Semiochemical studies of *Gonipterus sp. 2* (Curculionidae) and pheromone-based mass-trapping of *Nudaurelia clarki* (Saturniidae) in South Africa

By

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Declaration of Authorship

I, Luki-Marié Scheepers, declare that the dissertation, which I hereby submit for the degree of Masters in Science in Chemistry at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed,

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Preface

The forestry industry in South Africa is reliant on non-native *Eucalyptus* and pine trees. These non-native plantations unfortunately also create new environments where insect populations are not naturally controlled by predators. Defoliation due to insects can cause substantial economic loss when the populations of these insects become unnaturally high. Such outbreaks require sustainable and effective management programs. This dissertation focuses on two such insect pests of the South African forestry industry, namely *Gonipterus* species 2 (Coleoptera: Curculionidae) and *Nudaurelia clarki* (Lepidoptera: Saturniidae), that require development of sustainable pest management strategies.

Gonipterus sp. 2 (Coleoptera: Curculionidae) invaded South African *Eucalyptus* plantations in 1916 from Southeastern Australia. Weevil larva and adult stages are exclusive *Eucalyptus* defoliators and can cause considerable economic losses in plantations. *Gonipterus* sp. 2 is one of five unnamed *Gonipterus* species in the *Gonipterus* species complex, but this species is the only unnamed species to have invaded non-native regions, including South Africa, Italy and France. Biological control with the egg parasitoid, *Anaphes nitens* (Hymenoptera: Mymaridae), has been effective to reduce *Gonipterus* sp. 2 populations in the past, but recent outbreaks suggest a decline in its effectivity in South Africa. Alternative pest management practices are needed for the continued population suppression of this devastating weevil pest.

Nudaurelia clarki (Lepidoptera: Saturniidae) is endemic to South Africa where it naturally feeds on an array of native hosts. After the introduction of pine trees in South Africa, this moth underwent host range expansion to become a sporadic pest in pine plantations. Large populations of *N. clarki* larvae feed gregariously on pine needles and cause stunted growth of trees, because they damage the growing branch tips. Many biological control agents have been investigated, including the use of viruses against larvae and chacma baboons and pigs that feed on pupae and adult moths. However, the sporadic nature of the infestations of *N. clarki* cannot warrant such continuous labor-intensive and costly pest management practices. Development of a

monitoring method for *N. clarki* moths is necessary and can facilitate targeted and better timing of insecticide applications.

Pheromones have been used as a tool for integrated pest management strategies for around 60 years. These species-specific natural volatiles can be used to manipulate movement of pests into traps. Pheromones are also used in mass-trapping, mating-disruption and push-and-pull strategies. The development of pheromones for *Gonipterus* sp. 2 and *N. clarki* will provide the opportunity to integrate pheromone-based tools with existing management practices for the most efficient, sustainable management of these plantation pests.

In this dissertation, chapter one and two focusses on reviewing the non-Scolytinae weevil pheromone literature. Chapter three aims to explore and identify potential pheromones for *Gonipterus* sp. 2 in South Africa. Chapter four explores the known pheromone of *Nudaurelia cytherea* for monitoring *N. clarki*. *Nudaurelia cytherea* and *N. clarki* are closely related, however uncertainty with regards to the species identity and its pheromone application for *N. clarki* exists.

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Abbreviations and Symbols

Abbreviations

GC-EAD	Gas chromatography electroantennographic detection
GC-MS	Gas chromatography mass spectrometer
GC-FID	Gas chromatography flame ionization detection
TIC	Total ion chromatogram
NIST	National Institute for Standards and Technology
IPM	Integrated pest management
SPME	Solid phase microextraction
DHS	Dynamic headspace sample/sampling
GLV	Green leaf volatile
DMDS	Dimethyl disulfide
EAG	Electroantennography
FID	Flame ionization detector
RR	Red rubber polyisoprene
PDMS	Polydimethylsiloxane
PE	Polyethylene
COI	Cytochrome oxidase I
KZN	Kwa-ZuluNatal
Rmatch	Reverse match factor

Symbols

I_K	Kovat's retention index
m/z	Mass to charge ratio
SD	Standard deviation
V	Volts
M1A1R1	Male one, first antenna, first replicate
F3A2R2	Female three, second antenna, second replicate

