_3$, 0.25), 264 ($M^+-OCH_2CH_3$, 16.2), 222 (11.3), 180 (11.2), 155 (7.0), 138 (5.6), 123 (13.5), 111 (20.6), 97 (38.6), 88 (45.6), 83 (49.0), 69 (69.0), 55 (100), 41 (76.4). Spectral data were consistent with the literature.[6]

References

1. Naik, S., Bhattacharjya, G., Talukdar, B. & Patel, B. K. Chemoselective acylation of amines in aqueous media. *European J. Org. Chem.* **2004**, 1254–1260 (2004).
2. Wee, S. L. & Tan, K. H. Female sexual response to male rectal volatile constituents in the fruit fly, *Bactrocera carambolae* (Diptera: Tephritidae). *Appl. Entomol. Zool.* **40**, 365–372 (2005).
3. Kitching, W. *et al.* Chemistry of fruit flies. Composition of the rectal gland secretion of (male) *Dacus cucumis* (cucumber fly) and *Dacus halfordiae*. Characterization of (*Z,Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. *J. Org. Chem.* **54**, 3893–3902 (1989).
4. Kartika, T., Shimizu, N. & Yoshimura, T. Identification of esters as novel aggregation pheromone components produced by the male powder-post beetle, *Lyctus africanus* Lesne (Coleoptera: Lyctinae). *PLoS One* **10**, e0141799 (2015).
5. Maegawa, T., Otake, K., Goto, A. & Fujioka, H. Direct conversion of acetals to esters with high regioselectivity via *O,P*-acetals. *Org. Biomol. Chem.* **9**, 5648–5651 (2011).
6. Denton, R. M., Tang, X. & Przeslak, A. Catalysis of phosphorus(V)-mediated transformations: dichlorination reactions of epoxides under Appel conditions. *Org. Lett.* **12**, 4678–4681 (2010).

Appendix II:

Supplementary information for

Attraction and Electrophysiological Response to Identified Rectal Gland Volatiles in *Bactrocera frauenfeldi* (Schiner)

Saeedeh Noushini[1,3*], Jeanneth Perez[2,3], Soo Jean Park[1,3], Danielle Holgate[1], Vivian Mendez Alvarez[2,3], Ian Jamie[1,3], Joanne Jamie[1, 3], Phillip Taylor[2,3]

[1] Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia.

[2] Applied BioSciences, Macquarie University, Sydney, NSW 2109, Australia.

[3] Australian Research Council Industrial Transformation Training Centre for Fruit Fly Biosecurity Innovation, Macquarie University, Sydney, NSW 2109, Australia.

Corresponding author:
Saeedeh Noushini, Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia
E-mail:
ORCID: 0000-0001-5558-1656

General Procedures for Synthesis

All reagents were purchased from Sigma-Aldrich and used without further purification. All solvents were anhydrous or analytical grade (Sigma-Aldrich) and used without further purification. The reaction progress was monitored by GC-MS (using the procedure given in the main text). Solvents were removed under reduced pressure using a Büchi Rotavapor R-200 and Büchi B-490 heating bath set to 40 °C. Mixtures were further dried under high vacuum using an Alcatel Pascal 2005SD vacuum pump. NMR spectra were recorded on a Bruker AVANCE-400 instrument (^{1}H NMR: 400 MHz, ^{13}C NMR: 101 MHz) using $CDCl_3$ and C_6D_6. The ^{1}H NMR chemical shifts were referenced to the residual protonated solvent peaks at δH 7.26 for chloroform-d and 7.15 for benzene-d_6. ^{13}C NMR chemical shifts were referenced to the central solvent peaks of bulk solvent at δC 77.16 for chloroform-d and 127.68 for benzene-d_6. *J* values are given in Hz.

Synthesis of *N*-(2-methylbutyl)acetamide (**1**).

The synthesis was conducted using the method of Naik et al.[1] To a mixture of 2-methylbutylamine (4.4 g, 50 mmol) in water (50 mL) was added acetic anhydride (7.7 g, 75 mmol). The clear reaction mixture was stirred at room temperature for 0.5 hour and the completion of the reaction at this time was determined by GC-MS. The clear reaction mixture was extracted with ethyl acetate (3 × 50 mL). The combined organic layer was washed with 5% aqueous sodium bicarbonate solution (150 mL), dried over sodium sulfate and concentrated under reduced pressure to give the crude product, which was purified by vacuum distillation (150 – 160 °C, 20 mm Hg) to afford *N*-(2-methylbutyl)acetamide (**1**) as a clear liquid (5.6 g, 86% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.83 (6 H, m, $CHCH_2C\mathbf{H}_3$ and $CH_2C\mathbf{H}_3$), 1.07 (1 H, apparent sep, *J* = 6.6, $C\mathbf{H}CH_3$), 1.29 – 1.53 (2 H, m, $C\mathbf{H}_2CH_3$), 1.93 (3 H, s, $C\mathbf{H}_3CO$), 2.94 – 3.13 (2 H, m, $NC\mathbf{H}_2$), 6.33 (1 H, bs, N**H**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 11.2, 17.1, 23.1, 27.0, 34.8, 45.4, 170.6. GC-MS (EI) *m/z* (%) 129 (M^+, 8), 100 ($M^+-CH_2CH_3$, 38), 72 ($M^+-CHCH(CH_3)CH_2CH_3$, 100). This compound is known, but spectral data are not available in the literature.

Synthesis of *N*-(3-methylbutyl)acetamide (**2**).

Using a similar reaction, work-up and purification conditions to *N*-(2-methylbutyl)acetamide (**1**) (above), 3-methylbutylamine (5.0 g, 57 mmol) in water (50 mL) was acetylated with acetic anhydride (8.7 g, 86 mmol) to produce *N*-(3-methylbutyl)acetamide (**2**) as a clear liquid (5.4 g, 73% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85 (6 H, d, *J* = 6.6, $CH(C\mathbf{H}_3)_2$), 1.33 (2 H, m, $C\mathbf{H}_2CH_3$), 1.56 (1 H, non, *J* =

6.7, C**H**), 1.92 (3 H, s, C**H**$_3$CO), 3.18 (2 H, m, NC**H**$_2$), 6.21 (1 H, bs, N**H**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 22.4, 23.1, 25.8, 38.0, 38.3, 170.4. GC-MS (EI) *m/z* (%) 129 (M^+, 5), 114 (M^+-CH_3, 12), 73 ($M^+-CH_2CH_2CH(CH_3)_2$, 100). MS data match with those in the literature.[2] NMR data are not available in the literature.

Synthesis of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**).

1,10-Undecadien-6-ol. Following the method of Kitching et al.,[3] Grignard reaction followed by hydrolysis was conducted to give 1,10-undecadien-6-ol. In brief, a flame-dried argon-flushed two-necked round bottom flask was charged with magnesium (1.8 g, 74 mmol), a single crystal of iodine, and a magnetic stirrer bar, and fitted with a condenser. Dry diethyl ether (60 mL) was added and the suspension was brought to reflux. 5-Bromopent-1-ene (10 g, 67 mmol) in diethyl ether (30 mL) was added dropwise and then the colourless suspension was stirred at reflux for 4 hours. The colourless suspension was cooled to 0 °C and ethyl formate (2.6 g, 34 mmol) was added. The suspension was warmed to room temperature, stirred for 1 hour, then quenched with saturated ammonium chloride and extracted with diethyl ether (3 × 15 mL). The combined organic layers were washed with saturated aqueous brine and dried over magnesium sulfate. After solvent removal by rotary evaporation, the yellow oil was refluxed in 15% aqueous potassium hydroxide solution for 3 hours. The solution was cooled to room temperature and extracted with diethyl ether (3 × 20 mL). Solvent was removed under reduced pressure to give the crude product as a yellow oil, which was purified by distillation (110 – 115 °C, 10 mm Hg) to afford 1,10-undecadien-6-ol as a colourless oil (3.7 g, 60% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.81 (2 H, ddt, *J* = 17, 10.3, 6.7 Hz, C**H**=), 5.01 (2 H, dq, *J* = 17.1, 1.7 Hz, C**H**$_2$=), 4.91 – 5.01 (2 H, m, C**H**$_2$=), 3.61 (1 H, bs, C**H**OH), 2.00 – 2.13 (4 H, m, C**H**$_2$CH=CH$_2$), 1.26 – 1.61 (9 H, m, including OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.7 (CH=), 114.6 (CH$_2$=), 71.7 (CHOH), 36.9 (CH_2), 33.7 (CH_2), 24.9 (CH_2). GC-MS (EI) *m/z* (%) 84 (12.3), 81 (100), 80 (10), 79 (19.5), 69 (9.2), 68 (9.3), 67 (20.6), 58 (9.5), 57 (30.2), 55 (72.3), 54 (27.7), 53 (9.4), 43 (32.1), 42 (9), 41 (43.8). Spectral data were consistent with the literature.[3]

Undeca-1,10-dien-6-one. To a solution of 1,10-undecadien-6-ol (3.99 g, 23.7 mmol) in acetone (10 mL) at -10 °C, freshly prepared Jones reagent (2.7 g of chromium trioxide in 4 mL of sulfuric acid and 12 mL of distilled water) was added dropwise and the reaction monitored by GC-MS. After completion of the reaction (2 hours), the green suspension was filtered through a pad of Celite. The filtrate was washed with saturated aqueous

sodium bicarbonate (17 mL), extracted with diethyl ether (4 × 50 mL) and washed with water (50 mL) and saturated aqueous brine (50 mL), then dried over magnesium sulfate. Concentration by rotary evaporation yielded undeca-1,10-dien-6-one as a colourless oil (2.9 g, 75% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.80 (2 H, ddt, *J* = 17.2, 10.3, 6.7 Hz, C**H**=), 4.87 – 4.97 (4 H, m, C**H**$_2$=), 2.33 (4 H, t, *J* = 7.5 Hz, C**H**$_2$CO), 1.98 (4 H, m, C**H**$_2$CH=CH$_2$), 1.60 (4 H, quin, *J* = 7.3 Hz, CH$_2$C**H**$_2$CH$_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 210.9 (CO), 138.0 (CH=), 115.2 (CH$_2$=), 41.9 (CH$_2$), 33.1 (CH$_2$), 22.8 (CH$_2$). GC-MS (EI) *m/z* (%) 112 (14.6), 97 (30), 84 (27.8), 83 (10.6), 70 (14.1), 69 (59.5), 58 (48.7), 55 (49.6), 43 (24.5), 41 (100). Spectral data were consistent with the literature.[3]

2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane. $Hg(OAc)_2$ (1.9 g, 6.1 mmol) was added to a stirred solution of undeca-1,10-dien-6-one (0.5 g, 3 mmol) in 1% aqueous perchloric acid: tetrahydrofuran (15 mL:15 mL) and the solution was stirred for 15 hours. Benzyltriethylammonium chloride (2.4 g, 10.5 mmol) in 10% aqueous sodium hydroxide (15 mL) and dichloromethane (5 mL) was added followed by sodium borohydride (0.09 g, 2.3 mmol) in 10% aqueous sodium hydroxide (5 mL). The grey suspension was stirred and monitored by GC-MS. After completion of the reaction (20 minutes), the grey suspension was filtered through a pad of Celite, which was then washed with 30 mL of diethyl ether. The aqueous phase was then extracted with diethyl ether (3 × 30 mL) and the combined organic layer (from Celite wash and extraction) were washed with saturated aqueous brine (50 mL) and dried over magnesium sulfate. After solvent removal by rotary evaporation the product was purified by Kugelrohr distillation (bp 110 °C; 30 mm Hg). According to the literature [3] under this condition a mixture of *E,E* diastereomer with some *E,Z* and no *Z,Z* isomer is obtained. These configurational isomers produced different MS fragmentation patterns that were matched with those in the literature.[3]

*(*E,E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane* (**3**). ^{13}C NMR (101 MHz, C_6D_6) δ 95.75 (CO_2), 64.8 (CO), 35.33 (CH$_2$), 32.90 (CH$_2$), 21.92 (CH$_3$), 19.03 (CH$_2$). GC-MS (EI) *m/z* (%) 184 (M^+, 5.6), 169 (M^+-CH_3, 1.9), 140 (M^+-CH_3CHO, 11.6), 125 (8.2), 115 ($M^+-CH_3CH_2CH_2CHCH^-$, 92.4), 114 (43.2), 113 (8.6), 112 ($M^+-CH_2CHCH(OH)CH_3$, 100), 97 (68.4), 84 (15.7), 83 (23.8), 73 (24.8), 71 (18.5), 70 (15.8), 69 (54.9), 58 (18.2), 55 (52.1), 43 (69.4), 42 (35.9), 41 (56.1).

*(*E,Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane* (**3**). GC-MS (EI) *m/z* (%) 184 (M^+, 8.1), 115 (M^+−$CH_3CH_2CH_2CHCH^-$, 100), 114 (37), 112 (M^+−$CH_2CHCH(OH)CH_3$, 39.5), 97 (73.1), 83 (11.8), 73 (27.5), 71 (12.4), 69 (59.9), 55 (41.8), 43 (38.6), 42 (24.6), 41 (38.4).

Ethyl Palmitoleate (**18**). A mixture of palmitoleic acid (0.50 g, 1.9 mmol), ethanol (10 mL) and concentrated sulfuric acid (2 drops) was heated to reflux for 1.5 hours. After cooling, diethyl ether (10 mL) and 5% *w/v* aqueous sodium bicarbonate (10 mL) were added to the reaction mixture. The organic layer was separated and washed with 5% *w/v* aqueous sodium bicarbonate (3 × 10 mL) and dried over sodium sulfate. The solvent was removed under reduced pressure, yielding the crude product, which was purified by distillation to give ethyl palmitoleate as a colourless liquid (0.11 g, 19% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.32 – 5.35 (2 H, m, C**H**=C**H**), 4.11 (2 H, q, *J* = 7.2 Hz, OC**H₂**CH_3), 2.28 (2 H, t, *J* = 7.5 Hz, C**H₂**COOEt), 1.98 – 2.01 (4 H, m, C**H₂**CH=CHC**H₂**), 1.59 – 1.63 (2 H, m, C**H₂**CH_2COOEt), 1.23 – 1.30 (19 H, m, C**H₂**), 0.88 (3 H, t, *J* = 6.9 Hz, CH_2C**H₃**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.0 (C=O), 130.1 (CH), 129.9 (CH), 60.2 (OCH_2), 34.5 (CH_2), 31.9 (CH_2), 29.87 (CH_2), 29.82 (CH_2), 29.3 (CH_2), 29.26 (CH_2), 29.23 (CH_2), 29.1 (CH_2), 27.36 (CH_2), 27.30 (CH_2), 25.1 (CH_2), 22.8 (CH_2), 14.4 (CH_3), 14.2 (CH_3). GC-MS (EI) *m/z* (%) 282 (M^+, 3.8), 236 (M^+−OCH_2CH_3, 14.3), 218 (1.4), 207 (1.4), 194 (M^+−$CH_2COOCH_2CH_3$, 15.0), 179 (1.65), 165 (2.8), 152 (M^+−$(CH_2)_4COOCH_2CH_3$, 14.9), 138 (6.9), 123 (11.9), 101 (31.8), 88 (50.6), 83 (44.9), 69 (64.1), 55 (100), 41 (81.8). Spectral data were not available in the literature.

Ethyl Elaidate (**22**). Using similar conditions to above, elaidic acid (0.45 g, 1.6 mmol), was esterified with ethanol (10 mL) in the presence of concentrated sulfuric acid (2 drops), quenched with sodium bicarbonate and diethyl ether and purified by distillation to afford ethyl elaidate as a colourless oil (113 mg, 23% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 – 5.28 (2 H, m, C**H**=C**H**), 4.11 (2 H, q, *J* = 7.1 Hz, OC**H₂**CH_3), 2.27 (2 H, t, *J* = 7.6 Hz, C**H₂**COOEt), 1.95 – 1.96 (4 H, m, C**H₂**CH=CHC**H₂**), 1.57 – 1.60 (2 H, m, C**H₂**CH_2COOEt), 1.23 – 1.28 (23 H, m, CH_2), 0.87 (3 H, t, *J* = 6.7 Hz, CH_2CH_2C**H₃**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.0 (C=O), 130.6 (CH), 130.4 (CH), 60.3 (OCH_2), 34.5 (CH_2), 32.78 (CH_2), 32.73 (CH_2), 32.0 (CH_2), 29.8 (CH_2), 29.7 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.36 (CH_2), 29.30 (CH_2), 29.1 (CH_2), 25.1 (CH_2), 22.8 (CH_2), 14.4 (CH_3), 14.2 (CH_3). GC-MS (EI) *m/z* (%) 310 (M^+, 3.5), 281 (M^+−CH_2CH_3, 0.25), 264 (M^+−OCH_2CH_3, 16.2), 222 (11.3), 180 (11.2), 155 (7.0), 138 (5.6), 123 (13.5), 111 (20.6), 97 (38.6), 88

(45.6), 83 (49.0), 69 (69.0), 55 (100), 41 (76.4). Spectral data were consistent with the literature.[4]

Y-Maze apparatus

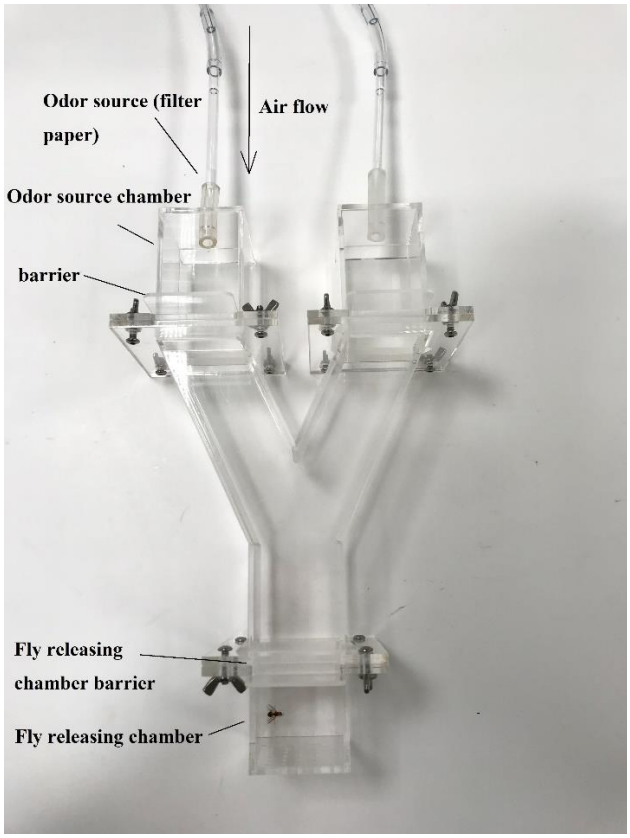


Figure 9. Y-Maze apparatus used in this study

References

1. Naik, S., Bhattacharjya, G., Talukdar, B. & Patel, B. K. Chemoselective acylation of amines in aqueous media. *European J. Org. Chem.* **2004**, 1254–1260 (2004).
2. Wee, S. L. & Tan, K. H. Female sexual response to male rectal volatile constituents in the fruit fly, *Bactrocera carambolae* (Diptera: Tephritidae). *Appl. Entomol. Zool.* **40**, 365–372 (2005).
3. Kitching, W. *et al.* Chemistry of fruit flies. Composition of the rectal gland secretion of (male) *Dacus cucumis* (cucumber fly) and *Dacus halfordiae*. Characterization of (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. *J. Org. Chem.* **54**, 3893–3902 (1989).

4. Denton, R. M., Tang, X. & Przeslak, A. Catalysis of phosphorus(V)-mediated transformations: dichlorination reactions of epoxides under Appel conditions. *Org. Lett.* **12**, 4678–4681 (2010).

Appendix III:

Supplementary Material for

Rectal Gland Exudates and Emissions of *Bactrocera bryoniae*: Chemical Identification, Electrophysiological and Pheromonal Functions

Saeedeh Noushini[1,3*], Soo Jean Park[2,3], Ian Jamie[1,3], Joanne Jamie[1], Phillip Taylor[2,3]

[1] *Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia.*

[2] *Applied BioSciences, Macquarie University, Sydney, NSW 2109, Australia.*

[3] *Australian Research Council Industrial Transformation Training Centre for Fruit Fly Biosecurity Innovation, Macquarie University, Sydney, NSW 2109, Australia.*

Corresponding author:

Saeedeh Noushini, Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia

E-mail:

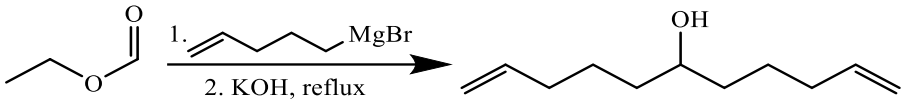
ORCID: 0000-0001-5558-1656

Synthesis of compounds.

All reagents were purchased from Sigma-Aldrich and used without further purification. All solvents were anhydrous or analytical grade (Sigma-Aldrich) and used without further purification. The reaction progress was monitored by GC-MS (using the procedure given in the main text). Solvents were removed under reduced pressure using a Büchi Rotavapor R-200 and Büchi B-490 heating bath set to 40 °C. Mixtures were further dried under high vacuum using an Alcatel Pascal 2005SD vacuum pump. NMR spectra were recorded on a Bruker AVANCE-400 instrument (^{1}H NMR: 400 MHz, ^{13}C NMR: 101 MHz) or a Bruker AVANCE-600 instrument equipped with a cryoprobe (^{1}H NMR: 600 MHz, ^{13}C NMR: 150 MHz) using $CDCl_3$ and C_6D_6. The ^{1}H NMR chemical shifts were referenced to the residual protonated solvent peaks at δH 7.26 for chloroform-d and 7.15 for benzene-d_6. ^{13}C NMR chemical shifts were referenced to the central solvent peaks of bulk solvent at δC 77.16 for chloroform-d and 127.68 for benzene-d_6. *J* values are given in Hz.

Synthesis of (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**4**).

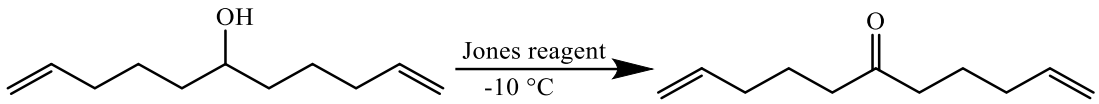
1,10-Undecadien-6-ol.



Following the method of Kitching et al.,[1] a Grignard reaction followed by hydrolysis was conducted to give 1,10-undecadien-6-ol. In brief, a flame-dried argon-flushed two-necked round bottom flask was charged with magnesium (1.8 g, 74 mmol), a single crystal of iodine, and a magnetic stirrer bar, and fitted with a condenser. Dry diethyl ether (60 mL) was added and the suspension was brought to reflux. 5-Bromopent-1-ene (10 g, 67 mmol) in diethyl ether (30 mL) was added dropwise and then the colourless suspension was stirred at reflux for 4 hours. The colourless suspension was cooled to 0 °C and ethyl formate (2.6 g, 34 mmol) was added. The suspension was warmed to room temperature, stirred for 1 hour, then quenched with saturated ammonium chloride and extracted with diethyl ether (3 × 15 mL). The combined organic layers were washed with saturated aqueous brine and dried over magnesium sulfate. After solvent removal by rotary evaporation, the yellow oil was heated to reflux in 15% aqueous potassium hydroxide solution for 3 hours. The solution was cooled to room temperature and extracted with

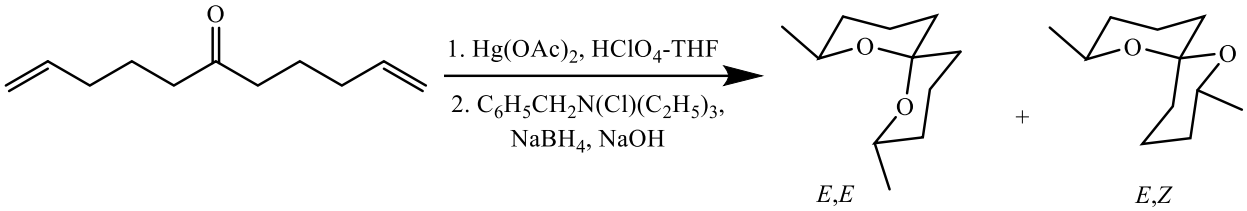
diethyl ether (3 × 20 mL). Solvent was removed under reduced pressure to give the crude product as a yellow oil, which was purified by distillation (110 – 115 °C, 10 mm Hg) to afford 1,10-undecadien-6-ol as a colourless oil (3.7 g, 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.81 (2 H, ddt, *J* = 17, 10.3, 6.7 Hz, C**H**=), 5.01 (2 H, dq, *J* = 17.1, 1.7 Hz, C**H**$_2$=), 4.91 – 5.01 (2 H, m, C**H**$_2$=), 3.61 (1 H, bs, C**H**OH), 2.00 – 2.13 (4 H, m, C**H**$_2$CH=CH$_2$), 1.26 – 1.61 (9 H, m, including OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.7 (CH=), 114.6 (CH_2=), 71.7 (CHOH), 36.9 (CH_2), 33.7 (CH_2), 24.9 (CH_2). GC-MS (EI) *m/z* (%) 84 (12.3), 81 (100), 80 (10), 79 (19.5), 69 (9.2), 68 (9.3), 67 (20.6), 58 (9.5), 57 (30.2), 55 (72.3), 54 (27.7), 53 (9.4), 43 (32.1), 42 (9), 41 (43.8). Spectral data were consistent with the literature.[1]

Undeca-1,10-dien-6-one.



To a solution of 1,10-undecadien-6-ol (3.99 g, 23.7 mmol) in acetone (10 mL) at -10 °C, freshly prepared Jones reagent (2.7 g of chromium trioxide in 4 mL of sulfuric acid and 12 mL of distilled water) was added dropwise and the reaction monitored by GC-MS. After completion of the reaction (2 hours), the green suspension was filtered through a pad of Celite. The filtrate was washed with saturated aqueous sodium bicarbonate (17 mL), extracted with diethyl ether (4 × 50 mL) and washed with water (50 mL) and saturated aqueous brine (50 mL), then dried over magnesium sulfate. Concentration by rotary evaporation yielded undeca-1,10-dien-6-one as a colourless oil (2.9 g, 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.80 (2 H, ddt, *J* = 17.2, 10.3, 6.7 Hz, C**H**=), 4.87 – 4.97 (4 H, m, C**H**$_2$=), 2.33 (4 H, t, *J* = 7.5 Hz, C**H**$_2$CO), 1.98 (4 H, m, C**H**$_2$CH=CH$_2$), 1.60 (4 H, quin, *J* = 7.3 Hz, CH$_2$C**H**$_2$CH$_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 210.9 (CO), 138.0 (CH=), 115.2 (CH_2=), 41.9 (CH_2), 33.1 (CH_2), 22.8 (CH_2). GC-MS (EI) *m/z* (%) 112 (14.6), 97 (30), 84 (27.8), 83 (10.6), 70 (14.1), 69 (59.5), 58 (48.7), 55 (49.6), 43 (24.5), 41 (100). Spectral data were consistent with the literature.[1]

2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane.



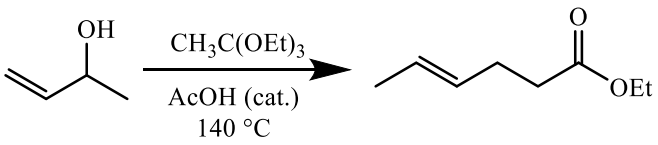
$Hg(OAc)_2$ (1.9 g, 6.1 mmol) was added to a stirred solution of undeca-1,10-dien-6-one (0.5 g, 3 mmol) in 1% aqueous perchloric acid:tetrahydrofuran (15 mL:15 mL) and the solution was stirred for 15 hours. Benzyltriethylammonium chloride (2.4 g, 10.5 mmol) in 10% aqueous sodium hydroxide (15 mL) and dichloromethane (5 mL) was added, followed by sodium borohydride (0.09 g, 2.3 mmol) in 10% aqueous sodium hydroxide (5 mL). The gray suspension was stirred and monitored by GC-MS. After completion of the reaction (20 minutes), the gray suspension was filtered through a pad of Celite, which was then washed with 30 mL of diethyl ether. The aqueous phase was then extracted with diethyl ether (3 × 30 mL) and the combined organic layers (from Celite wash and extraction) were washed with saturated aqueous brine (50 mL) and dried over magnesium sulfate. After solvent removal by rotary evaporation, the product was purified by Kügelrohr distillation (bp 110 °C; 30 mm Hg). In accordance with the literature [1], a mixture of *E,E* diastereomer with some *E,Z* and no *Z,Z* isomer was obtained. These two configurational isomers produced different MS fragmentation patterns that matched those in the literature.[1]

(*E,E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**4**). ^{13}C NMR (101 MHz, C_6D_6) δ 95.75 (CO_2), 64.8 (CO), 35.33 (CH_2), 32.90 (CH_2), 21.92 (CH_3), 19.03 (CH_2). GC-MS (EI) *m/z* (%) 184 (M^+, 5.6), 169 (M^+-CH_3, 1.9), 140 (M^+-CH_3CHO, 11.6), 125 (8.2), 115 ($CH_3(C_5H_7O)=OH^+$, 92.4), 114 (43.2), 113 (8.6), 112 ($CH_3(C_5H_7O)=CH_2$, 100), 97 (68.4), 84 (15.7), 83 (23.8), 73 (24.8), 71 (18.5), 70 (15.8), 69 (54.9), 58 (18.2), 55 (52.1), 43 (69.4), 42 (35.9), 41 (56.1).

(*E,Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane. GC-MS (EI) *m/z* (%) 184 (M^+, 8.1), 115 ($CH_3(C_5H_7O)=OH^+$, 100), 114 (37), 112 ($CH_3(C_5H_7O)=CH_2$, 39.5), 97 (73.1), 83 (11.8), 73 (27.5), 71 (12.4), 69 (59.9), 55 (41.8), 43 (38.6), 42 (24.6), 41 (38.4).

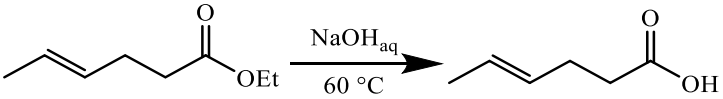
Synthesis of 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]undecane (**5**).

(*E*)-Ethyl hex-4-enoate.



Following the method of Tay et al.,[2] ortho-ester Johnson-Claisen rearrangement was conducted to give (*E*)-ethyl hex-4-enoate. In brief, 3-buten-2-ol (6 g, 84.3 mmol), triethyl orthoacetate (20.3 g, 125.4 mmol) and acetic acid (0.1 g, 2.5 mmol) were heated at 140 °C for 4 hours. The consumption of starting material at this time was determined by GC-MS. The reaction mixture was then cooled to room temperature and ethanol (20 mL) and water (20 ml) was added. The aqueous layer was extracted with diethyl ether (3 × 10 mL). The combined organic layers were stirred with hydrochloric acid (1 M aq, 20 mL) at room temperature for 30 minutes, then washed with saturated aqueous brine (20 mL) and dried over magnesium sulfate. Concentration by rotary evaporation yielded (*E*)-ethyl hex-4-enoate as a clear yellow oil (10.3 g, 86%). GC-MS (EI) *m/z* (%) 142 (6.6), 97 (26.3), 96 (14.9), 88 (22.3), 71 (58.1), 70 (15.6), 69 (83.0), 68 (100), 67 (29.8), 60 (32.6), 55 (74.6), 43 (19.3), 42 (17.3), 41 (82.55). Experimental spectra were consistent with literature data.[3]

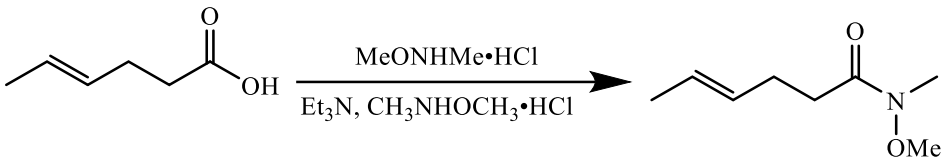
(*E*)-Hex-4-enoic acid.



Minor modification was made to the method of Tay et al.[2] to form (*E*)-hex-4-enoic acid. A solution of sodium hydroxide in 1:1 water:methanol (3.6 M, 100 mL) was added to a solution of (*E*)-ethyl hex-4-enoate (10.31 g, 72.5 mmol) in tetrahydrofuran (50 mL). The reaction mixture was stirred at 60 °C. The consumption of starting material at this time was determined by GC-MS. The reaction mixture was then cooled to room temperature and diethyl ether (40 mL) and sodium hydroxide (1 M aq, 40 mL) was added. The aqueous layer was extracted with diethyl ether (3 × 40 mL) and the combined organic layers were washed with sodium hydroxide (1 M aq, 3 × 20 mL). The combined aqueous washes were acidified to pH 1 using hydrochloric acid (1 M aq) and extracted with diethyl ether (3 × 40 mL). The combined organic layers were washed with saturated aqueous brine, dried over magnesium sulfate and the solvent removed by rotary evaporation to yield (*E*)-hex-4-enoic

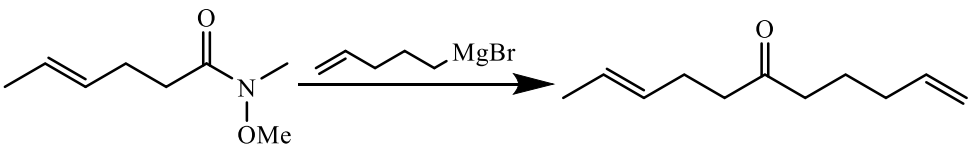
acid as a clear oil (7.5 g, 91%), which was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.50 – 5.42 (2 H, m, $CH_3C\mathbf{H}C\mathbf{H}$ CH_2), 2.44 – 2.40 (2 H, m, $C\mathbf{H}_2$), 2.35 – 2.29 (2 H, m, CH_2), 1.67 – 1.65 (3 H, m, CH_3). Experimental spectra were consistent with literature data.[4]

(*E*)-*N*-Methoxy-*N*-methylhex-4-enamide.



To a solution of (*E*)-hex-4-enoic acid (4 g, 35 mmol) in dichloromethane (100 mL) at 0 °C was added distilled trimethylamine (10 g, 100 mmol), followed by the addition of *N*,*O*-dimethylhydroxylamine hydrochloride (3.4 g, 35 mmol) in one portion. After stirring for 10 minutes, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (6.7 g, 35 mmol) was added in two equal portion over 5 minutes and the heterogeneous mixture was warmed to room temperature. After stirring for 12 hours, water (100 mL) was added and the aqueous layer was extracted with dichloromethane (3 × 30 mL). The combined organic layers were washed consecutively with hydrochloric acid (1 M aq, 200 mL), saturated aqueous sodium bicarbonate (100 mL) and saturated aqueous brine (50 mL), and dried over magnesium sulfate. After solvent removal by rotary evaporation, the product was purified by Kügelrohr distillation (bp 95-105 °C; 15 mm Hg) to yield (*E*)-*N*-methoxy-*N*-methylhex-4-enamide as a clear oil (2.45 g, 45%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.50 – 5.47 (2 H, m, $CH_3C\mathbf{H}C\mathbf{H}$ CH_2), 3.69 (3 H, s, $OC\mathbf{H}_3$), 3.19 (3 H, s, $NC\mathbf{H}_3$), 2.51 – 2.47 (2 H, m, $C\mathbf{H}_2$), 2.34 – 2.29 (2 H, m, $C\mathbf{H}_2$), 1.65 (3 H, d, J = 6.6, $C\mathbf{H}_3$). Experimental spectra were consistent with literature data.[4]

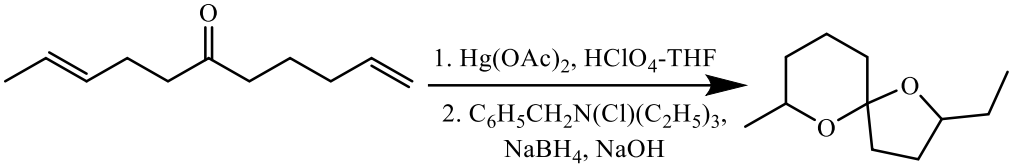
(*E*)-Undeca-1,9-dien-6-one.



To a pre-cooled (–10 °C) Grignard reagent prepared from 5-bromo-1-pentene (0.6 g, 4 mmol), magnesium (1.0 g, 4.4 mmol) and a single crystal of iodine in diethyl ether (4 mL), was added (*E*)-undeca-1,9-dien-6-one (0.6 g, 4 mmol) in diethyl ether (8 mL) over 10

minutes. The suspension was then slowly warmed to room temperature and stirred for 24 hours. Diethyl ether (20 mL) and saturated aqueous ammonium chloride (20 mL) was added to the suspension and the aqueous layer was extracted with diethyl ether (2 × 10 mL). The combined organic layers were washed with saturated aqueous brine (10 mL) and dried over magnesium sulfate. Solvent removal by rotary evaporation yielded a crude yellow oil, which was purified by flash column chromatography (0:100–10:90 ethyl acetate:*n*-hexanes) to yield (*E*)-undeca-1,9-dien-6-one as a clear oil (0.2 g, 30%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.74 – 5.64 (1 H, m, $CH_2C\mathbf{H}CH_2$), 5.40 – 5.29 (2 H, m, C**H**C**H**), 4.96 – 4.88 (2 H, m, CHC$\mathbf{H}_2$), 2.39 – 2.31 (4 H, m, C$\mathbf{H}_2$COC$\mathbf{H}_2$), 2.20 – 2.15 (2 H, m, CHC$\mathbf{H}_2CH_2$), 2.00 – 1.95 (2 H, m, CHC$\mathbf{H}_2CH_2$), 1.60 (5 H, m, $CH_2C\mathbf{H}_2CH_2$ and C$\mathbf{H}_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 210.5 (C=O), 138.0 (CH), 129.6 (CH), 125.8 (CH), 115.1 (CH_2), 42.6 (CH_2), 42.0 (CH_2), 33.1 (CH_2), 26.8 (CH_2), 22.7 (CH_2), 17.8 (CH_3). This compound is known, but spectral data are not available in the literature.

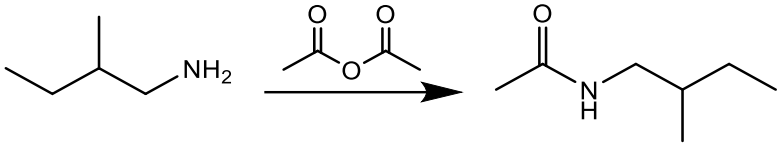
2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane.



Using a similar reaction and work-up conditions to 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, oxymercuration-reduction was performed to convert (*E*)-undeca-1,9-dien-6-one (0.1 g, 6 mmol) to 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane. Under this condition, a mixture of *E*,*E* and *E*,*Z* isomers of 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane was formed together with *E*,*E* and *E*,*Z* isomers of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (clear oil, 0.4 g, 36%).

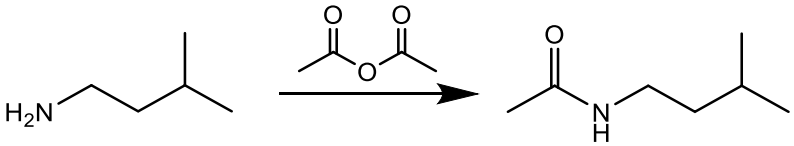
(*E*,*E*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**5**). GC-MS (EI) *m/z* (%) 184 (M^+, 0.9), 169 (M−CH_3, 0.5), 155 (M−C_2H_5, 7.6), 140 (M−C_2H_4O, 2.47), 126 (2.3), 115 (M–$C_5H_9^-$, 41.13), 114 (11.9), 113 (4.13), 112 (M–C_4H_8O, 30.7), 97 (51.82), 95 (10.2), 85 (51.7), 83 (23.4), 73 (22.9), 71 (14.5), 70 (14.7), 69 (64.3), 67 (9.7), 55 (100), 58 (16,2), 43 (89.2), 42 (44.6), 41 (79.3). Experimental spectra were consistent with literature data.[5]

Synthesis of *N*-(2-methylbutyl)acetamide (**6**).