Chapter 1

Studying and applying pheromones of non-Scolytinae Curculionidae: Biology and Methods

Abstract

Pheromone identifications are usually driven by economic losses caused by excessive insect feeding. For the large family of Curculionidae, the number of pests is expected to increase as many factors force these weevils out of their natural habitats into flourishing plantations and crops. Pheromones are increasingly popular for use in pest management strategies independently, or together with existing control measures. Biological differences between Curculionidae species often require species-specific adaptations and optimizations of sampling methodology to successfully identify pheromone components. Correct pheromone identification is paramount to its successful implementation in pest control. Pheromone blends from each species must mimic natural blend ratios and release rates. Beyond this, implementations of pheromones are also species-specific and must be optimized for each pest individually. This review aims to provide an overview of current knowledge on non-Scolytinae Curculionidae pheromones. The broad range of damage caused by these insects in different plant-based industries are summarized, together with the stage of pheromone development for each species. A guideline to pheromone sampling was constructed from previous literature, with examples of case-specific considerations when biological differences between species were encountered. The importance of understanding the chemical ecology in related species to know which factors to consider for pheromone sampling and successful pheromone-based trapping implementation for non-Scolytinae Curculionidae, is also highlighted. These considerations are an essential history-based guide for future pheromone identification and implementation endeavors.

Introduction

Introduction of Curculionidae

Many species in the Curculionidae family are phytophagous as adults or in their larval state. It is due to that reason that they can become economically damaging plant pests that have to be managed (Garnas et al. 2016; Tewari et al. 2014). The exploitation of pheromone communication in Curculionidae aids foresters and farmers in integrative pest management regimes. These interventions can provide a way to sustainably control pest infestations affecting plant-reliant industries. Successful implementation, however, relies on a thorough understanding of each pest's chemical ecology.

The first pheromone identification procedure from a non-Scolytinae Curculionidae

The first pheromone from a non-Scolytinae Curculionidae weevil was isolated and identified by Tumlinson et al. (1969). This pheromone was obtained from the boll weevil, *Anthonomus grandis* Boheman (Tumlinson et al. 1969), a serious pest of cotton in the USA (Francke and Dettner 2005). The isolation process required solvent extraction and distillation of 54.7 kg of frass obtained from 4.5 million weevils of mixed sexes. Samples were fractionated using column chromatography and each fraction was screened for behavioral responses in laboratory bioassays. Structure elucidations of biologically active components were determined with methods including MS, NMR and IR (Tumlinson et al. 1969). The biologically active male-specific pheromone is multicomponent blend that must be applied in a specific ratio to maximize the efficiency of attracting these pests (Tumlinson et al. 1969). Subsequent studies showed that an adjusted blend is equally effective, but cuts synthesis costs in commercial applications (Hedin et al. 1979).

Challenges associated with pheromone control of Curculionidae populations

After the discovery of the boll weevil pheromone, many similar economically important Curculionidae species were investigated to elucidate their pheromones (reviewed in Ambrogi et al. 2009; Hardie and Minks 1999; Tewari et al. 2014). Thus far, pheromones have been identified from 45 non-Scolytinae Curculionidae species

and some of these pheromones are being used in management strategies, as referenced in Figure 2.3 and Supplementary Table 2.1. Despite these successes, challenges with regards to the initial identification of these pheromones and their subsequent implementation in pest control interventions still remain. It may be possible to simplify new pheromone identifications for the Curculionidae family by studying these systems and the lessons that were learned from them.

The process of identifying pheromones for a new weevil species is complex. The process requires a good understanding of the chemical ecology of the studied species. Knowledge of the biology of a new undescribed pest may not be available, which makes the process of predicting when, where and how to sample for pheromones difficult. Researchers thus often depend on knowledge from known systems that may not be applicable to the studied weevil, even if these are closely related (Giblin-Davis et al. 1996; Ramirez-Lucas et al. 1996; Unelius et al. 2013). After the identification of putative pheromone constituents, the next challenge is the correct formulation and presentation of an artificial pheromone signal within pheromone lures. Lures have to release pheromones in the correct blend ratios (Hibbard and Webster 1993; Phillips et al. 1989; Unelius et al. 2013; Zarbin et al. 2003) and release rates (Hallett et al. 1993; Hardee et al. 1974; Piñero and Prokopy 2003) that are biologically active for their target species.