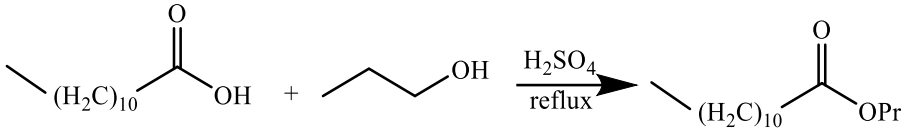
The synthesis was conducted using the method of Naik et al.[6] To a mixture of 2-methylbutylamine (4.4 g, 50 mmol) in water (50 mL) was added acetic anhydride (7.7 g, 75 mmol). The clear reaction mixture was stirred at room temperature for 0.5 hour and the completion of the reaction at this time was determined by GC-MS. The clear reaction mixture was extracted with ethyl acetate (3 × 50 mL). The combined organic layer was washed with 5% aqueous sodium bicarbonate solution (150 mL), dried over sodium sulfate and concentrated under reduced pressure to give the crude product, which was purified by vacuum distillation (150 – 160 °C, 20 mm Hg) to afford *N*-(2-methylbutyl)acetamide (**5**) as a clear liquid (5.6 g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.83 (6 H, m, $CHCH_2C\mathbf{H}_3$ and $CH_2C\mathbf{H}_3$), 1.07 (1 H, apparent sep, *J* = 6.6, C**H**CH_3), 1.29 – 1.53 (2 H, m, C**H**$_2CH_3$), 1.93 (3 H, s, C**H**$_3CO$), 2.94 – 3.13 (2 H, m, NC**H**$_2$), 6.33 (1 H, bs, N**H**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 11.2, 17.1, 23.1, 27.0, 34.8, 45.4, 170.6. GC-MS (EI) *m/z* (%) 129 (M^+, 8), 100 ($M^+-CH_2CH_3$, 38), 72 ($M^+-CHCH(CH_3)CH_2CH_3$, 100). This compound is known, but spectral data are not available in the literature.

Synthesis of *N*-(3-methylbutyl)acetamide (**7**).



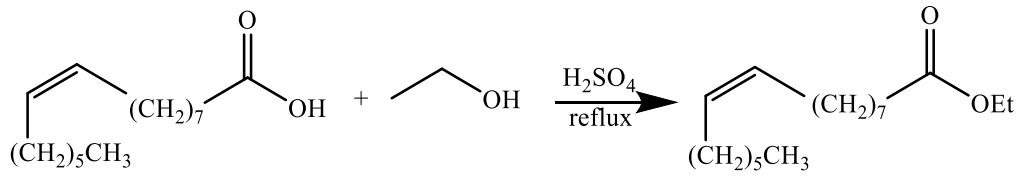
Using a similar reaction, work-up and purification conditions to *N*-(2-methylbutyl)acetamide (**5**) (above), 3-methylbutylamine (5.0 g, 57 mmol) in water (50 mL) was acetylated with acetic anhydride (8.7 g, 86 mmol) to produce *N*-(3-methylbutyl)acetamide (**6**) as a clear liquid (5.4 g, 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85 (6 H, d, *J* = 6.6, $CH(C\mathbf{H}_3)_2$), 1.33 (2 H, m, C**H**$_2CH_3$), 1.56 (1 H, non, *J* = 6.7, C**H**), 1.92 (3 H, s, C**H**$_3CO$), 3.18 (2 H, m, NC**H**$_2$), 6.21 (1 H, bs, N**H**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 22.4, 23.1, 25.8, 38.0, 38.3, 170.4. GC-MS (EI) *m/z* (%) 129 (M^+, 5), 114 (M^+-CH_3, 12), 73 ($M^+-CH_2CH_2CH(CH_3)_2$, 100). MS data was in agreement with the literature.[7] NMR data are not available in the literature.

Synthesis of propyl laurate (**17**).



A mixture of lauric acid (1.0 g, 5 mmol), 1-propanol (10 mL) and concentrated sulfuric acid (2 drops) was heated to reflux for 1.5 hours. After cooling, diethyl ether (10 mL) and 5% *w/v* aqueous sodium bicarbonate (10 mL) were added to the reaction mixture. The organic layer was separated and washed with 5% *w/v* aqueous sodium bicarbonate (3 × 10 mL) and dried over sodium sulfate. The solvent was removed under reduced pressure, yielding the crude product, which was purified by distillation to give ethyl palmitoleate as a colourless liquid (0.44 g, 38%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.87 (3 H, t, *J* = 7.0 Hz, $CH_2C\mathbf{H}_3$), 0.93 (3 H, t, *J* = 7.4 Hz, $OCH_2CH_2C\mathbf{H}_3$), 1.25-1.29 (16 H, m, $C\mathbf{H}_2$), 1.57-1.66 (4 H, m, $C\mathbf{H}_2CH_2COOPr$, $CH_3C\mathbf{H}_2CH_2OCO$), 2.29 (2 H, t, *J* = 7.5 Hz, $C\mathbf{H}_2COOPr$), 4.02 (2 H, t, *J* = 6.7 Hz, $C\mathbf{H}_2OCO$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 10.5 (CH_3), 14.2 (CH_3), 22.1 (CH_2), 22.8 (CH_2), 25.1 (CH_2), 29.3 (CH_2), 29.40 (CH_2), 29.47 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 32.0 (CH_2), 34.5 (CH_2), 65.9 (OCH_2), 174.1 (C=O). GC-MS (EI) *m/z* (%) 242 (M^+, 4.3), 213 (1.4), 201 ($M^+-CH_2CH_2CH_3$, 27.5), 183 ($M^+-OCH_2CH_2CH_3$, 25.7), 171 (6.6), 157 (6.8), 143 (3.3), 129 (8.7), 115 (21.8), 102 (McLafferty rearrangement product, 32.5), 97 (7.7), 85 (12.2), 73 (39.3), 61 (100), 57 (30.9), 43 (80.2). Experimental spectra were consistent with literature data.[8]

Synthesis of ethyl palmitoleate (**23**).



Using similar conditions to above, palmitoleic acid (0.50 g, 1.9 mmol), was esterified with ethanol (10 mL) in the presence of concentrated sulfuric acid (2 drops), quenched with sodium bicarbonate and diethyl ether and purified by distillation to afford ethyl palmitoleate as a colourless oil (0.11 g, 19%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.32 – 5.35 (2 H, m, C**H**=C**H**), 4.11 (2 H, q, *J* = 7.2 Hz, $OC\mathbf{H}_2CH_3$), 2.28 (2 H, t, *J* = 7.5 Hz, $C\mathbf{H}_2COOEt$), 1.98 – 2.01 (4 H, m, $C\mathbf{H}_2CH{=}CHC\mathbf{H}_2$), 1.59 – 1.63 (2 H, m, $C\mathbf{H}_2CH_2COOEt$), 1.23 – 1.30 (19 H, m, $C\mathbf{H}_2$), 0.88 (3 H, t, *J* = 6.9 Hz, $CH_2C\mathbf{H}_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.0 (C=O), 130.1 (CH), 129.9 (CH), 60.2 (OCH_2), 34.5

(CH_2), 31.9 (CH_2), 29.87 (CH_2), 29.82 (CH_2), 29.3 (CH_2), 29.26 (CH_2), 29.23 (CH_2), 29.1 (CH_2), 27.36 (CH_2), 27.30 (CH_2), 25.1 (CH_2), 22.8 (CH_2), 14.4 (CH_3), 14.2 (CH_3). GC-MS (EI) *m/z* (%) 282 (M^+, 3.8), 236 ($M^+-OCH_2CH_3$, 14.3), 218 (1.4), 207 (1.4), 194 ($M^+-CH_2COOCH_2CH_3$, 15.0), 179 (1.65), 165 (2.8), 152 ($M^+-(CH_2)_4COOCH_2CH_3$, 14.9), 138 (6.9), 123 (11.9), 101 (31.8), 88 (McLafferty rearrangement product, 50.6), 83 (44.9), 69 (64.1), 55 (100), 41 (81.8). Spectral data were not available in the literature.

Y-Tube apparatus

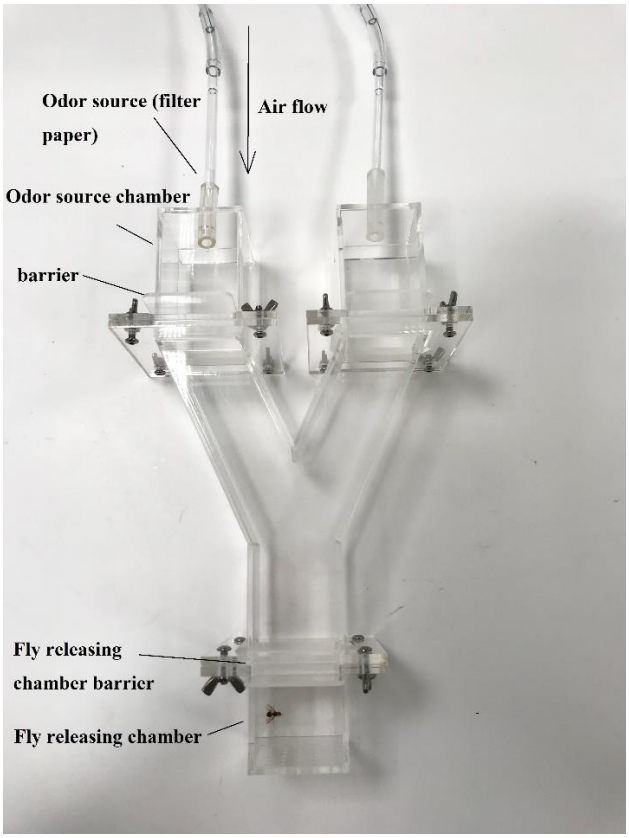


Figure S1. Y-Tube apparatus used in this study

References

1. Kitching, W. *et al.* Chemistry of fruit flies. Composition of the rectal gland secretion of (male) *Dacus cucumis* (cucumber fly) and *Dacus halfordiae*. Characterization of (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. *J. Org. Chem.* **54**, 3893–3902 (1989).

2. Tay, G. C., Sizemore, N. & Rychnovsky, S. D. Stereoselection in intramolecular Diels-Alder reactions of 2-cyano-1-azadienes: indolizidine and quinolizidine synthesis. *Org. Lett.* **18**, 3050–3053 (2016).
3. Adachi, Y., Kamei, N., Yokoshima, S. & Fukuyama, T. Total synthesis of (−)-histrionicotoxin. *Org. Lett.* **13**, 4446–4449 (2011).
4. Harris, J. R., Waetzig, S. R. & Woerpel, K. A. Palladium(II)-catalyzed cyclization of unsaturated hydroperoxides for the synthesis of 1,2-dioxanes. *Org. Lett.* **11**, 3290–3293 (2009).
5. Schwartz, B. D. *et al.* Spiroacetal biosynthesis in insects from Diptera to Hymenoptera: The giant ichneumon wasp *Megarhyssa nortoni nortoni* Cresson. *J. Am. Chem. Soc.* **130**, 14853–14860 (2008).
6. Naik, S., Bhattacharjya, G., Talukdar, B. & Patel, B. K. Chemoselective acylation of amines in aqueous media. *European J. Org. Chem.* **2004**, 1254–1260 (2004).
7. Wee, S. L. & Tan, K. H. Female sexual response to male rectal volatile constituents in the fruit fly, *Bactrocera carambolae* (Diptera: Tephritidae). *Appl. Entomol. Zool.* **40**, 365–372 (2005).
8. Santos, M. *et al.* Poly(3-hydroxyoctanoate) depolymerase from *Pseudomonas fluorescens* GK13: Catalysis of ester-forming reactions in non-aqueous media. *J. Mol. Catal. B Enzym.* **77**, 81–86 (2012).

Appendix IV:

Supplementary material for

Behavioural and electrophysiological responses to rectal gland secretions and headspace volatiles emitted by *Bactrocera kraussi* (Hardy) (Tephritidae)

Saeedeh Noushini[1,3*], Soo Jean Park[2,3], Jeanneth Perez[2,3], Danielle Holgate[1], Vivian Mendez Alvarez[2,3], Ian Jamie[1,3], Joanne Jamie[1*], Phillip Taylor[2,3]

[1] *Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia.*

[2] *Applied BioSciences, Macquarie University, Sydney, NSW 2109, Australia.*

[3] *Australian Research Council Industrial Transformation Training Centre for Fruit Fly Biosecurity Innovation, Macquarie University, Sydney, NSW 2109, Australia.*

Corresponding author:

Saeedeh Noushini, Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia

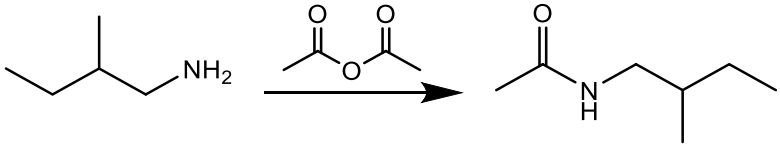
E-mail:

ORCID: 0000-0001-5558-1656

Synthesis of compounds

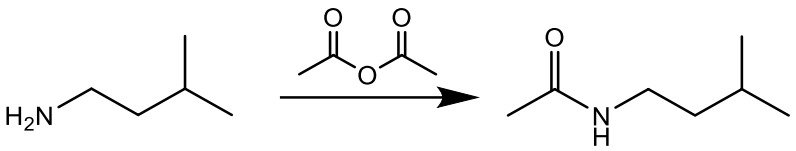
All reagents were purchased from Sigma-Aldrich and used without further purification. All solvents were anhydrous or analytical grade (Sigma-Aldrich) and used without further purification. The reaction progress was monitored by GC-MS (using the procedure given in the main text). Solvents were removed under reduced pressure using a Büchi Rotavapor R-200 and Büchi B-490 heating bath set to 40 °C. Mixtures were further dried under high vacuum using an Alcatel Pascal 2005SD vacuum pump. NMR spectra were recorded on a Bruker AVANCE-400 instrument (^{1}H NMR: 400 MHz, ^{13}C NMR: 101 MHz) or a Bruker AVANCE-600 instrument equipped with a cryoprobe (^{1}H NMR: 600 MHz, ^{13}C NMR: 150 MHz) using $CDCl_3$ and C_6D_6. The ^{1}H NMR chemical shifts were referenced to the residual protonated solvent peaks at δH 7.26 for chloroform-d and 7.15 for benzene-d_6. ^{13}C NMR chemical shifts were referenced to the central solvent peaks of bulk solvent at δC 77.16 for chloroform-d and 127.68 for benzene-d_6. *J* values are given in Hz.

Synthesis of *N*-(2-methylbutyl)acetamide (**5**).



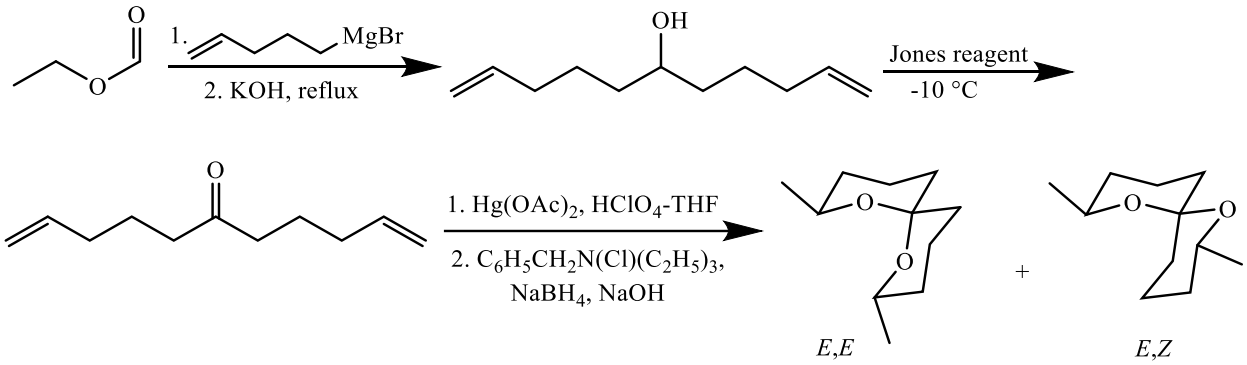
The synthesis was conducted using the method of Naik et al.[1] To a mixture of 2-methylbutylamine (4.4 g, 50 mmol) in water (50 mL) was added acetic anhydride (7.7 g, 75 mmol). The clear reaction mixture was stirred at room temperature for 0.5 hour and the completion of the reaction at this time was determined by GC-MS. The clear reaction mixture was extracted with ethyl acetate (3 × 50 mL). The combined organic layer was washed with 5% aqueous sodium bicarbonate solution (150 mL), dried over sodium sulfate and concentrated under reduced pressure to give the crude product, which was purified by vacuum distillation (150 – 160 °C, 20 mm Hg) to afford *N*-(2-methylbutyl)acetamide (**5**) as a clear liquid (5.6 g, 86%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.83 (6 H, m, $CHCH_2C\mathbf{H}_3$ and $CH_2C\mathbf{H}_3$), 1.07 (1 H, apparent sep, *J* = 6.6, C**H**CH_3), 1.29 – 1.53 (2 H, m, C**H**$_2CH_3$), 1.93 (3 H, s, C**H**$_3CO$), 2.94 – 3.13 (2 H, m, NC**H**$_2$), 6.33 (1 H, bs, N**H**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 11.2, 17.1, 23.1, 27.0, 34.8, 45.4, 170.6. GC-MS (EI) *m/z* (%) 129 (M^+, 8), 100 ($M^+-CH_2CH_3$, 38), 72 ($M^+-CHCH(CH_3)CH_2CH_3$, 100). This compound is known, but spectral data are not available in the literature.

Synthesis of *N*-(3-methylbutyl)acetamide (**6**).



Using a similar reaction, work-up and purification conditions to *N*-(2-methylbutyl)acetamide (**5**) (above), 3-methylbutylamine (5.0 g, 57 mmol) in water (50 mL) was acetylated with acetic anhydride (8.7 g, 86 mmol) to produce *N*-(3-methylbutyl)acetamide (**6**) as a clear liquid (5.4 g, 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85 (6 H, d, *J* = 6.6, CH(C**H**$_3$)$_2$), 1.33 (2 H, m, C**H**$_2$CH$_3$), 1.56 (1 H, non, *J* = 6.7, C**H**), 1.92 (3 H, s, C**H**$_3$CO), 3.18 (2 H, m, NC**H**$_2$), 6.21 (1 H, bs, N**H**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 22.4, 23.1, 25.8, 38.0, 38.3, 170.4. GC-MS (EI) *m/z* (%) 129 (M^+, 5), 114 (M^+-CH_3, 12), 73 ($M^+-CH_2CH_2CH(CH_3)_2$, 100). MS data was in agreement with the literature.[2] NMR data are not available in the literature.

Synthesis of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**2**).



1,10-Undecadien-6-ol. Following the method of Kitching et al.,[3] Grignard reaction followed by hydrolysis was conducted to give 1,10-undecadien-6-ol. In brief, a flame-dried argon-flushed two-necked round bottom flask was charged with magnesium (1.8 g, 74 mmol), a single crystal of iodine, and a magnetic stirrer bar, and fitted with a condenser. Dry diethyl ether (60 mL) was added and the suspension was brought to reflux. 5-Bromopent-1-ene (10 g, 67 mmol) in diethyl ether (30 mL) was added dropwise and then the colourless suspension was stirred at reflux for 4 hours. The colourless suspension was cooled to 0 °C and ethyl formate (2.6 g, 34 mmol) was added. The suspension was warmed to room temperature, stirred for 1 hour, then quenched with saturated ammonium chloride and extracted with diethyl ether (3 × 15 mL). The combined organic layers were washed with saturated aqueous brine and dried over magnesium sulfate. After solvent removal by rotary evaporation, the yellow oil was refluxed in 15% aqueous potassium hydroxide solution for 3 hours. The solution was cooled to room temperature and extracted with

diethyl ether (3 × 20 mL). Solvent was removed under reduced pressure to give the crude product as a yellow oil, which was purified by distillation (110 – 115 °C, 10 mm Hg) to afford 1,10-undecadien-6-ol as a colourless oil (3.7 g, 60% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.81 (2 H, ddt, *J* = 17, 10.3, 6.7 Hz, C**H**=), 5.01 (2 H, dq, *J* = 17.1, 1.7 Hz, C**H**$_2$=), 4.91 – 5.01 (2 H, m, C**H**$_2$=), 3.61 (1 H, bs, C**H**OH), 2.00 – 2.13 (4 H, m, C**H**$_2$CH=CH$_2$), 1.26 – 1.61 (9 H, m, including OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.7 (CH=), 114.6 (CH$_2$=), 71.7 (CHOH), 36.9 (CH$_2$), 33.7 (CH$_2$), 24.9 (CH$_2$). GC-MS (EI) *m/z* (%) 84 (12.3), 81 (100), 80 (10), 79 (19.5), 69 (9.2), 68 (9.3), 67 (20.6), 58 (9.5), 57 (30.2), 55 (72.3), 54 (27.7), 53 (9.4), 43 (32.1), 42 (9), 41 (43.8). Spectral data were consistent with the literature.[3]

Undeca-1,10-dien-6-one. To a solution of 1,10-undecadien-6-ol (3.99 g, 23.7 mmol) in acetone (10 mL) at -10 °C, freshly prepared Jones reagent (2.7 g of chromium trioxide in 4 mL of sulfuric acid and 12 mL of distilled water) was added dropwise and the reaction monitored by GC-MS. After completion of the reaction (2 hours), the green suspension was filtered through a pad of Celite. The filtrate was washed with saturated aqueous sodium bicarbonate (17 mL), extracted with diethyl ether (4 × 50 mL) and washed with water (50 mL) and saturated aqueous brine (50 mL), then dried over magnesium sulfate. Concentration by rotary evaporation yielded undeca-1,10-dien-6-one as a colourless oil (2.9 g, 75% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.80 (2 H, ddt, *J* = 17.2, 10.3, 6.7 Hz, C**H**=), 4.87 – 4.97 (4 H, m, C**H**$_2$=), 2.33 (4 H, t, *J* = 7.5 Hz, C**H**$_2$CO), 1.98 (4 H, m, C**H**$_2$CH=CH$_2$), 1.60 (4 H, quin, *J* = 7.3 Hz, CH$_2$C**H**$_2$CH$_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 210.9 (CO), 138.0 (CH=), 115.2 (CH$_2$=), 41.9 (CH$_2$), 33.1 (CH$_2$), 22.8 (CH$_2$). GC-MS (EI) *m/z* (%) 112 (14.6), 97 (30), 84 (27.8), 83 (10.6), 70 (14.1), 69 (59.5), 58 (48.7), 55 (49.6), 43 (24.5), 41 (100). Spectral data were consistent with the literature.[3]

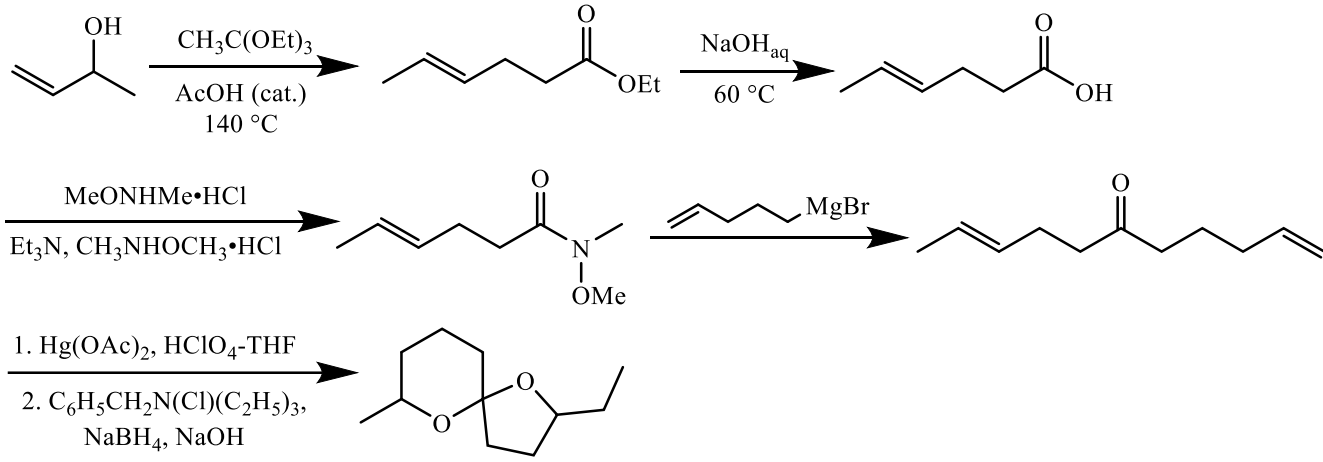
2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane. $Hg(OAc)_2$ (1.9 g, 6.1 mmol) was added to a stirred solution of undeca-1,10-dien-6-one (0.5 g, 3 mmol) in 1% aqueous perchloric acid: tetrahydrofuran (15 mL:15 mL) and the solution was stirred for 15 hours. Benzyltriethylammonium chloride (2.4 g, 10.5 mmol) in 10% aqueous sodium hydroxide (15 mL) and dichloromethane (5 mL) was added followed by sodium borohydride (0.09 g, 2.3 mmol) in 10% aqueous sodium hydroxide (5 mL). The grey suspension was stirred and monitored by GC-MS. After completion of the reaction (20 minutes), the grey suspension was filtered through a pad of Celite, which was then washed with 30 mL of diethyl ether. The aqueous phase was then extracted with diethyl ether (3 × 30 mL) and the combined

organic layer (from Celite wash and extraction) were washed with saturated aqueous brine (50 mL) and dried over magnesium sulfate. After solvent removal by rotary evaporation the product was purified by Kugelrohr distillation (bp 110 °C; 30 mm Hg). According to the literature [3] under this condition a mixture of *E,E* diastereomer with some *E,Z* and no *Z,Z* isomer is obtained. These configurational isomers produced different MS fragmentation patterns that were matched with those in the literature.[3]

(*E,E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**2**). ^{13}C NMR (101 MHz, C_6D_6) δ 95.75 (CO_2), 64.8 (CO), 35.33 (CH_2), 32.90 (CH_2), 21.92 (CH_3), 19.03 (CH_2). GC-MS (EI) *m/z* (%) 184 (M^+, 5.6), 169 (M^+-CH_3, 1.9), 140 (M^+-CH_3CHO, 11.6), 125 (8.2), 115 ($CH_3(C_5H_7O)=OH^+$, 92.4), 114 (43.2), 113 (8.6), 112 ($CH_3(C_5H_7O)=CH_2$, 100), 97 (68.4), 84 (15.7), 83 (23.8), 73 (24.8), 71 (18.5), 70 (15.8), 69 (54.9), 58 (18.2), 55 (52.1), 43 (69.4), 42 (35.9), 41 (56.1).

(*E,Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane. GC-MS (EI) *m/z* (%) 184 (M^+, 8.1), 115 ($CH_3(C_5H_7O)=OH^+$, 100), 114 (37), 112 ($CH_3(C_5H_7O)=CH_2$, 39.5), 97 (73.1), 83 (11.8), 73 (27.5), 71 (12.4), 69 (59.9), 55 (41.8), 43 (38.6), 42 (24.6), 41 (38.4).

Synthesis of 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**3**).



*(*E*)-Ethyl hex-4-enoate*. Following the method of Tay et al.,[4] ortho-ester Johnson-Claisen rearrangement was conducted to give (*E*)-ethyl hex-4-enoate. In brief, 3-Buten-2-ol (6 g, 84.3 mmol), triethylorthoacetate (20.3 g, 125.4 mmol) and acetic acid (0.1 g, 2.5 mmol) was heated at 140 °C for 4 hours. The consumption of starting material at this time was determined by GC-MS. The reaction mixture was then cooled to room temperature and ethanol (20 ml) and water (20 ml) was added. The aqueous layer was extracted with diethyl ether (3 × 10 mL). The combined organic layers were stirred with hydrochroric acid solution (1 M aq, 20 ml) at room temperature for 30 minutes, then washed with brine (20

ml) and dried over magnesium sulfate. Concentration by rotary evaporation yielded (*E*)-ethyl hex-4-enoate as a clear yellow oil (10.3 g, 86%). GC-MS (EI) *m/z* (%) 142 (6.6), 97 (26.3), 96 (14.9), 88 (22.3), 71 (58.1), 70 (15.6), 69 (83.0), 68 (100), 67 (29.8), 60 (32.6), 55 (74.6), 43 (19.3), 42 (17.3), 41 (82.55). Experimental spectra were consistent with literature data.[5]

*(*E*)-Hex-4-enoic acid.* Minor modification was made to the method of Tay et al.[4] to form (*E*)-hex-4-enoic acid. A solution of sodium hydroxide in 1:1 water:methanol (3.6 M, 100ml) was added to a solution of (*E*)-ethyl hex-4-enoate(10.31 g, 72.5 mmol) in tetrahydrofuran (50 ml). The reaction mixture was stirred at 60 °C. The consumption of starting material at this time was determined by GC-MS. The reaction mixture was then cooled to room temperature and diethyl ether (40ml) and sodium hydroxide (1M aq, 40 ml) was added. The aqueous layer was extracted with diethyl ether (3 × 40 mL) and the combined organic layers were washed with sodium hydroxide (1M aq, 3 × 20 ml). The combined aqueous washes were acidified to pH = 1 using hyrdochoric acid (1M aq), extracted with diethyl ether (3 × 40 mL), then combined organic layers were washed with brine, dried over magnesium sulfate and the solvent removed by rotary evaporation yielded (*E*)-hex-4-enoic acid as a clear oil which was used in the next step without further purification (7.5 g, 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.50 – 5.42 (2 H, m, $CH_3C\mathbf{H}C\mathbf{H}$ CH_2), 2.44 – 2.40 (2 H, m, $C\mathbf{H}_2$), 2.35 – 2.29 (2 H, m, CH_2), 1.67 – 1.65 (3 H, m, CH_3). Experimental spectra were consistent with literature data.[6]

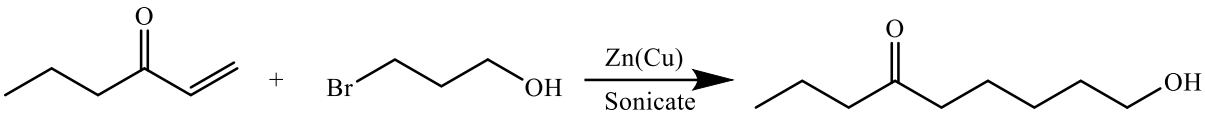
*(*E*)-*N*-*Methoxy*-N-*methylhex-4-enamide.* To a solution of (*E*)-hex-4-enoic acid (4 g, 35 mmol) in dichloromethane (100 ml) at 0 °C was added distilled trimethylamine (10 g, 100mmol) followed by the addition of *N*,*O*-dimethylhydroxylamine hydrochloride (3.4 g, 35 mmol) in one portion. After stirring for 10 minutes, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (6.7 g, 35 mmol) was added in two equal portion over 5 minutes and the heterogeneous mixture was warmed to room temperature. After stirring for 12 hours, water (100ml) was added and the aqueous layer was extracted with dichloromethane (3 × 30 ml). the combined organic layers were washed with hydrochloric acid (1M aq, 200 ml), sodium bicarbonate (100 ml), brine (50 ml) and dried over magnesium sulfate. After solvent removal by rotary evaporation the product was purified by Kugelrohr distillation (bp 95-105 °C; 15 mm Hg) to yield (*E*)-*N*-Methoxy-*N*-methylhex-4-enamide as a clear oil (2.45 g, 45%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.50 – 5.47 (2 H, m, $CH_3C\mathbf{H}C\mathbf{H}$ CH_2), 3.69 (3 H, s, $OC\mathbf{H}_3$), 3.19 (3 H, s, $NC\mathbf{H}_3$), 2.51 – 2.47 (2

H, m, C**H**$_2$), 2.34 – 2.29 (2 H, m, C**H**$_2$), 1.65 (3 H, d, *J* = 6.6, C**H**$_3$). Experimental spectra were consistent with literature data.[6]

(*E*)-Undeca-1,9-dien-6-one. To a pre-cooled (–10 °C) Grignard regent prepared from 5-bromo-1-pentene(0.6 g, 4 mmol), magnesium (1.0 g, 4.4 mmol) and a single crystal of iodine in diethyl ether (4 ml) was added (*E*)-undeca-1,9-dien-6-one (0.6 g, 4 mmol) in diethyl ether (8ml) over 10 minutes, then slowly warmed to room temperature and stirred for 24 hours. Then diethyl ether (20 mL) and saturated ammonium chloride (20 mL) was added and the aqueous layer was extracted with diethyl ether (2 x 10 mL). The combined organic layers were washed with brine (10 mL) and dried over magnesium sulfate. Solvent removal by rotary evaporation yielded a crude yellow oil which then purified by flash column chromatography (0:100–10:90 ethyl acetate:hexanes) to yield (*E*)-undeca-1,9-dien-6-one as a clear oil (0.2 g, 30%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.74 – 5.64 (1 H, m, $CH_2C\mathbf{H}CH_2$), 5.40 – 5.29 (2 H, m, C**H**C**H**), 4.96 – 4.88 (2 H, m, CHC**H**$_2$), 2.39 – 2.31 (4 H, m, C**H**$_2$COC**H**$_2$), 2.20 – 2.15 (2 H, m, CHC**H**$_2$CH$_2$), 2.00 – 1.95 (2 H, m, CHC**H**$_2$CH$_2$), 1.60 (5 H, m, CH$_2$C**H**$_2$CH$_2$ and C**H**$_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 210.5 (C=O), 138.0 (CH), 129.6 (CH), 125.8 (CH), 115.1 (CH_2), 42.6 (CH_2), 42.0 (CH_2), 33.1 (CH_2), 26.8 (CH_2), 22.7 (CH_2), 17.8 (CH_3). This compound is known, but spectral data are not available in the literature.

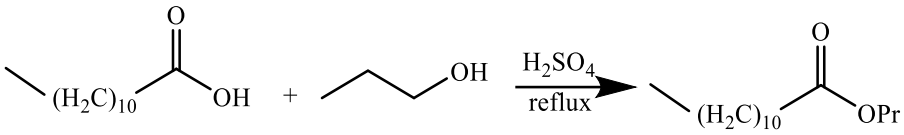
2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane. Using a similar reaction and work-up conditions to 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, oxymercuration-reduction was performed to convert (*E*)-undeca-1,9-dien-6-one (0.1 g, 6 mmol) to 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane. Under this condition, a mixture of *E,E* and *E,Z* isomers of 2-ethyl-7-methyl-1,6-dioxaspiro[4.5]decane was formed together with *E,E* and *E,Z* isomers of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (clear oil, 0.4 g, 36%).
(*E,E*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane (**5**). GC-MS (EI) *m/z* (%) 184 (M^+, 0.9), 169 ($M-CH_3$, 0.5), 155 ($M-CH_2CH_3$, 7.6), 140 ($M-CH_3CHO$, 2.47), 126 (2.3), 115 ($CH_3(C_5H_7O)=OH^+$, 41.13), 114 (11.9), 113 (4.13), 112 ($M-CH_2CHCH(OH)CH_3$, 30.7), 97 (51.82), 95 (10.2), 85 (51.7), 83 (23.4), 73 (22.9), 71 (14.5), 70 (14.7), 69 (64.3), 67 (9.7), 55 (100), 58 (16,2), 43 (89.2), 42 (44.6), 41 (79.3). Experimental spectra were consistent with literature data.[7]

Synthesis of 6-oxanon-1-ol (**11**).



The synthesis was conducted using the method of singh et al.[8] A mixture of ethanol-water (9:1, 24 ml), zinc dust (1.0 g, 16 mmol), copper(I) iodide (0.9 g, 4.8 mmol), 3-bromo-1-propanol (880 mg, 6.4 mmol) in ethanol (4 ml) and 1-hexen-3-one (620 mg, 6.4 mmol) in ethanol (4 ml) at 0 °C were sonicated for 7.5 hours, and reaction progress was monitored by GC-MS. The completion of the reaction at this time was determined by GC-MS. The reaction was then quenched with brine and filtered. Concentration by rotary evaporation yielded the crude product that was taken up in diethyl ether (50 ml), washed with water (2 × 20 ml), and brine (2 × 20 ml), and dried over Na_2SO_4. Solvent was removed under reduced pressure, yielding the crude productwhich was purified by flash column chromatography (eluted twice with 0-10% ethyl acetate in *n*-hexane) to afford 6-oxanon-1-ol as a colourless oil (112 mg, 11%). GC-MS (EI) m/z (% of base peak) 158 (M^+, 1.1), 140 (M^+-H_2O, 2.1), 115 (8.2), 112 (3.5), 99 (7.3), 97 (26.1), 86 (32.0), 79 (10.0), 73 (11.4), 71 (66.9), 69 (70.1), 58 (52.1), 55 (34.7), 43 (100), 41 (72.5). Experimental spectra were consistent with literature data.[9]

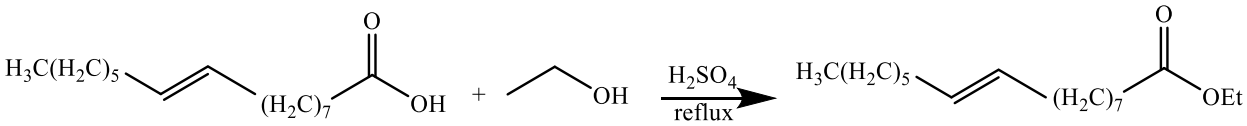
Synthesis of propyl laurate (**15**).



A mixture of lauric acid (1.0 g, 5 mmol), 1-propanol (10 mL) and concentrated sulfuric acid (2 drops) was heated to reflux for 1.5 hours. After cooling, diethyl ether (10 mL) and 5% *w/v* aqueous sodium bicarbonate (10 mL) were added to the reaction mixture. The organic layer was separated and washed with 5% *w/v* aqueous sodium bicarbonate (3 × 10 mL) and dried over sodium sulfate. The solvent was removed under reduced pressure, yielding the crude product, which was purified by distillation to give ethyl palmitoleate as a colourless liquid (0.44 g, 38% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.87 (3 H, t, *J* = 7.0 Hz, $CH_2C\mathbf{H}_3$), 0.93 (3 H, t, *J* = 7.4 Hz, $OCH_2CH_2C\mathbf{H}_3$), 1.25-1.29 (16 H, m, $C\mathbf{H}_2$), 1.57-1.66 (4 H, m, $C\mathbf{H}_2CH_2COOPr$, $CH_3C\mathbf{H}_2CH_2OCO$), 2.29 (2 H, t, *J* = 7.5 Hz, $C\mathbf{H}_2COOPr$), 4.02 (2 H, t, *J* = 6.7 Hz, $C\mathbf{H}_2OCO$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 10.5 (CH_3), 14.2 (CH_3), 22.1 (CH_2), 22.8 (CH_2), 25.1 (CH_2), 29.3 (CH_2), 29.40 (CH_2), 29.47 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 32.0 (CH_2), 34.5 (CH_2), 65.9 (OCH_2), 174.1 (C=O). GC-MS (EI) *m/z*

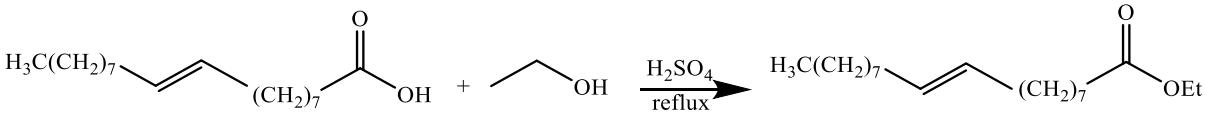
(%) 242 (M^+, 4.3), 213 (1.4), 201 ($M^+-CH_2CH_2CH_3$, 27.5), 183 ($M^+-OCH_2CH_2CH_3$, 25.7), 171 (6.6), 157 (6.8), 143 (3.3), 129 (8.7), 115 (21.8), 102 (McLafferty rearrangement product, 32.5), 97 (7.7), 85 (12.2), 73 (39.3), 61 (100), 57 (30.9), 43 (80.2). Experimental spectra were consistent with literature data.[10]

Synthesis of ethyl palmitoleate (**23**).