The biology of the weevil being targeted can affect trapping success rates because some weevil species can only be lured when they are sexually mature and responsive to pheromone signals (Booth et al. 1983). The factors affecting interactions between conspecifics need to be identified in field conditions, because *in situ* observations are not always mirrored in field conditions where attraction and repulsion matters (Hedin et al. 1979). In addition, correct placement of traps and the timing of the trapping interventions is crucial (Drmić et al. 2017; Hallett et al. 1993; Piñero and Prokopy 2003; Reddy et al. 2012; Tooke 1953). In some cases, the intervention efficacy can also be dependent on the population density of the pest (Alpizar et al. 2002; Drmić et al. 2017; Oehlschlager et al. 2002).

Aim of review

Pheromones can be useful for managing weevils, especially if used in integrated pest management (IPM) strategies (Larsson 2016; Nadel et al. 2012). However,

successful elucidation of new effective pheromone formulations for species where pheromone blends are unknown remains a major challenge. This review aims to investigate the chemical communication of those weevil species for which pheromones are already known to guide future pheromone-elucidating studies. Furthermore, the review considers the methods that ensured successful pheromone identification and explores the reasons why these procedures were successful for these non-Scolytinae Curculionidae weevils. The Scolytinae subfamily is excluded, as species in the subfamily have biology and pheromone functionality that forms part of a specialized group which have been reviewed elsewhere (Gitau et al. 2013; Wood 1982).

Life cycle and control implications

Why do weevils produce pheromones?

Pheromone communication provides insects with an adaptive advantage (Gitau et al. 2013; Pureswaran et al. 2006). Many adult weevils produce and rely on long-range pheromone signals like aggregation pheromones (Ambrogi et al. 2009; Tewari et al. 2014) and sex pheromones (Tewari et al. 2014). These signals may increase a species' fitness through a number of ways (Gitau et al. 2013). For example, sex pheromones benefit a species by reducing time required to find a mating partner and therefore risk of predation (Pureswaran et al. 2006). Aggregation pheromones also help beetles find suitable food sources and help with overcoming host defenses (Gitau et al. 2013).

The part of the life cycle when a weevil species relies on pheromone communication should be known for successful isolation and identification of the pheromone blend. This requires an understanding of the life cycle in each studied species. Below, a general life-cycle of the non-Scolytinae Curculionidae is discussed, followed by an elaboration on variations on this scheme for different species. Parameters that may impact on pheromone production during the lifecycle and the implications for pheromone-based control measures are also explored.

The General life cycle of Curculionidae

The life cycle of most Curculionidae follow a general scheme that starts with a female that lays fertilized eggs on the host plant or in the soil. Eggs then develop into

larvae and these larvae go through a number of instars before they are ready to pupate. Beetles emerge from pupae that have matured over time either in the host plant or in the soil. In some species, adult weevils have a prepubescent period before mating (Ruiz-Montiel et al. 2009) and in others this does not occur (Giblin-Davis et al. 2000; Kamiya et al. 2015). Once mated, eggs are laid by the female and the cycle repeats.

Variations in the life cycles of Curculionidae

Variations with regards to the life cycle occur between species (Table 1.1). For example, the developmental time required for larvae to emerge from eggs can range from seven days (Wolcott 1936) for the citrus root weevil, *Diaprepes abbreviatus* Linnaeus, to 15 days for the sugar beet weevil, *Bothynoderes punctiventris* Germar (Drmić 2016). The number of larval instars may also differ between species. For example, the *Eucalyptus* snout beetle, *Gonipterus* sp. complex has four larval instars (Tooke 1953), while the citrus root weevil has sixteen larval instars (Wolcott 1936).