Using similar conditions to above, palmitoleic acid (0.50 g, 1.9 mmol), was esterified with ethanol (10 mL) in the presence of concentrated sulfuric acid (2 drops), quenched with sodium bicarbonate and diethyl ether and purified by distillation to afford ethyl palmitoleate as a colourless oil (0.11 g, 19%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.32 – 5.35 (2 H, m, C**H**=C**H**), 4.11 (2 H, q, *J* = 7.2 Hz, OC**H₂**CH_3), 2.28 (2 H, t, *J* = 7.5 Hz, C**H₂**COOEt), 1.98 – 2.01 (4 H, m, C**H₂**CH=CHC**H₂**), 1.59 – 1.63 (2 H, m, C**H₂**CH_2COOEt), 1.23 – 1.30 (19 H, m, C**H₂**), 0.88 (3 H, t, *J* = 6.9 Hz, CH_2C**H₃**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.0 (C=O), 130.1 (CH), 129.9 (CH), 60.2 (OCH_2), 34.5 (CH_2), 31.9 (CH_2), 29.87 (CH_2), 29.82 (CH_2), 29.3 (CH_2), 29.26 (CH_2), 29.23 (CH_2), 29.1 (CH_2), 27.36 (CH_2), 27.30 (CH_2), 25.1 (CH_2), 22.8 (CH_2), 14.4 (CH_3), 14.2 (CH_3). GC-MS (EI) *m/z* (%) 282 (M^+, 3.8), 236 ($M^+-OCH_2CH_3$, 14.3), 218 (1.4), 207 (1.4), 194 ($M^+-CH_2COOCH_2CH_3$, 15.0), 179 (1.65), 165 (2.8), 152 ($M^+-(CH_2)_4COOCH_2CH_3$, 14.9), 138 (6.9), 123 (11.9), 101 (31.8), 88 (50.6), 83 (44.9), 69 (64.1), 55 (100), 41 (81.8). Spectral data were not available in the literature

Synthesis of ethyl elaidate (**24**).



Using similar conditions to above, elaidic acid (0.45 g, 1.6 mmol), was esterified with ethanol (10 mL) in the presence of concentrated sulfuric acid (2 drops), quenched with sodium bicarbonate and diethyl ether and purified by distillation to afford ethyl elaidate as a colourless oil (113 mg, 23%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 – 5.28 (2 H, m, C**H**=C**H**), 4.11 (2 H, q, *J* = 7.1 Hz, OC**H₂**CH_3), 2.27 (2 H, t, *J* = 7.6 Hz, C**H₂**COOEt), 1.95 – 1.96 (4 H, m, C**H₂**CH=CHC**H₂**), 1.57 – 1.60 (2 H, m, C**H₂**CH_2COOEt), 1.23 – 1.28 (23 H, m, CH_2), 0.87 (3 H, t, *J* = 6.7 Hz, CH_2CH_2C**H₃**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.0

(C=O), 130.6 (CH), 130.4 (CH), 60.3 (OCH_2), 34.5 (CH_2), 32.78 (CH_2), 32.73 (CH_2), 32.0 (CH_2), 29.8 (CH_2), 29.7 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.36 (CH_2), 29.30 (CH_2), 29.1 (CH_2), 25.1 (CH_2), 22.8 (CH_2), 14.4 (CH_3), 14.2 (CH_3). GC-MS (EI) *m/z* (%) 310 (M^+, 3.5), 281 ($M^+-CH_2CH_3$, 0.25), 264 ($M^+-OCH_2CH_3$, 16.2), 222 (11.3), 180 (11.2), 155 (7.0), 138 (5.6), 123 (13.5), 111 (20.6), 97 (38.6), 88 (45.6), 83 (49.0), 69 (69.0), 55 (100), 41 (76.4). Spectral data were consistent with the literature.[11]

Y-Tube apparatus

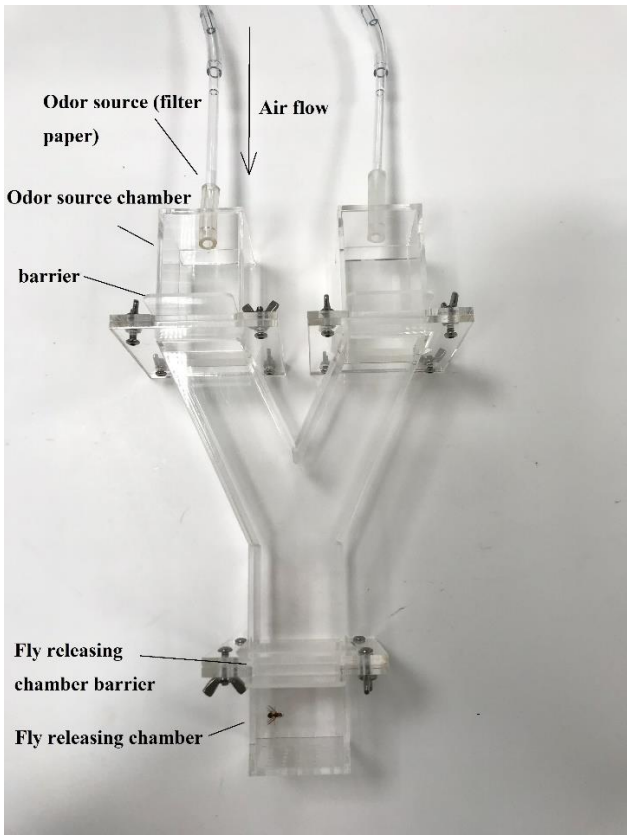


Figure S1. Y-Tube apparatus used in this study

References

1. Naik, S., Bhattacharjya, G., Talukdar, B. & Patel, B. K. Chemoselective acylation of amines in aqueous media. *European J. Org. Chem.* **2004**, 1254–1260 (2004).
2. Wee, S. L. & Tan, K. H. Female sexual response to male rectal volatile constituents in the fruit fly, *Bactrocera carambolae* (Diptera: Tephritidae). *Appl. Entomol. Zool.* **40**, 365–372 (2005).

3. Kitching, W. *et al.* Chemistry of fruit flies. Composition of the rectal gland secretion of (male) *Dacus cucumis* (cucumber fly) and *Dacus halfordiae*. Characterization of (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. *J. Org. Chem.* **54**, 3893–3902 (1989).
4. Tay, G. C., Sizemore, N. & Rychnovsky, S. D. Stereoselection in intramolecular Diels-Alder reactions of 2-cyano-1-azadienes: indolizidine and quinolizidine synthesis. *Org. Lett.* **18**, 3050–3053 (2016).
5. Adachi, Y., Kamei, N., Yokoshima, S. & Fukuyama, T. Total synthesis of (−)-histrionicotoxin. *Org. Lett.* **13**, 4446–4449 (2011).
6. Harris, J. R., Waetzig, S. R. & Woerpel, K. A. Palladium(II)-catalyzed cyclization of unsaturated hydroperoxides for the synthesis of 1,2-dioxanes. *Org. Lett.* **11**, 3290–3293 (2009).
7. Schwartz, B. D. *et al.* Spiroacetal biosynthesis in insects from Diptera to Hymenoptera: The giant ichneumon wasp *Megarhyssa nortoni nortoni* Cresson. *J. Am. Chem. Soc.* **130**, 14853–14860 (2008).
8. Singh, J., Kaur, J., Nayyar, S., Bhandari, M. & Kad, G. L. Ultrasound mediated synthesis of a few naturally occurring compounds. *Indian J. Chem. - Sect. B Org. Med. Chem.* **40**, 386–390 (2001).
9. Perkins, M. V., Fletcher, M. T., Kitching, W., Drew, R. A. I. & Moore, C. J. Chemical studies of rectal gland secretions of some species of *Bactrocera dorsalis* complex of fruit flies (diptera: Tephritidae). *J. Chem. Ecol.* **16**, 2475–2487 (1990).
10. Santos, M. *et al.* Poly(3-hydroxyoctanoate) depolymerase from *Pseudomonas fluorescens* GK13: Catalysis of ester-forming reactions in non-aqueous media. *J. Mol. Catal. B Enzym.* **77**, 81–86 (2012).
11. Denton, R. M., Tang, X. & Przeslak, A. Catalysis of phosphorus(V)-mediated transformations: dichlorination reactions of epoxides under Appel conditions. *Org. Lett.* **12**, 4678–4681 (2010).

Appendix V:

Supplementary Information for

Sampling technique biases in the analysis of fruit fly volatiles: A case study of Queensland fruit fly

Saeedeh Noushini[1,3*], Soo Jean Park[2,3], Ian Jamie[1,3], Joanne Jamie[1], Phillip Taylor[2,3]

[1] Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia.

[2] Applied BioSciences, Macquarie University, Sydney, NSW 2109, Australia.

[3] Australian Research Council Industrial Transformation Training Centre for Fruit Fly Biosecurity Innovation, Macquarie University, Sydney, NSW 2109, Australia.

Corresponding author:
Saeedeh Noushini, Department of Molecular Sciences, Macquarie University, Sydney, NSW 2109, Australia
E-mail:
ORCID: 0000-0001-5558-1656

1. Sample collection design

Table S1. Sample collection design.

Method	Rectal gland solvent extract		Headspace sampling	
	Intact gland	Crushed gland	Dynamic headspace	Static headspace
Sampling matrix	DCM, ethanol, hexane	Hexane	Tenax, Porapak	PDMS, PDMS/DVB, PA

2. Statistics

2.1. Effect of solvent on rectal gland extractions

Table S2. *P*-value obtained from ANOVA for solvent comparisons in rectal gland extractions. Amide 1: *N*-(2-methylbutyl)acetamide, Amide 2: *N*-(3-methylbutyl)acetamide, Amide 3: *N*-(2-methylbutyl)propanamide, Amide 4: *N*-(3-methylbutyl)propanamide, Amide 5: *N*-(2-methylbutyl)isobutyrate, Amide 6: *N*-(3-methylbutyl)isobutyrate, Spiroacetal 1: 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, Spiroacetal 2: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 3: (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 4: (*E*,*E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 5: (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane.

	$P_{\text{hexane-DCM}}$	$P_{\text{hexane-EtOH}}$	$P_{\text{DCM-EtOH}}$
Amide 1	0.566	0.987	0.577
Amide 2	< 0.001	0.270	< 0.001
Amide 3	0.004	0.945	0.005
Amide 4	< 0.001	0.051	< 0.001
Amide 5	0.887	0.975	0.862
Amide 6	0.008	0.988	0.008
Ethyl isobutyrate	0.465	0.538	0.178
Ethyl-2-methylbutanoate	0.975	0.947	0.921
Diethyl succinate	0.992	0.995	0.998
Methyl laurate	0.904	0.955	0.948
Ethyl laurate	0.457	< 0.001	< 0.001
Methyl myristate	0.842	0.903	0.748
Ethyl myristate	0.019	< 0.001	< 0.001
Ethyl myristoleate	0.941	0.028	0.033
Methyl palmitoleate	0.796	0.945	0.743
Ethyl palmitate/palmitoleate	0.037	< 0.001	< 0.001
Ethyl oleate/elaidate	0.016	0.150	< 0.001
Spiroacetal 1	0.749	0.801	0.946
Spiroacetal 2	< 0.001	< 0.001	0.001
Spiroacetal 3	0.2430	0.087	0.585
Spiroacetal 4	0.961	0.975	0.986
Spiroacetal 5	0.846	0.907	0.938

2.1.1. Interactions between solvents and compounds from male and female rectal gland extractions:

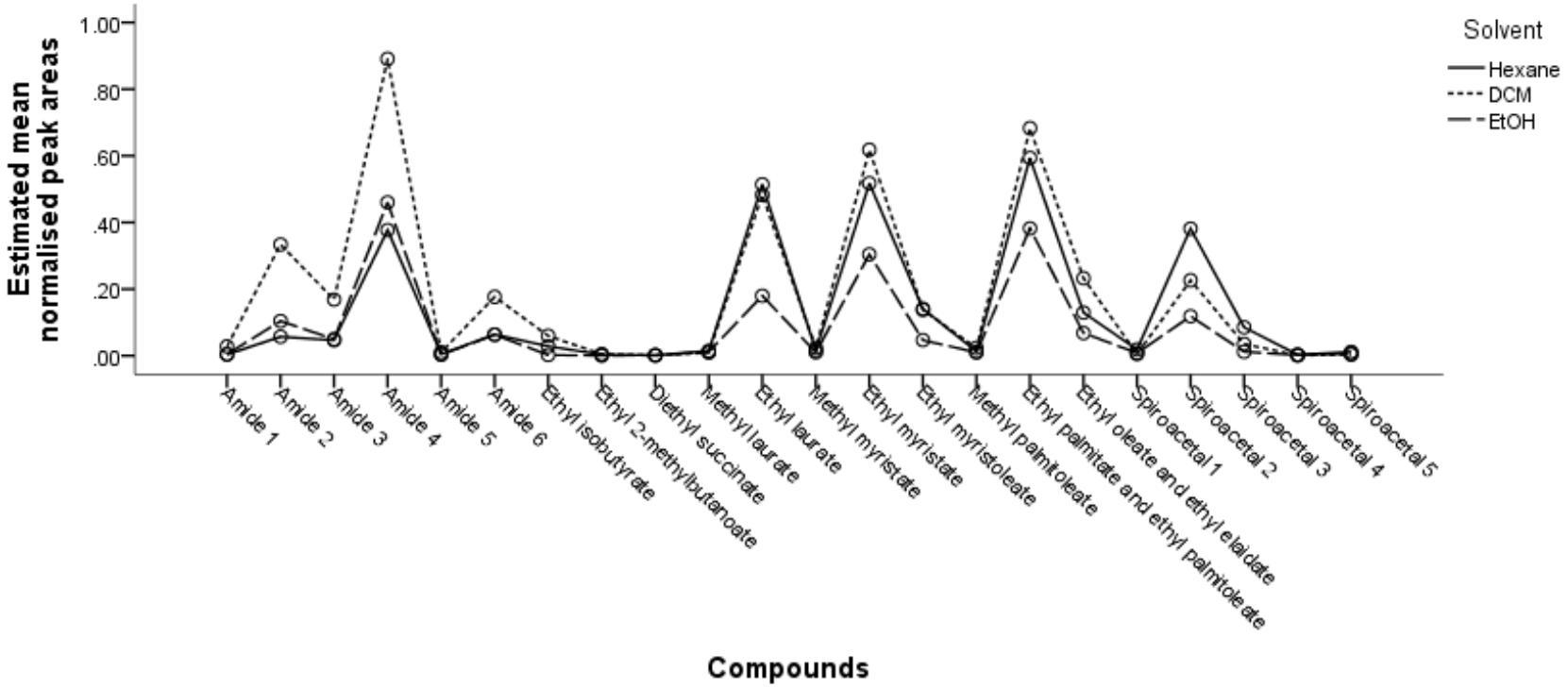


Figure S10. Graphical display of solvent x compound interactions for rectal gland extractions. Amide 1: *N*-(2-methylbutyl)acetamide, Amide 2: *N*-(3-methylbutyl)acetamide, Amide 3: -(*N* 2-methylbutyl)propanamide, Amide 4: *N*-(3-methylbutyl)propanamide, Amide 5: *N*-(2-methylbutyl)isobutyrate, Amide 6: *N*-(3-methylbutyl)isobutyrate, Spiroacetal 1: 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, Spiroacetal 2: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 3: (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 4: (*E*,*E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 5: (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane.

2.2. Effect of crushing on rectal gland extractions

Table S3. *P*-value obtained from ANOVA for crushing rectal gland extractions. Amide 1: *N*-(2-methylbutyl)acetamide, Amide 2: *N*-(3-methylbutyl)acetamide, Amide 3: *N*-(2-methylbutyl)propanamide, Amide 4: *N*-(3-methylbutyl)propanamide, Amide 5: *N*-(2-methylbutyl)isobutyrate, Amide 6: *N*-(3-methylbutyl)isobutyrate, Spiroacetal 1: 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, Spiroacetal 2: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 3: (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 4: (*E*,*E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 5: (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane.

	$P_{\text{crushed-intact}}$
Amide 1	0.850
Amide 2	0.013
Amide 3	0.061
Amide 4	< 0.001
Amide 5	0.796
Amide 6	< 0.001
Ethyl isobutyrate	0.715
Ethyl-2-methylbutanoate	0.903
Diethyl succinate	0.998
Methyl laurate	0.978
Ethyl laurate	0.030
Methyl myristate	0.990
Ethyl myristate	0.003
Ethyl myristoleate	0.302
Methyl palmitoleate	0.937
Ethyl palmitate/palmitoleate	< 0.001
Ethyl oleate/elaidate	0.627
Spiroacetal 1	0.918
Spiroacetal 2	< 0.001
Spiroacetal 3	0.101
Spiroacetal 4	0.859
Spiroacetal 5	0.968

2.2.1. Interactions between crushing and compounds from male and female rectal gland extractions:

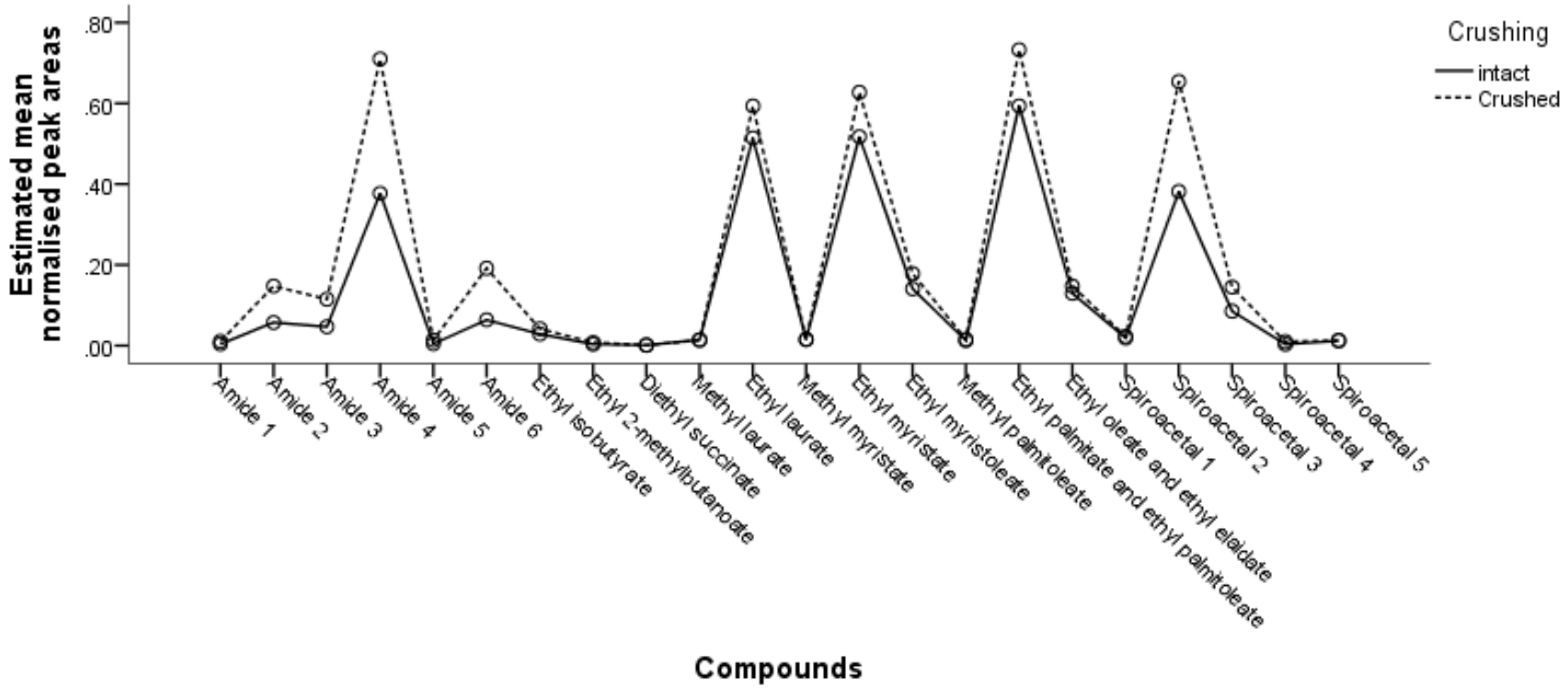


Figure S2. Graphical display of crushing x compound interactions for rectal gland extractions. Amide 1: *N*-(2-methylbutyl)acetamide, Amide 2: *N*-(3-methylbutyl)acetamide, Amide 3: -(*N* 2-methylbutyl)propanamide, Amide 4: *N*-(3-methylbutyl)propanamide, Amide 5: *N*-(2-methylbutyl)isobutyrate, Amide 6: *N*-(3-methylbutyl)isobutyrate, Spiroacetal 1: 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, Spiroacetal 2: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 3: (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 4: (*E*,*E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 5: (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane.

2.3. Effect of SPME fibers in static headspace sampling

Table S4. *P*-value obtained from ANOVA for SPME fiber comparisons in static headspace sampling. Amide 1: *N*-(2-methylbutyl)acetamide, Amide 2: *N*-(3-methylbutyl)acetamide, Amide 3: *N*-(2-methylbutyl)propanamide, Amide 4: *N*-(3-methylbutyl)propanamide, Amide 5: *N*-(2-methylbutyl)isobutyrate, Amide 6: *N*-(3-methylbutyl)isobutyrate, Spiroacetal 1: 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, Spiroacetal 2: (*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 3: (*E,E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 4: (*E,E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 5: (*E,E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane.

	$P_{\text{PDMS-PDMS/DVB}}$	$P_{\text{PDMS-PA}}$	$P_{\text{PA-PDMS/DVB}}$
Amide 1	0.948	0.919	0.865
Amide 2	0.2241	0.512	0.575
Amide 3	0.581	0.805	0.760
Amide 4	0.390	0.519	0.829
Amide 5	0.600	0.249	0.529
Amide 6	0.432	0.298	0.798
Ethyl isobutyrate	0.002	< 0.001	< 0.001
Ethyl-2-methylbutanoate	0.005	0.187	< 0.001
Diethyl succinate	0.756	0.561	0.786
Methyl laurate	0.414	0.796	0.283
Ethyl laurate	0.102	0.028	0.566
Methyl myristate	0.520	0.946	0.976
Ethyl myristate	0.069	0.293	0.440
Ethyl myristoleate	0.084	0.166	0.732
Methyl palmitoleate	0.428	0.474	0.939
Ethyl palmitate/palmitoleate	0.163	0.370	0.022
Spiroacetal 1	0.306	0.055	0.367
Spiroacetal 2	0.078	0.009	0.394
Spiroacetal 3	0.070	0.002	0.191
Spiroacetal 4	0.095	0.025	0.569
Spiroacetal 5	0.637	0.151	0.334

2.3.1. Interactions between fibers and compounds in static headspace:

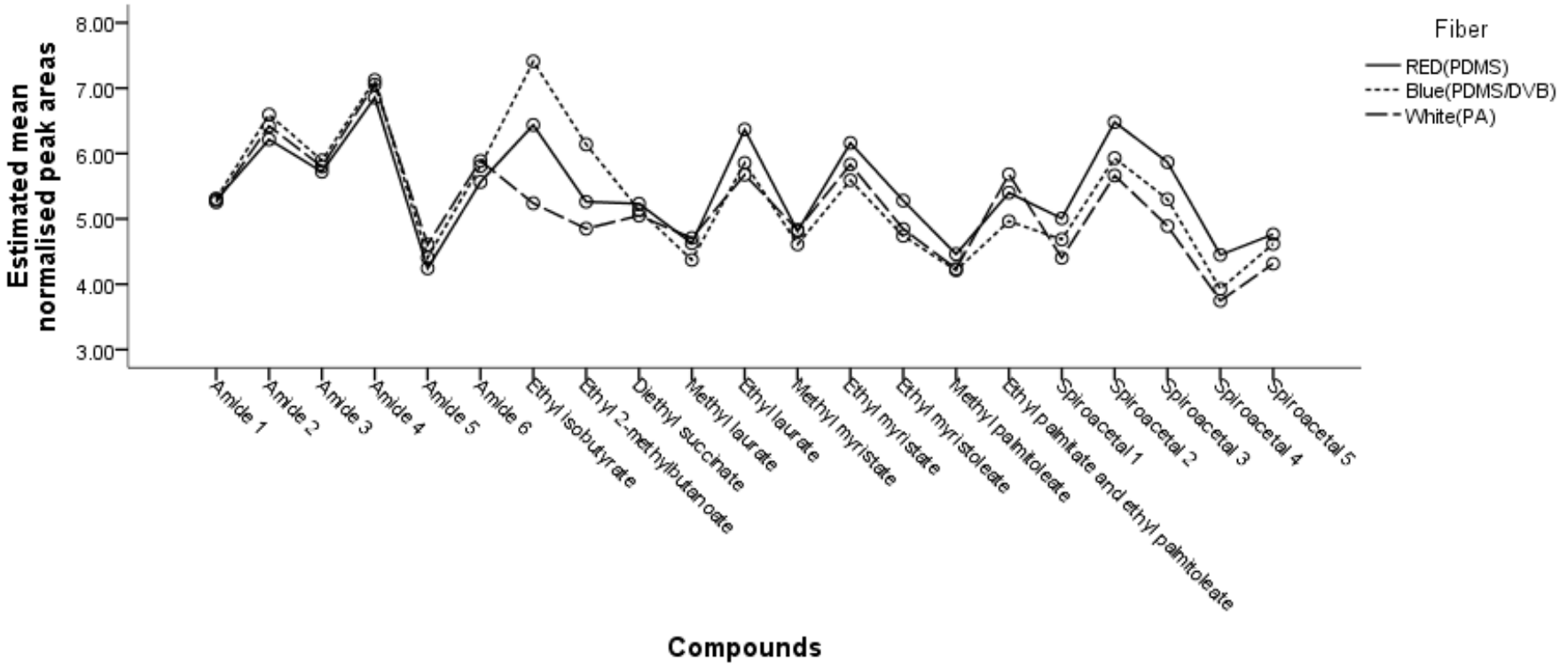


Figure S3. Graphical display of fiber x compound interactions for static headspace sampling. Amide 1: *N*-(2-methylbutyl)acetamide, Amide 2: *N*-(3-methylbutyl)acetamide, Amide 3: -(*N* 2-methylbutyl)propanamide, Amide 4: *N*-(3-methylbutyl)propanamide, Amide 5: *N*-(2-methylbutyl)isobutyrate, Amide 6: *N*-(3-methylbutyl)isobutyrate, Spiroacetal 1: 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, Spiroacetal 2: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 3: (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 4: (*E*,*E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 5: (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecan.

2.4. Effect of sorbent material and time in dynamic headspace sampling

2.4.1. Interactions between sorbents, time and compounds from male and female headspace:

Table S5. *P*-value obtained from ANOVA for duration of sampling (time) comparisons in dynamic headspace using Tenax and Porapak. Amide 1: *N*-(2-methylbutyl)acetamide, Amide 2: *N*-(3-methylbutyl)acetamide, Amide 3: *N*-(2-methylbutyl)propanamide, Amide 4: *N*-(3-methylbutyl)propanamide, Amide 5: *N*-(2-methylbutyl)isobutyrate, Amide 6: *N*-(3-methylbutyl)isobutyrate, Spiroacetal 1: 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, Spiroacetal 2: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 3: (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 4: (*E*,*E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 5: (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane.

	Sorbent	$P_{10\text{-}20}$	$P_{10\text{-}40}$	$P_{10\text{-}60}$	$P_{10\text{-}90}$	$P_{20\text{-}40}$	$P_{20\text{-}60}$	$P_{20\text{-}90}$	$P_{40\text{-}60}$	$P_{40\text{-}90}$	$P_{60\text{-}90}$
Amide 1	Tenax	0.996	0.802	0.781	0.786	0.835	0.814	0.818	0.979	0.983	0.996
	Porapak	0.997	0.940	0.911	0.901	0.942	0.913	0.903	0.971	0.961	0.990
Amide 2	Tenax	0.267	0.152	< 0.001	< 0.001	0.754	< 0.001	0.003	< 0.001	0.007	0.304
	Porapak	0.739	0.203	0.057	0.027	0.348	0.116	0.060	0.525	0/347	0.760
Amide 3	Tenax	0.879	0.563	0.343	0.377	0.670	0.427	0.464	0.712	0.760	0.949
	Porapak	0.976	0.733	0.606	0.544	0.756	0.627	0.564	0.861	0.790	0.928
Amide 4	Tenax	0.111	0.002	< 0.001	< 0.001	0.147	< 0.001	< 0.001	< 0.001	< 0.001	0.084
	Porapak	0.729	0.003	< 0.001	< 0.001	0.008	< 0.001	< 0.001	0.008	0.001	0.488
Amide 5	Tenax	0.996	0.988	0.929	0.929	0.992	0.932	0.933	0.940	0.941	0.999
	Porapak	0.999	0.986	0.921	0.925	0.987	0.923	0.926	0.936	0.940	0.996
Amide 6	Tenax	0.848	0.713	0.077	0.193	0.860	0.114	0.267	0.161	0.351	0.638
	Porapak	0.918	0.601	0.217	0.205	0.674	0.258	0.244	0.477	0.547	0.974
Ethyl isobutyrate	Tenax	0.994	0.996	0.997	0.994	0.998	0.998	0.989	0.999	0.990	0.991
	Porapak	0.982	0.973	0.971	0.970	0.992	0.989	0.988	0.997	0.996	0.999
Methyl laurate	Tenax	0.895	0.791	0.486	0.673	0.894	0.572	0.772	0.666	0.876	0.783
	Porapak	0.930	0.885	0.750	0.737	0.954	0.817	0.804	0.861	0.849	0.987
Ethyl laurate	Tenax	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	Porapak	0.010	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.056	0.835	0.034

Methyl myristate	Tenax	0.998	0.865	0.617	0.337	0.868	0.619	0.742	0.645	0.429	0.645
	Porapak	0.977	0.766	0.542	0.562	0.744	0.524	0.543	0.756	0.778	0976
Ethyl myristate	Tenax	0.001	< 0.001	< 0.001	< 0.001	0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	Porapak	0.114	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.044	0.998	0.043
Ethyl myristoleate	Tenax	0.637	0.133	0.004	< 0.001	0.303	0.017	< 0.001	0.172	< 0.001	< 0.001
	Porapak	0.973	0.001	< 0.001	< 0.001	0.001	< 0.001	< 0.001	0.418	0.618	0.756
Methyl palmitoleate	Tenax	0.919	0.893	0.881	0.859	0.974	0.962	0.939	0.988	0.966	0.977
	Porapak	0.952	0.920	0.906	0.952	0.969	0.954	0.999	0.985	0.968	0.953
Ethyl palmitate and ethyl palmitoleate	Tenax	0.918	0.003	< 0.001	< 0.001	0.004	< 0.001	< 0.001	0.075	< 0.001	< 0.001
	Porapak	0.860	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.209	0.513	0.548
Spiroacetal 1	Tenax	0.973	0.924	0.522	0.827	0.951	0.544	0.800	0.586	0.753	0.390
	Porapak	0.901	0.220	0.098	0.146	0.270	0.125	0.183	0.667	0.820	0.840
Spiroacetal 2	Tenax	0.558	0.002	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.191	< 0.001	< 0.001
	Porapak	0.844	< 0.001	< 0.001	0.016	< 0.001	< 0.001	0.011	0/646	0.006	0.001
Spiroacetal 3	Tenax	0.420	0.798	0.292	< 0.001	0.581	0.063	0.001	0.190	< 0.001	< 0.001
	Porapak	0.986	< 0.001	< 0.001	0,019	< 0.001	< 0.001	0.018	0.180	0.249	0.013
Spiroacetal 4	Tenax	0.886	0.863	0.755	0.948	0.977	0.867	0.937	0.914	0.889	0.805
	Porapak	0.871	0.769	0.698	0.0.846	0.895	0.821	0.974	0.924	0.921	0.846
Spiroacetal 5	Tenax	0.695	0.218	0.121	0.290	0.401	0.247	0.505	0.750	0.863	0.623
	Porapak	0.871	0.240	0.209	0.590	0.311	0.274	0.707	0.936	0.524	0.473

Table S6. *P*-value obtained from ANOVA for type of sorbent comparisons in dynamic headspace. Amide 1: *N*-(2-methylbutyl)acetamide, Amide 2: *N*-(3-methylbutyl)acetamide, Amide 3: *N*-(2-methylbutyl)propanamide, Amide 4: *N*-(3-methylbutyl)propanamide, Amide 5: *N*-(2-methylbutyl)isobutyrate, Amide 6: *N*-(3-methylbutyl)isobutyrate, Spiroacetal 1: 2,7-dimethyl-1,6-dioxaspiro[4.5]decane, Spiroacetal 2: (*E*,*E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 3: (*E*,*E*)-2-ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 4: (*E*,*E*)-2-ethyl-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane, Spiroacetal 5: (*E*,*E*)-2-propyl-8-methyl-1,7-dioxaspiro[5.5]undecane.

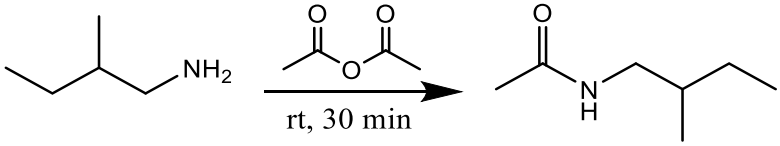
	$P_{\text{Tenax-Porapak}}$				
	10 min	20 min	40 min	60 min	90 min
Amide 1	0.993	0.962	0.854	0.862	0.876
Amide 2	0.891	0.361	0.765	0.001	0.042
Amide 3	0.969	0.872	0.782	0.638	0.752
Amide 4	0.642	0.088	0.604	< 0.001	0.029
Amide 5	0.996	0.993	0.998	0.997	1.000
Amide 6	0.900	0.830	0.976	0.508	0.874
Ethyl isobutyrate	1.000	0.987	0.977	0.974	0.964
Methyl laurate	0.984	0.949	0.888	0.690	0.915
Ethyl laurate	0.912	0.098	0.232	0.001	< 0.001
Methyl myristate	0.973	0.948	0.925	0.940	0.678
Ethyl myristate	0.871	0.050	0.003	0.489	< 0.001
Ethyl myristoleate	0.533	0.289	0.249	0.551	< 0.001
Methyl palmitoleate	0.977	0.989	0.995	0.998	0.928
Ethyl palmitate/palmitoleate	0.225	0.254	0.816	0.769	< 0.001
Spiroacetal 1	0.876	0.806	0.198	0.241	0.067
Spiroacetal 2	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Spiroacetal 3	0.814	0.306	< 0.001	< 0.001	< 0.001
Spiroacetal 4	0.965	0.951	0.869	0.904	0.862
Spiroacetal 5	0.911	0.906	0.956	0.854	0.682

3. Synthesis of compounds

3.1. General procedures

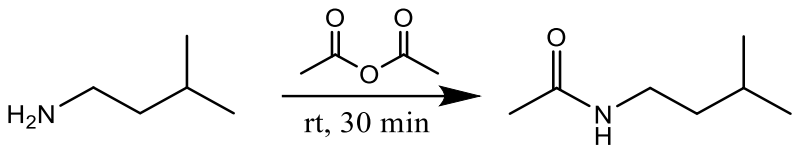
All reagents were purchased from Sigma-Aldrich and used without further purification. All solvents were anhydrous or analytical grade (Sigma-Aldrich) and used without further purification. The reaction progress was monitored by GC-MS (using the procedure given in the main text). Solvents were removed under reduced pressure using a Büchi Rotavapor R-200 and Büchi B-490 heating bath set to 40 °C. Mixtures were further dried under high vacuum using an Alcatel Pascal 2005SD vacuum pump. NMR spectra were recorded on a Bruker AVANCE-400 instrument (^{1}H NMR: 400 MHz, ^{13}C NMR: 101 MHz) or a Bruker AVANCE-600 instrument equipped with a cryoprobe (^{1}H NMR: 600 MHz, ^{13}C NMR: 150 MHz) using $CDCl_3$ and C_6D_6. The ^{1}H NMR chemical shifts were referenced to the residual protonated solvent peaks at δH 7.26 for chloroform-d and 7.15 for benzene-d_6. ^{13}C NMR chemical shifts were referenced to the central solvent peaks of bulk solvent at δC 77.16 for chloroform-d and 127.68 for benzene-d_6. *J* values are given in Hz.

3.2. Synthesis of *N*-(2-methylbutyl)acetamide (**6**).



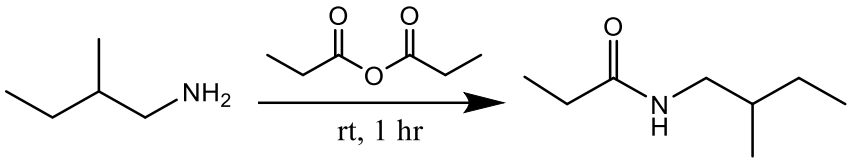
The synthesis was conducted using the method of Naik et al.[1] To a mixture of 2-methylbutylamine (4.4 g, 50 mmol) in water (50 mL) was added acetic anhydride (7.7 g, 75 mmol). The clear reaction mixture was stirred at room temperature for 0.5 hour and the completion of the reaction at this time was determined by GC-MS. The clear reaction mixture was extracted with ethyl acetate (3 × 50 mL). The combined organic layer was washed with 5% aqueous sodium bicarbonate solution (150 mL), dried over sodium sulfate and concentrated under reduced pressure to give the crude product, which was purified by vacuum distillation (150 – 160 °C, 20 mm Hg) to afford *N*-(2-methylbutyl)acetamide (**6**) as a clear liquid (5.6 g, 86% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.83 (6 H, m, $CHCH_2C\mathbf{H}_3$ and $CH_2C\mathbf{H}_3$), 1.07 (1 H, apparent sep, *J* = 6.6, C**H**CH_3), 1.29 – 1.53 (2 H, m, C**H**$_2CH_3$), 1.93 (3 H, s, C**H**$_3$CO), 2.94 – 3.13 (2 H, m, NC**H**$_2$), 6.33 (1 H, bs, N**H**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 11.2, 17.1, 23.1, 27.0, 34.8, 45.4, 170.6. GC-MS (EI) *m/z* (%) 129 (M^+, 8), 100 ($M^+-CH_2CH_3$, 38), 72 ($M^+-CHCH(CH_3)CH_2CH_3$, 100). This compound is known, but spectral data are not available in the literature.

3.3. Synthesis of *N*-(3-methylbutyl)acetamide (**7**).



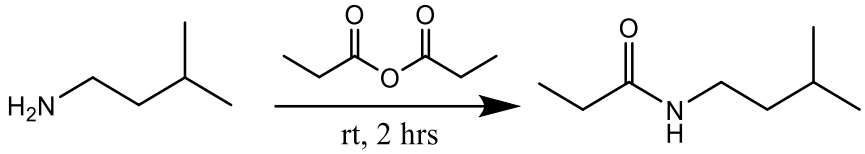
Using a similar reaction, work-up and purification conditions to *N*-(2-methylbutyl)acetamide (**6**) (above), 3-methylbutylamine (5.0 g, 57 mmol) in water (50 mL) was acetylated with acetic anhydride (8.7 g, 86 mmol) to produce *N*-(3-methylbutyl)acetamide (**7**) as a clear liquid (5.4 g, 73% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85 (6 H, d, *J* = 6.6, $CH(C\mathbf{H}_3)_2$), 1.33 (2 H, m, $C\mathbf{H}_2CH_3$), 1.56 (1 H, non, *J* = 6.7, C**H**), 1.92 (3 H, s, $C\mathbf{H}_3CO$), 3.18 (2 H, m, $NC\mathbf{H}_2$), 6.21 (1 H, bs, N**H**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 22.4, 23.1, 25.8, 38.0, 38.3, 170.4. GC-MS (EI) *m/z* (%) 129 (M^+, 5), 114 (M^+-CH_3, 12), 73 ($M^+-CH_2CH_2CH(CH_3)_2$, 100). MS data match with those in the literature.[2] NMR data are not available in the literature.