The larvae grow until their last instar and form pupae often hidden within the host plant (Alpizar et al. 2002) or in the soil (Mody et al. 1975). Pupae develop faster during periods of warmer climate conditions (Drmić 2016; Lapointe 2000; Santolamazza-Carbone et al. 2006), and the rate of pupal development can be different between species. For example, the larvae of *Curculio caryae* Horn develop to a pharate adult stage in the soil in the first two years, where after development to the adult stage requires another year in the soil (Collins 1996). In contrast, the pupae of *Eucalyptus* snout weevils need between 30 to 40 days to develop to adults (Tooke 1953) (Table 1.1).

Pheromones have been identified from mostly adult Curculionidae weevils (reviewed by Ambrogi et al. 2009; Tewari et al. 2014). It has been established that males generally produce long-range pheromones that function as aggregation signals (Ambrogi et al. 2009; Tewari et al. 2014). These volatiles are of particular importance for control measures that aim to mimic these attraction properties. When females produce volatile pheromones, they are usually short-range sex pheromones (Cork and Lobos 2003; Heath et al. 1986; Ravi and Palaniswami 2002). Non-volatile contact pheromones have also been identified from both adults and larval states (Mody et al.

1975; Mutis et al. 2009). Such studies are few because larval pheromones are not especially suitable for application in known pest management strategies.

Control interventions and how they are affected by the biological variations in different Curculionidae species

Certain life stages of Curculionidae are hidden inside the host and are difficult to target with control interventions (Table 1.1). Hidden pests are often not detected until high levels of damage have been reached (Chambers et al. 1996). For example, the banana pseudostem weevil may live up to 200 days concealed in fallen or rotten banana stems and infestations are only noticed from the damage they cause (Ravi and Palaniswami 2002) (Table 1.1). The larvae of citrus root weevils can cause severe damage to roots in only five weeks (CABI 2019). The damage is also exacerbated through some fungal pathogens (Graham et al. 1996). Both species have larvae that are hidden and these larvae are considered more damaging than the adult beetles. Sensitive pheromone-based monitoring strategies that detect adults may aid in these cases, as hidden larvae cannot be targeted with pheromone control tactics (Chambers et al. 1996; Lapointe et al. 2012; Ravi and Palaniswami 2002).

Weevil species that are exposed during certain life stages are more easily targeted with pest control tactics (see references in Table 1.1).. For example, citrus root and plum weevil larvae crawl on the soil for up to three hours before burrowing into the soil for pupation purposes (Akotsen-Mensah et al. 2012). Control measures were developed to specifically target those larvae (Akotsen-Mensah et al. 2012; Jones and Schroeder 1983). The beetles of the economic important grain-, maize-, bearded- and corkwood weevils move around outside their host plant in search of mating partners (Table 1.1). They are also relatively easy to target and capture with pheromones at this stage (Faustini et al. 1982; Kamiya et al. 2015; Reis et al. 2018; Walgenbach et al. 1983).

Developmental time of different life stages vary between species and this impacts how control measures must be implemented (Table 1.1). For instance, the larvae of the pecan weevil may enter a diapause period that lasts up to two years before they develop into pupae (Tedders and Wood 1994). Adults of this species mate and lay eggs within a relatively short time frame of four to six days and often emerge synchronously to do so (Hall, 2018). This causes huge larval population outbreaks that

occur when eggs hatch a month later. Control measures can be difficult to implement for univoltine weevil species such as these (Simpson et al. 1996), because they emerge in short periods of time, which are often difficult to predict in varying environmental circumstances. Sensitive pheromone-based monitoring strategies have been implemented for such systems with variable success rates (Tedders and Wood 1994).

Beetle species that are polyphagous (Simpson et al. 1996) are relatively more difficult, and subsequently more expensive, to control when compared to those species that feed on single plant species (Budenberg et al. 1993; Rannestad et al. 2011). Polyphagous weevils for which pheromones are known include the citrus root weevil, *Diaprepes abbreviatus*, the plum curculio, *Conotrachelus nenuphar*, the palm weevil, *Rhynchophorus spp.* (Hallett et al. 1993) and the maize weevil, *Sitophilus zeamais* (Hallett et al. 1993; Walgenbach et al. 1983) (Table 1.1). Control of these weevils is difficult because they have multiple hosts that can serve as population reservoirs in the surrounding vegetation (Simpson et al. 1996). Area-wide applications of pheromone-based control measures are usually advised in such circumstances (Drmić et al. 2017; Haney et al. 2009; Reddy et al. 2012). In contrast, weevils that feed on a single host plant species, like the banana weevil *Cosmopolites sordidus*, are simpler to control because only one crop needs to be targeted with control strategies (Budenberg et al. 1993; Rannestad et al. 2011) (Table 1.1).