3.4. Synthesis of *N*-(2-methylbutyl) propanamide (**9**).



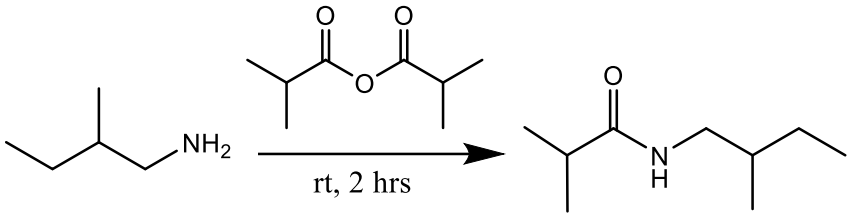
Using a similar reaction, work-up and purification conditions as above, 2-methylbutylamine (4.4 g, 50 mmol) in water (50 mL) was acetylated with propionic anhydride (9.7 g, 75 mmol) to produce *N*-(2-methylbutyl) propanamide (**9**) as a clear liquid (5.4 g, 63% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.82 (6 H, m, overlapped $CH(CH_3)CH_2C\mathbf{H}_3$ and $COCH_2C\mathbf{H}_3$), 1.06 (4 H, m, overlapped C**H**C$\mathbf{H}_3$), 1.41 (2 H, m, $CHC\mathbf{H}_2CH_3$), 2.15 (2 H, q, *J* = 7.6, $CH_3C\mathbf{H}_2CO$), 3.04 (2 H, m, $HNC\mathbf{H}_2$), 6.06 (1 H, bs, NH); ^{13}C NMR (101 MHz, $CDCl_3$) δ 10.1, 11.2, 17.1, 27.0, 29.7, 34.9, 45.1, 174.1; GC-MS (EI) *m/z* (%) 143 (M^+, 10), 86 ($\cdot CH_3CH_2CH(CH_3)CH_2NH^+$, 75), 57 ($CH_3CH_2CHCH_3^+$, 100). This compound is known, but spectral data are not available in the literature.

3.5. Synthesis of *N*-(3-methylbutyl)propanamide (**10**).



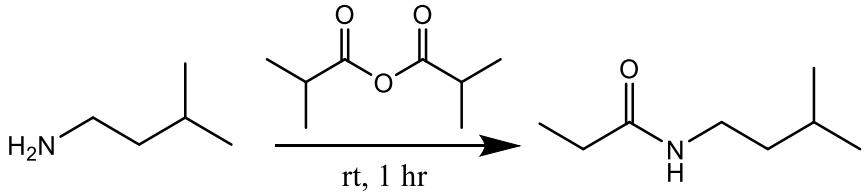
Using a similar reaction, work-up and purification conditions to above, 3-methylbutylamine (4.4 g, 51 mmol) in water (50 mL) was acetylated with propionic anhydride (10.0 g, 77 mmol) to produce *N*-(3-methylbutyl)propanamide (**10**) as a clear liquid (5.7 g, 79% yield).^{1}H NMR (400 MHz, $CDCl_3$) δ 0.80 (6 H, m, CH(C**H**$_3$)$_2$), 1.04 (3 H, t, *J* = 7.6, CH_2C**H**$_3$), 1.29 (2 H, m, C**H**$_2$ CH), 1.51 (1 H, non, *J* = 6.7, C**H**), 2.11 (2 H, q, *J* = 7.6, C**H**$_2CH_3$), 3.15 (2 H, m, HNC**H**$_2$), 6.38 (1 H, bs, N**H**); ^{13}C NMR (101 MHz, $CDCl_3$) δ 10.0 ; 22.9, 25.2, 29.7, 36.7, 37.5, 174.6; GC-MS (EI) *m/z* (%) 143 (M^+, 8), 128 (M^+-CH_3, 10), 114 ($M^+-CH_2CH_3$, 9) 57 ($(CH_3)_2CHCH_2^+$, 100). This compound is known, but spectral data are not available in the literature.

3.6. Synthesis of *N*-(2-methylbutyl)isobutyrate (**11**).



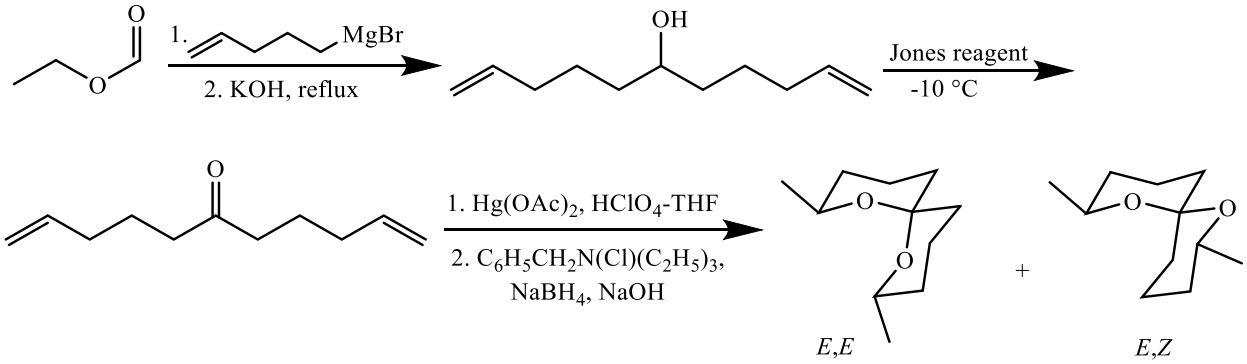
Using a similar reaction, work-up and purification conditions as above, 2-methylbutylamine (4.4 g, 50 mmol) in water (50 mL) was acetylated with isobutyric anhydride (11.8 g, 75 mmol) to produce *N*-(2-methylbutyl)isobutyrate (**11**) as colorless needles (6.8 g, 87% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.81 (6 H, d, *J* = 6.6, C**H**$_3$CHCH_2C**H**$_3$), 1.04 (6 H, d, *J* = 6.9, COCH(C**H**$_3$)$_2$), 1.30 (2 H, m, C**H**$_2$CH), 1.52 (1 H, oct, *J* = 6.9, C**H**$(CH_3)_2$), 2.31 (1 H, sep, *J* = 6.6, C**H**CO), 3.27 (2 H, m, HNC**H**$_2$), 6.21 (1 H, bs, N**H**); ^{13}C NMR (100 MHz, $CDCl_3$) δ 19.7, 22.9, 25.2, 35.4, 36.7, 37.8, 177.0; GC-MS (EI) *m/z* (%) 157 (M^+, 8), 142 (M^+-CH_3, 12), 114 ($M^+-CH(CH_3)_2$, 16), 101 (M^+-CH_3 and $CH(CH_3)_2$, 50), 71 ($\cdot CH_3CH_2CON^+$, 100). This compound is known, but spectral data are not available in the literature.

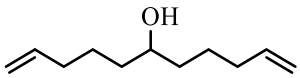
3.7. Synthesis of *N*-(3-methylbutyl)isobutyrate (**12**).



Using a similar reaction, work-up and purification conditions as above, 3-methylbutylamine (5.2 g, 60 mmol) in water (50 mL) was acetylated with isobutyric anhydride (14.2 g, 90 mmol) for 1 hour to produce *N*-(3-methylbutyl)isobutyrate (**12**) as a clear liquid (5.0 g, 53% yield).[1]H NMR (400 MHz, $CDCl_3$) δ 0.90 (6 H, m, *J* = 6.6, $CHCH_2C\mathbf{H}_3$ and $CH_2CH(C\mathbf{H}_3)CH_2$), 1.13 (1 H, m, $C\mathbf{H}(CH_3)CH_2$) 1.16 (6 H, d, *J* = 6.9, $CH(C\mathbf{H}_3)_2$), 2.37 (1 H, non, *J* = 6.9, $C\mathbf{H}(CH_3)_2$), 3.12 (2 H, m, $HNC\mathbf{H}_2$), 5.65 (1 H, bs, N**H**); ^{13}C NMR (100 MHz, $CDCl_3$) δ 11.4, 17.2, 19.8, 27.1, 35.0, 35.9, 45.0, 177.2; GC-MS (EI) *m/z* (%) 157 (M^+, 10), 114 (M^+- (CH_3 and CH_2CH_3), 45), 43 (($CH_3)_2CH^+$, 100). This compound is known, but spectral data are not available in the literature.

3.8. Synthesis of 2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**14**).

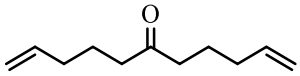




1,10-Undecadien-6-ol

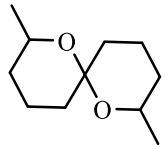
Following the method of Kitching et al.,[3] a Grignard reaction followed by hydrolysis was conducted to give 1,10-undecadien-6-ol. In brief, a flame-dried argon-flushed two-necked round bottom flask was charged with magnesium (1.8 g, 74 mmol), a single crystal of iodine, and a magnetic stirrer bar, and fitted with a condenser. Dry diethyl ether (60 mL) was added and the suspension was brought to reflux. 5-Bromopent-1-ene (10 g, 67 mmol) in diethyl ether (30 mL) was added dropwise and then the colourless suspension was

stirred at reflux for 4 hours. The colourless suspension was cooled to 0 °C and ethyl formate (2.6 g, 34 mmol) was added. The suspension was warmed to room temperature, stirred for 1 hour, then quenched with saturated ammonium chloride and extracted with diethyl ether (3 × 15 mL). The combined organic layers were washed with saturated aqueous brine and dried over magnesium sulfate. After solvent removal by rotary evaporation, the yellow oil was refluxed in 15% aqueous potassium hydroxide solution for 3 hours. The solution was cooled to room temperature and extracted with diethyl ether (3 × 20 mL). Solvent was removed under reduced pressure to give the crude product as a yellow oil, which was purified by distillation (110 – 115 °C, 10 mm Hg) to afford 1,10-undecadien-6-ol as a colourless oil (3.7 g, 60% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.81 (2 H, ddt, *J* = 17, 10.3, 6.7 Hz, C**H**=), 5.01 (2 H, dq, *J* = 17.1, 1.7 Hz, C**H**$_2$=), 4.91 – 5.01 (2 H, m, C**H**$_2$=), 3.61 (1 H, bs, C**H**OH), 2.00 – 2.13 (4 H, m, C**H**$_2$CH=CH$_2$), 1.26 – 1.61 (9 H, m, including OH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.7 (CH=), 114.6 (CH_2=), 71.7 (CHOH), 36.9 (CH_2), 33.7 (CH_2), 24.9 (CH_2). GC-MS (EI) *m/z* (%) 84 (12.3), 81 (100), 80 (10), 79 (19.5), 69 (9.2), 68 (9.3), 67 (20.6), 58 (9.5), 57 (30.2), 55 (72.3), 54 (27.7), 53 (9.4), 43 (32.1), 42 (9), 41 (43.8). Spectral data were consistent with the literature.[3]



Undeca-1,10-dien-6-one

To a solution of 1,10-undecadien-6-ol (3.99 g, 23.7 mmol) in acetone (10 mL) at -10 °C, freshly prepared Jones reagent (2.7 g of chromium trioxide in 4 mL of sulfuric acid and 12 mL of distilled water) was added dropwise and the reaction monitored by GC-MS. After completion of the reaction (2 hours), the green suspension was filtered through a pad of Celite. The filtrate was washed with saturated aqueous sodium bicarbonate (17 mL), extracted with diethyl ether (4 × 50 mL) and washed with water (50 mL) and saturated aqueous brine (50 mL), then dried over magnesium sulfate. Concentration by rotary evaporation yielded undeca-1,10-dien-6-one as a colourless oil (2.9 g, 75% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.80 (2 H, ddt, *J* = 17.2, 10.3, 6.7 Hz, C**H**=), 4.87 – 4.97 (4 H, m, C**H**$_2$=), 2.33 (4 H, t, *J* = 7.5 Hz, C**H**$_2$CO), 1.98 (4 H, m, C**H**$_2$CH=CH$_2$), 1.60 (4 H, quin, *J* = 7.3 Hz, CH$_2$C**H**$_2$CH$_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 210.9 (CO), 138.0 (CH=), 115.2 (CH_2=), 41.9 (CH_2), 33.1 (CH_2), 22.8 (CH_2). GC-MS (EI) *m/z* (%) 112 (14.6), 97 (30), 84 (27.8), 83 (10.6), 70 (14.1), 69 (59.5), 58 (48.7), 55 (49.6), 43 (24.5), 41 (100). Spectral data were consistent with the literature.[3]



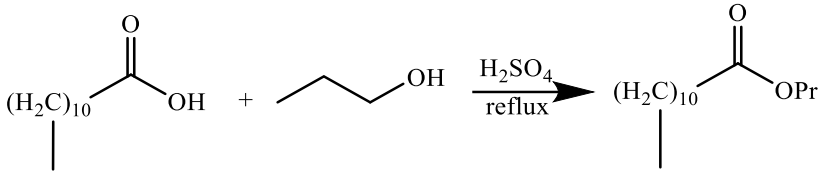
2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane

$Hg(OAc)_2$ (1.9 g, 6.1 mmol) was added to a stirred solution of undeca-1,10-dien-6-one (0.5 g, 3 mmol) in 1% aqueous perchloric acid: tetrahydrofuran (15 mL:15 mL) and the solution was stirred for 15 hours. Benzyltriethylammonium chloride (2.4 g, 10.5 mmol) in 10% aqueous sodium hydroxide (15 mL) and dichloromethane (5 mL) was added followed by sodium borohydride (0.09 g, 2.3 mmol) in 10% aqueous sodium hydroxide (5 mL). The gray suspension was stirred and monitored by GC-MS. After completion of the reaction (20 minutes), the gray suspension was filtered through a pad of Celite, which was then washed with 30 mL of diethyl ether. The aqueous phase was then extracted with diethyl ether (3 × 30 mL) and the combined organic layer (from Celite wash and extraction) were washed with saturated aqueous brine (50 mL) and dried over magnesium sulfate. After solvent removal by rotary evaporation the product was purified by Kugelrohr distillation (bp 110 °C; 30 mm Hg). According to the literature [3] under this condition a mixture of *E,E* diastereomer with some *E,Z* and no *Z,Z* isomer is obtained. These configurational isomers produced different MS fragmentation patterns that were matched with those in the literature.[3]

(*E,E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (**14**). ^{13}C NMR (101 MHz, C_6D_6) δ 95.75 (CO_2), 64.8 (CO), 35.33 (CH_2), 32.90 (CH_2), 21.92 (CH_3), 19.03 (CH_2). GC-MS (EI) *m/z* (%) 184 (M^+, 5.6), 169 (M^+-CH_3, 1.9), 140 (M^+-CH_3CHO, 11.6), 125 (8.2), 115 ($CH_3(C_5H_7O)=OH^+$, 92.4), 114 (43.2), 113 (8.6), 112 ($CH_3(C_5H_7O)=CH_2$, 100), 97 (68.4), 84 (15.7), 83 (23.8), 73 (24.8), 71 (18.5), 70 (15.8), 69 (54.9), 58 (18.2), 55 (52.1), 43 (69.4), 42 (35.9), 41 (56.1).

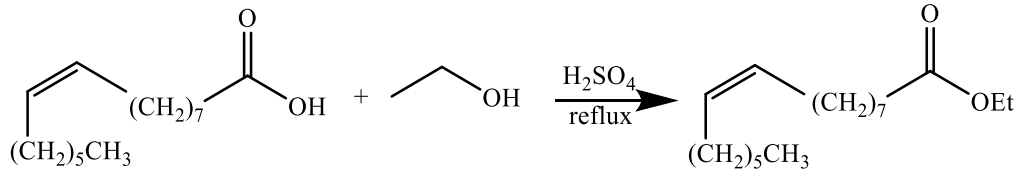
(*E,Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane. GC-MS (EI) *m/z* (%) 184 (M^+, 8.1), 115 ($CH_3(C_5H_7O)=OH^+$, 100), 114 (37), 112 ($CH_3(C_5H_7O)=CH_2$
, 39.5), 97 (73.1), 83 (11.8), 73 (27.5), 71 (12.4), 69 (59.9), 55 (41.8), 43 (38.6), 42 (24.6), 41 (38.4).

3.9. Propyl laurate (**21**).



A mixture of lauric acid (1.0 g, 5 mmol), 1-propanol (10 mL) and concentrated sulfuric acid (2 drops) was heated to reflux for 1.5 hours. After cooling, diethyl ether (10 mL) and 5% *w/v* aqueous sodium bicarbonate (10 mL) were added to the reaction mixture. The organic layer was separated and washed with 5% *w/v* aqueous sodium bicarbonate (3 × 10 mL) and dried over sodium sulfate. The solvent was removed under reduced pressure, yielding the crude product, which was purified by distillation to give ethyl palmitoleate as a colourless liquid (0.44 g, 38% yield). ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.87 (3 H, t, *J* = 7.0 Hz, $CH_2C\mathbf{H}_3$), 0.93 (3 H, t, *J* = 7.4 Hz, $OCH_2CH_2C\mathbf{H}_3$), 1.25-1.29 (16 H, m, $C\mathbf{H}_2$), 1.57-1.66 (4 H, m, $C\mathbf{H}_2CH_2COOPr$, $CH_3C\mathbf{H}_2CH_2OCO$), 2.29 (2 H, t, *J* = 7.5 Hz, $C\mathbf{H}_2COOPr$), 4.02 (2 H, t, *J* = 6.7 Hz, $C\mathbf{H}_2OCO$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 10.5 (CH_3), 14.2 (CH_3), 22.1 (CH_2), 22.8 (CH_2), 25.1 (CH_2), 29.3 (CH_2), 29.40 (CH_2), 29.47 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 32.0 (CH_2), 34.5 (CH_2), 65.9 (OCH_2), 174.1 (C=O). GC-MS (EI) *m/z* (%) 242 (M^+, 4.3), 213 (1.4), 201 ($M^+-CH_2CH_2CH_3$, 27.5), 183 ($M^+-OCH_2CH_2CH_3$, 25.7), 171 (6.6), 157 (6.8), 143 (3.3), 129 (8.7), 115 (21.8), 102 (32.5), 97 (7.7), 85 (12.2), 73 (39.3), 61 (100), 57 (30.9), 43 (80.2). Spectral data were consistent with the literature.[4]

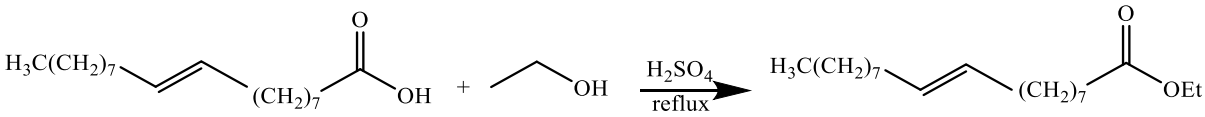
3.10. Ethyl palmitoleate (**25**).



Using similar conditions to above, palmitoleic acid (0.50 g, 1.9 mmol), was esterified with ethanol (10 mL) in the presence of concentrated sulfuric acid (2 drops), quenched with sodium bicarbonate and diethyl ether and purified by distillation to afford ethyl palmitoleate as a colourless oil (0.11 g, 19% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.32 – 5.35 (2 H, m, C**H**=C**H**), 4.11 (2 H, q, *J* = 7.2 Hz, $OC\mathbf{H}_2CH_3$), 2.28 (2 H, t, *J* = 7.5 Hz, $C\mathbf{H}_2COOEt$), 1.98 – 2.01 (4 H, m, $C\mathbf{H}_2CH=CHC\mathbf{H}_2$), 1.59 – 1.63 (2 H, m, $C\mathbf{H}_2CH_2COOEt$), 1.23 – 1.30 (19 H, m, $C\mathbf{H}_2$), 0.88 (3 H, t, *J* = 6.9 Hz, $CH_2C\mathbf{H}_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.0 (C=O), 130.1 (CH), 129.9 (CH), 60.2 (OCH_2), 34.5 (CH_2), 31.9 (CH_2), 29.87 (CH_2), 29.82 (CH_2), 29.3 (CH_2), 29.26 (CH_2), 29.23 (CH_2), 29.1

(CH_2), 27.36 (CH_2), 27.30 (CH_2), 25.1 (CH_2), 22.8 (CH_2), 14.4 (CH_3), 14.2 (CH_3). GC-MS (EI) *m/z* (%) 282 (M^+, 3.8), 236 (M^+−OCH_2CH_3, 14.3), 218 (1.4), 207 (1.4), 194 (M^+−$CH_2COOCH_2CH_3$, 15.0), 179 (1.65), 165 (2.8), 152 (M^+−$(CH_2)_4COOCH_2CH_3$, 14.9), 138 (6.9), 123 (11.9), 101 (31.8), 88 (50.6), 83 (44.9), 69 (64.1), 55 (100), 41 (81.8). Spectral data were not available in the literature.