Using pheromones to reduce the economic impact of Curculionidae weevils

Pest status generally drives pheromone identifications

The damage and subsequent economic loss caused by non-Curculionidae beetles and the inability of existing measures to control the population levels is the main reason why pheromones are sought after (Coulson and Stephen 2006; Nadel et al. 2012). As an example, severe crop damage was caused by the boll weevil due to several years of unsuccessful control measures that were followed by application of systemic insecticides that was banned in subsequent years (Haney et al. 2009; Szmedra et al. 1991). Economic loss in 1950 was estimated at an average of more than $200 million annually between 1892 and 1950 (Anonymous 1950). Once the pheromone was elucidated (Tumlinson et al. 1969), a pheromone trapping and

monitoring system were developed (Hardee et al. 1974; Hedin et al. 1979). The effect of the optimized pheromone-based intervention was evident a few years later, as the overall losses due to boll weevil damage dropped from $8.23 million to 0$, and the average crop production increased from $69 million to $399 million between 1991 to 1995 due to integrating pheromone-based monitoring for timely insecticide applications, followed by direct trapping of surviving adult weevils (Haney et al. 2009). An added benefit was that the number of costly insecticide applications was reduced by 60% (Haney et al. 2009; Szmedra et al. 1991). This example of successful control with pheromones remains a model for other Curculionidae species. The boll weevil pheromone (See Figure 2.3) is currently used in lure-and-kill-, mass-trapping- and monitoring strategies (Ambrogi et al. 2009; Haney et al. 2009; Hardee et al. 1974; Hedin et al. 1979).

Curculionidae (non-Scolytinae) are severe pests worldwide and economically important

An extensive range of agricultural industries suffer economic loss due to damage caused by Curculionidae weevils (see references in Table 1.3). These crops include those in the forestry industry, those in the fruit industry, and those in the grain and stored crops industries (Table 1.3). The way in which weevils induce damage varies among different weevil species. Some life stages feed on phloem or bark (Booth et al. 1983) and others damage the plant roots or foliage (Lapointe et al. 2012). The aim of this section is to identify the type of damage that is caused by non-Scolytinae Curculionidae and how the different species are currently managed through pheromone based pest control techniques (Ambrogi et al. 2009; Tewari et al. 2014).

Damage caused by non-Scolytinae Curculionidae

The type of damage caused by non-Scolytinae Curculionidae weevils varies between species (Figure 1.2, and references in Table 1.1). The type of damage can be used as a proxy to diagnose which species may be present and where or when pheromones are produced. More than three-quarters of the weevils for which pheromones have been described are borers (35 out of 43 species). As a group, the borers are diverse and include wood-borers (14 out of 43 species), and fruit-borers (13 out of 43 species). Pheromones are also known for one defoliator and legume-borer. The rest of the weevils with known pheromones include two ‘borers’, stored

products weevils (n = 3), root-feeders (n = 4) and pseudostem-borers (n = 5) (Figure 1.2). These findings show that a range of industries are affected by non-Scolytinae Curculionidae weevil pest species. It can be extrapolated that each weevil species has a different life style based on its host preference.

Adults in some species of non-Scolytinae Curculionidae have periods where they are exposed and periods where they are hidden (Booth et al. 1983; Giblin-Davis et al. 1996; Illescas-Riquelme et al. 2016; Phillips et al. 1984; Reis et al. 2018). By considering all the studied systems, it became evident that roughly half of non-Scolytinae Curculionidae species for which pheromones have been described are inconspicuous as adults (21 out of 43 species) and another nine species are hidden to some level as adults (Table 1.1, Figure 1.1). More than a quarter (12 out of 43 species) of the Curculionidae for which pheromones have been described are exposed and vulnerable to control measures. This information illustrates the possibility of successful pheromone implementation even to inconspicuous weevils.

Implementation of pheromones for non-Scolytinae Curculionidae pest management

Pheromones identified from non-Scolytinae Curculionidae weevils are in various stages of implementation in management strategies (Table 1.3). Roughly a quarter of all the studies on pheromones are still in the research phase, with different management tactics as focus (Figure 1.3). Most reported research is focused on developing pheromones for application as a mass-trapping strategy (Abdel-Azim et al. 2017; Illescas-Riquelme et al. 2016; Marques et al. 2011). Other research is focused on optimizing pheromone use in monitoring (Silva et al. 2018), as well as lure-and-infect strategies (Booth et al. 1983) (Figure 1.3, right). Only one research article has a focus on developing the pheromone as both mass-trapping and monitoring tool (Reis et al. 2018) (Figure 1.3, right). There are also studies that have integrated each respective pheromone successfully with other management strategies. Pheromone monitoring strategies are often applied together with insecticides (Blight and Wadhams 1987; Palanichamy et al. 2011; Phillips et al. 1989) (Figure 1.3, left). Lure-and-infect implementations are more common in recent literature (Lapointe et al. 2012; Padilla-Cubas et al. 2010). Mass-trapping has also been a favored pheromone-based pest management tactic in commercial applications against non-Scolytinae Curculionidae

pests, at least according to published literature (Abdel-Azim et al. 2017; Illescas-Riquelme et al. 2016; Marques et al. 2011) (Table 1.3).

Curculionidae weevils as vectors

Pheromone-based pest control has been essential in integrated management regimes for a few non-Scolytinae Curculionidae that vector important plant pathogens and secondary pests (Table 1.3). Palm weevils (Phillips et al. 1984) vector the *Bursaphelenchus cocophilis* Cobb. nematode which causes red ring disease in palm trees (Alpizar et al. 2002; Giblin-Davis et al. 1997; Oehlschlager et al. 2002). Palms can die within two to four months after being infected with the nematode (Giblin-Davis et al. 1996). In another system, a gram positive bacteria, *Pseudomonas fluorescens,* causes petrification of the agave plant, and may also be associated with agave weevils as it has been isolated from the body surface of these weevils (Ruiz-Montiel et al. 2008; Ruiz-Montiel et al. 2003). Citrus roots have also been shown to be more susceptible to *Phytophthora* infections due to galleries created in the roots by *Diaprepes abbreviatus* larvae (Graham et al. 1996). The damage caused by these secondary opportunistic pathogens has been so extensive that a name has been given to this problem: the *Diaprepes*-P*hytophthora* complex (Graham et al. 2003). In these scenarios it is more cost-effective to control the weevil vectors with the objective of preventing, rather than controlling, the diseases that they transfer to their hosts.

The Pheromone identification process

Pheromone production and isolation success

Adult weevils must be in the correct developmental stage for pheromone production to occur. Knowing the age of the beetles and if they are virgin or not, are therefore some of the most important factors to consider for success (Azuara-Domínguez et al. 2013; Giblin-Davis et al. 2000). This type of information is not always available especially when pheromones are sought for unstudied species. Weevils with unknown physiological states and ages are therefor often collected from field sites (Oehlschlager et al. 1995). Collecting beetles at different developmental stages or in large cohorts may improve the likelihood of successfully having some produce pheromone during the sampling process. If these procedures are not successful, beetles may have to be reared in captivity in order to know their exact ages and if they are mated or not (Ehounou and Ouali-N'goran 2018). However, development of

laboratory rearing techniques can be time-consuming and are not always successful (Ambrogi et al. 2012). Methods that were used in previous studies can be valuable for studies on species where the pheromone is still unknown.

Pheromone production and mating status or life cycle

Sampling virgin weevils may increase the probability of pheromone collection for some weevil species. Virgin male pecan weevils produce larger amounts of pheromone than mate males when they mount virgin females and the pheromone was sampled during this behavior (Hedin et al. 1997). In contrast, both the mated and virgin male maize weevils produce the same amount of pheromone (Walgenbach et al. 1983). Virgin weevils were sampled for pheromone in red pine weevils (Booth et al. 1983), plum weevils (Eller and Bartelt 1996) and New Guinea sugarcane weevils (Giblin-Davis et al. 2000) (Figure 1.4). In other studies, mating status was not considered, as only field collected individuals of unknown sex and age were sampled (Figure 1.4). These include banana weevils (Budenberg et al. 1993), cactus weevils (Tafoya et al. 2004) and cranberry weevils (Szendrei et al. 2011). These examples of successful pheromone isolations illustrate the variation in timing of pheromone production in different species.