3.11. Ethyl elaidate (**29**).



Using similar conditions to above, elaidic acid (0.45 g, 1.6 mmol), was esterified with ethanol (10 mL) in the presence of concentrated sulfuric acid (2 drops), quenched with sodium bicarbonate and diethyl ether and purified by distillation to afford ethyl elaidate as a colourless oil (113 mg, 23% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36 – 5.28 (2 H, m, C**H**=C**H**), 4.11 (2 H, q, *J* = 7.1 Hz, OC**H₂**CH_3), 2.27 (2 H, t, *J* = 7.6 Hz, C**H₂**COOEt), 1.95 – 1.96 (4 H, m, C**H₂**CH=CHC**H₂**), 1.57 – 1.60 (2 H, m, C**H₂**CH_2COOEt), 1.23 – 1.28 (23 H, m, CH_2), 0.87 (3 H, t, *J* = 6.7 Hz, CH_2CH_2C**H₃**). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.0 (C=O), 130.6 (CH), 130.4 (CH), 60.3 (OCH_2), 34.5 (CH_2), 32.78 (CH_2), 32.73 (CH_2), 32.0 (CH_2), 29.8 (CH_2), 29.7 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.36 (CH_2), 29.30 (CH_2), 29.1 (CH_2), 25.1 (CH_2), 22.8 (CH_2), 14.4 (CH_3), 14.2 (CH_3). GC-MS (EI) *m/z* (%) 310 (M^+, 3.5), 281 (M^+−CH_2CH_3, 0.25), 264 (M^+−OCH_2CH_3, 16.2), 222 (11.3), 180 (11.2), 155 (7.0), 138 (5.6), 123 (13.5), 111 (20.6), 97 (38.6), 88 (45.6), 83 (49.0), 69 (69.0), 55 (100), 41 (76.4). Spectral data were consistent with the literature.[5]

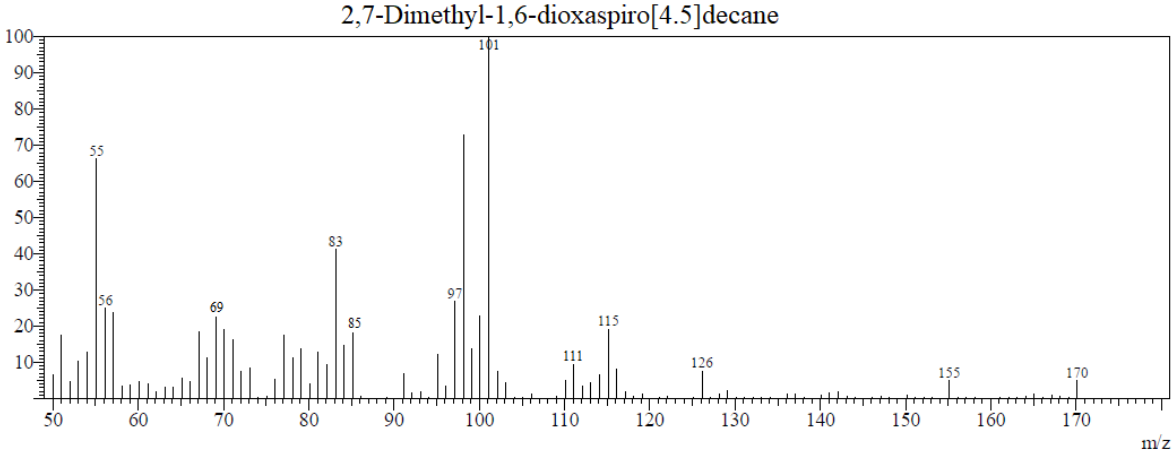
References

1. Naik, S., Bhattacharjya, G., Talukdar, B. & Patel, B. K. Chemoselective acylation of amines in aqueous media. *European J. Org. Chem.* **2004**, 1254–1260 (2004).
2. Wee, S. L. & Tan, K. H. Female sexual response to male rectal volatile constituents in the fruit fly, *Bactrocera carambolae* (Diptera: Tephritidae). *Appl. Entomol. Zool.* **40**, 365–372 (2005).
3. Kitching, W. *et al.* Chemistry of fruit flies. Composition of the rectal gland secretion of (male) *Dacus cucumis* (cucumber fly) and *Dacus halfordiae*. Characterization of (*Z*,*Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane. *J. Org. Chem.* **54**, 3893–3902 (1989).
4. Santos, M. *et al.* Poly(3-hydroxyoctanoate) depolymerase from *Pseudomonas fluorescens* GK13: Catalysis of ester-forming reactions in non-aqueous media. *J. Mol. Catal. B Enzym.* **77**, 81–86 (2012).
5. Denton, R. M., Tang, X. & Przeslak, A. Catalysis of phosphorus(V)-mediated transformations: dichlorination reactions of epoxides under Appel conditions. *Org. Lett.* **12**, 4678–4681 (2010).

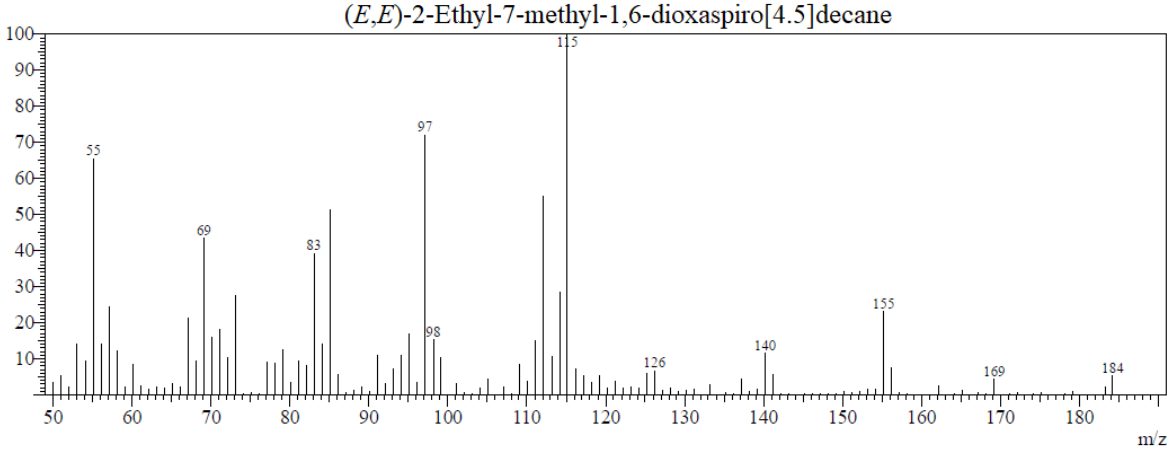
Appendix VI:

Mass spectra of spiroacetals identified in this study:

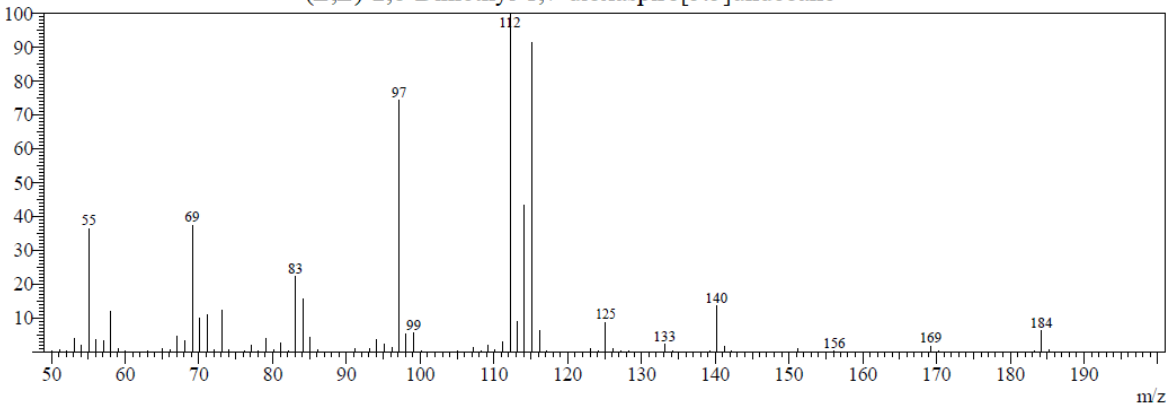
2,7-Dimethyl-1,6-dioxaspiro[4.5]decane



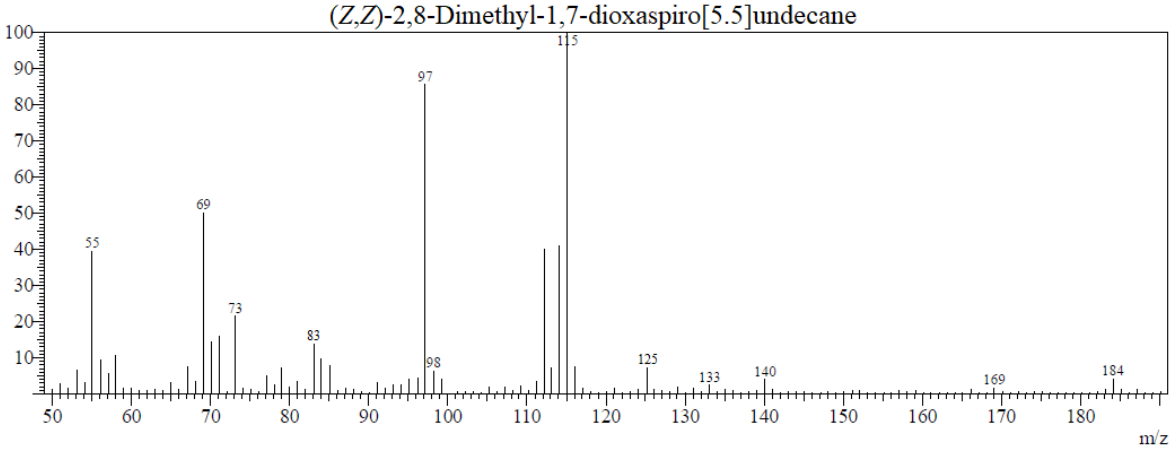
(*E*,*E*)-2-Ethyl-7-methyl-1,6-dioxaspiro[4.5]decane



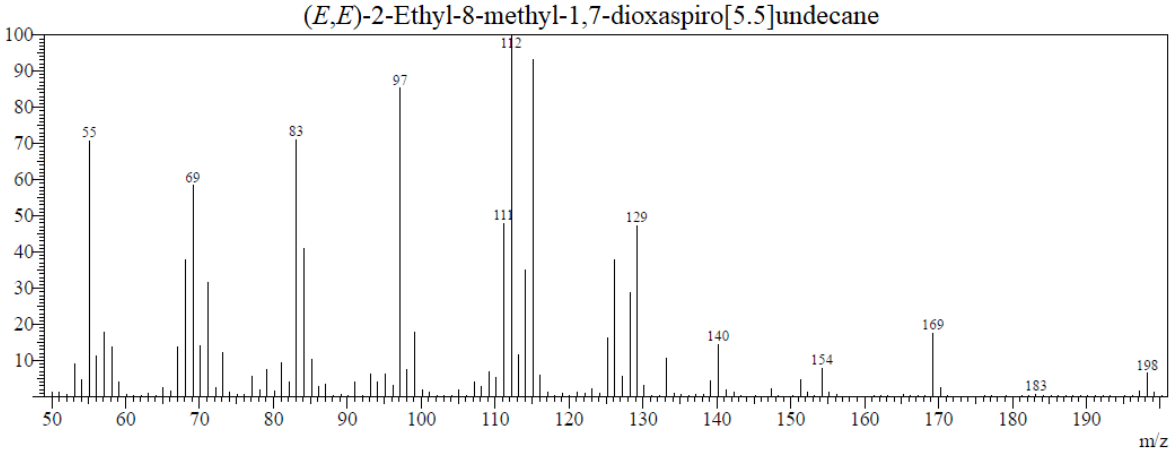
(*E*,*E*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane



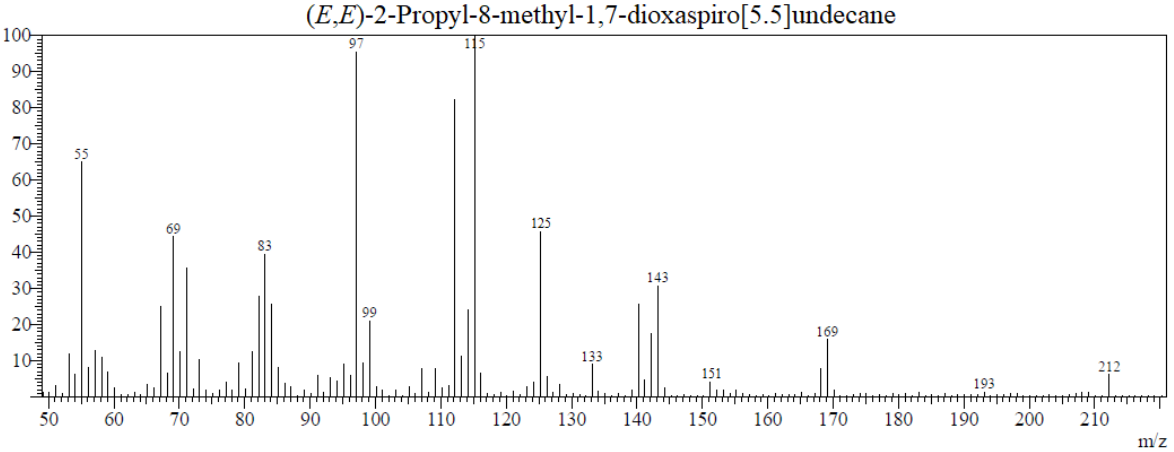
(*Z*,*Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane



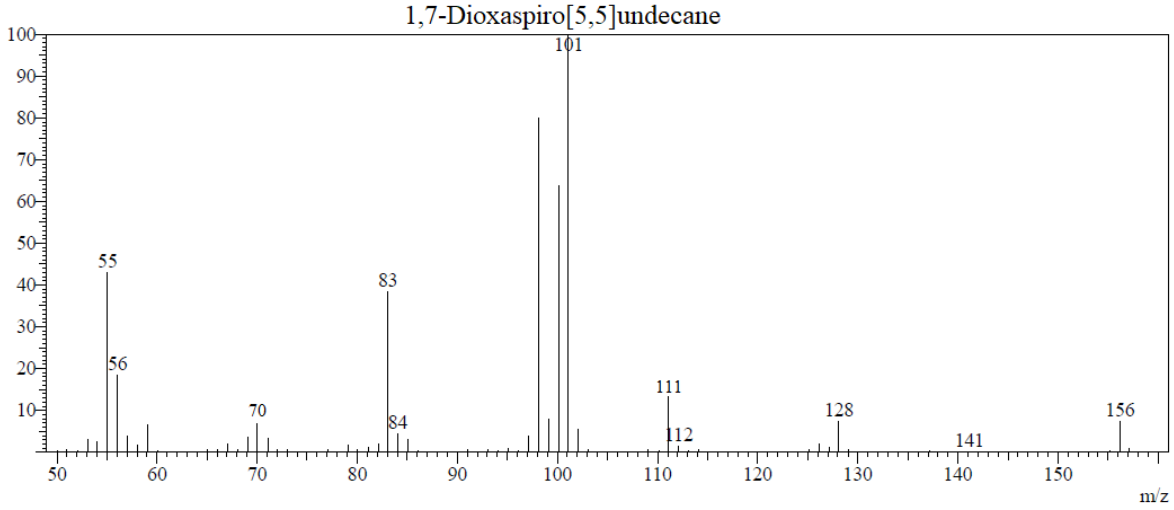
(*E*,*E*)-2-Ethyl-8-methyl-1,7-dioxaspiro[5.5]undecane



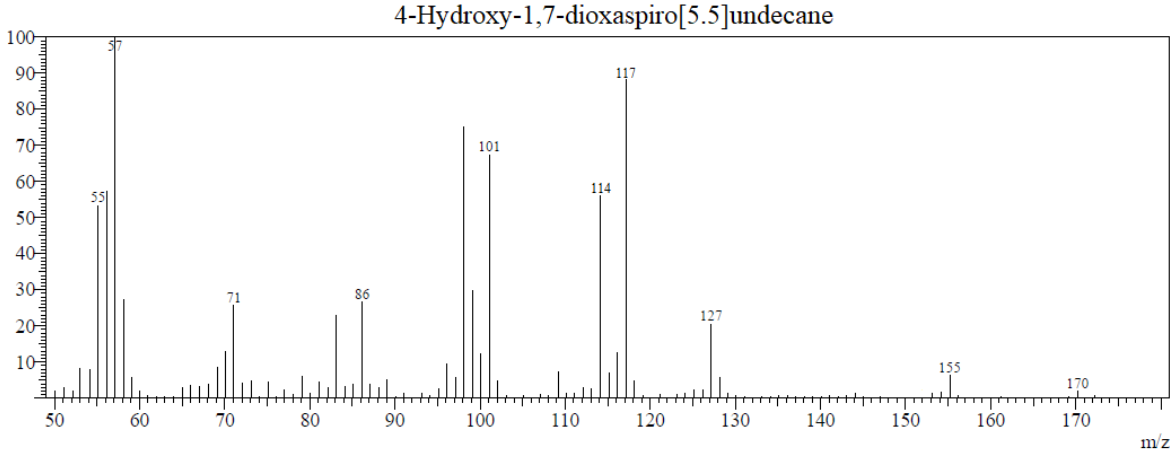
(*E*,*E*)-2-Propyl-8-methyl-1,7-dioxaspiro[5.5]undecane



1,7-Dioxaspiro[5,5]undecane



4-Hydroxy-1,7-dioxaspiro[5.5]undecane



Appendix VII:

List of conference presentations during PhD candidature:

Noushini S., Holgate D., Perez J., Park S. J., Jamie I., Jamie J., Taylor P. (2019) Volatile compounds from *Bactrocer*a fruit flies and correlation with GC-EAD responses. 47th IUPAC World Chemistry Congress (IUPAC 2019). July 7-12, 2019, Palais des Congres, Paris, France.

Noushini S., Perez J., Park S. J., Holgate D., Jamie I., Jamie J., Taylor P. (2019) Glandular secretion and emission profiles of *Bactrocera frauenfeldi* (Schiner): chemical, behavioural and electrophysiological studies. 7th Australian Biology of Tephritid Fruit Flies Conference. May 28-29, 2019, Shepparton, Victoria, australia

Noushini S., Holgate D., Park S. J., Perez J., Jamie I., Jamie J., Taylor P. (2018) Volatile compounds from *Bactrocer*a fruit flies and correlation with GC-EAD responses. Organic 18 (Organic Division Conference of the Royal Australian Chemical Institute). December 2-6. 2018, The University of Western Australia, Perth, Australia.

Noushini S., Perez J., Park S. J., Holgate D., Jamie I., Jamie J., Taylor P. (2018) Volatile Emissions of Fruit Flies as Chemical Lures. 6th Australian Biology of Tephritid Fruit Flies Meeting. March 6-7, 2018, CSIRO Black Mountain, Canberra, Australia.

Noushini S., Holgate D., Park S. J., Perez J., Jamie I., Jamie J., Taylor P. (2017) Identification and synthesis of putative pheromones from *Bactrocera* fruit flies. RACI Natural Products One-Day Symposium. September 22, 2017, Macquarie University, Sydney, Australia.

Noushini S., Holgate D., Park S. J., Perez J., Jamie I., Jamie J., Taylor P. (2017) Development of environmentally benign pheromone-based lures for fruit flies. Women in Science symposium. October 20, 2017, Macquarie University, Sydney, Australia.

Noushini S., Holgate D., Park S. J., Perez J., Jamie I., Jamie J., Taylor P. (2017) Identification and synthesis of putative pheromones from *Bactrocera* Fruit Flies. RACI 2017 Centenary Congress. July 23-28, 2017, Melbourne Convention and Exhibition Centre, Melbourne, Australia.