Behavior guides pheromone sampling

Success of sampling for specific pheromones can be improved if weevil behavior can be associated with pheromone production (Patrock 1986; Roseland et al. 1990). For instance, pheromone isolations from guava weevil males were improved through observations of the behavior associated with pheromone production occurring during the night (Palacio-Cortés et al. 2015; Zarbin et al. 2007). Other behaviors that potentially suggest the utilization of pheromones by certain species have been observed in the field. For example, it was observed that male red sunflower seed weevils, *Smicronyx fulvus* LeConte, were always present on sunflowers before females. This suggested that volatile chemicals possibly lure females and that these chemicals either were released by the males themselves or could have originated from the plant (Roseland et al. 1990). Some odd behavior was described for *Rhyncophorus cruentatus* in the late 1920's, where beetles aggregated during the day on newly painted cars (Giblin-Davis et al. 1996). The paint apparently contained constituents

similar to the natural components within the weevil pheromone (Giblin-Davis et al. 1996) (see Figure 2.3 for pheromone components).

For some weevil species, the presence of a pheromone can be confirmed through behaviors that include repetitive movements or possibly the approach angle of a lured mate (Collins 1996; Kamiya et al. 2015; Ravi and Palaniswami 2002; Zarbin et al. 2007). For example, *Odoiporus longicollis* females have a calling behavior that can be identified when females expose their ovipositors, when they rub them on a surface and when they knock on the substrate with their rostrum (Ravi and Palaniswami 2002). Conspecific males exhibited courtship behavior that was identified by males lifting their heads and extending their antennae followed by rapid movement towards the calling female. Males then probed females with their antennae and mounted the females (Ravi and Palaniswami 2002). These behaviors are generally only seen for weevils that produce contact or short-range pheromones (Mutis et al. 2009; Ravi and Palaniswami 2002).

There are examples where pheromones of non-Scolytinae weevils were successfully isolated and identified even though behavior was not studied beforehand (Oehlschlager et al. 1995; Palacio-Cortés et al. 2015). In these studies, single chromatographic peaks were found to elicit antennal responses during GC-EAD recordings when dynamic headspace extracts of sexed field collected *Rhynchophorus bilineatus* (Oehlschlager et al. 1995) and *Conotrachelus psidii* weevils (Palacio-Cortés et al. 2015) were collected and analyzed. Behavioral assays then confirmed attraction to 4-methyl-5-nonanol and papayanol in each species. Such cases are, however, not the general rule for success.

Many successful studies start with behavioral studies in controlled environments before the successful identification of sex- or aggregation pheromones (see references in Table 1.2). Examples include *Sitophilus granarius, Cyrtomon luridus* and *Odoiporus longicollis* weevil species (Faustini et al. 1982; Kamiya et al. 2015; Ravi and Palaniswami 2002). Their behaviors were investigated prior to pheromone isolation through pitfall bioassays using filter papers saturated with volatiles from fed, sexed conspecifics (Faustini et al. 1982), descriptive analysis of post-emergence mating behavior (Hedin et al. 1997; Kamiya et al. 2015) and y-tube olfactometers (Marques et al. 2011; Reis et al. 2018; Zarbin et al. 2007).

Different time intervals are needed for different species to respond to pheromones from their conspecifics. This is an important consideration for behavioral bioassays. In some cases, responses that are indicative of pheromone production occur within 5 minutes of behavioral testing (Sun et al. 2010) whereas in other cases weevils may respond to pheromones only after 15-30 minutes (Faustini et al. 1982; Marques et al. 2011). This emphasizes the importance of observations that can form part of a trial-and-error process simply because beetles do not always produce or respond to pheromones (Barnes and Capatos 1989). The challenge is that the correct behavioral response associated with a pheromone is usually unknown and needs to be observed and defined (Azuara-Domínguez et al. 2013). It is often difficult to be sure that pheromones are being produced and detected by the weevils even though they are observed in carefully controlled environments. The environment, for example, may cause behaviors like movement out of a saturated plot toward a trap, that can be confused with pheromone production of live weevils in traps (Piñero et al. 2001).

Examples of sampling explorations for pheromones of weevils

Components of pheromone blends may be produced at different localities on or in the weevil body. Many studies employed exploration of macerated body parts to determine where pheromones are produced (Budenberg et al. 1993; Chambers et al. 1996) (Table 1.2, Supplementary Table 1.1). From these investigations, it was concluded that the grain weevil does not have a clear storage compartment for its pheromone, but it is possibly produced near the abdomen or thorax (Chambers et al. 1996). In another study, the male banana weevil hindgut was found to be attractive to female conspecifics, where major pheromone components were found. However, the minor pheromone components could only be recovered from other body parts of the male (Budenberg et al. 1993). The site of pheromone production of different pheromone constituents is thus not always in the same site on/in a weevil.

Stimulant sources may be needed to induce pheromone production in weevils (Table 1.2). For example, the red pine weevil was investigated for pheromone presence by sampling feeding individuals, multiple times throughout the year (Booth et al. 1983). Pheromones were successfully isolated from headspace aerations of the hindguts and abdomen of these weevils, but only during spring and summer seasons when females were sexually mature (Booth et al. 1983). The food source may also be the stimulus necessary for pheromone production, as in the case of the guava- (Zarbin

et al. 2007) and agave weevils (Ruiz-Montiel et al. 2003). Other weevil species, however, do not need food as a stimulant to produce pheromones (Lapointe et al. 2012; Marques et al. 2011; Ravi and Palaniswami 2002; Tumlinson et al. 1969).

Sometimes it is unknown why individuals do not produce pheromones. For example, a comprehensive study was performed to investigate why agave weevil males of the same mating-, feeding and age status produced pheromone under the same light conditions, but others do not (Ruiz-Montiel et al. 2009). No conclusive evidence was found for this phenomenon. As such, it can be expected that a fraction of any population of weevils would not produce pheromone, even though conditions may be favorable. In these cases, it may be advantageous to sample a large group of weevils because it would maximize the chances of encountering individuals that release pheromone (Booth et al. 1983; Budenberg et al. 1993; Giblin-Davis et al. 2000; Oehlschlager et al. 1995; Perez et al. 1997; Unelius et al. 2013) (Table 1.2).

Pheromone sampling techniques

Sampling techniques should be chosen carefully to ensure that the true compound ratios are accurately represented in the sample that is analyzed. Sampling groups of male conspecifics in containers may lead to inaccurate estimated ratios of pheromone components (Hibbard and Webster 1993). This may be due to changes in pheromone component release rates in containers that saturate with the pheromone (Phillips et al. 1989) and it may cause confusion or aggressive behavior of the weevils (Ravi and Palaniswami 2002). It is thus important to consider the advantages and disadvantages of different techniques (Brezolin et al. 2018; Millar and Haynes 1998).

There are three main sampling methods that are employed for sampling pheromones from Curculionidae, namely dynamic and static headspace sampling, as well as solvent extraction directly from the insect tissues (Table 1.2). Dynamic headspace samples of insects may be obtained by passing a constant purified airflow through a sampling vessel and subsequently trapping the volatiles on a variety of sorbent materials (Table 1.2).

Static headspace samples can be obtained by sampling with a solid phase microextraction (SPME) fiber (Romero-Frías et al. 2016; Ruiz-Montiel et al. 2009) or from extracting absorbents with solvents placed within rearing chambers (Faustini et al. 1982). In contrast to dynamic headspace sampling, no air is pumped through these

chambers. Instead, weevils are left in the sealed environment for a time interval. Samples are obtained by exposing the solid sorbent of choice to the accumulated volatiles in the chamber. The surfaces of rearing chambers or the chambers used in static sampling can also be washed with solvents to obtain samples (Hedin et al. 1997; Szendrei et al. 2011).

Some studies obtained solvent extract samples from either whole insects (Collins 1996; Tumlinson et al. 1969; Yang et al. 2017) or just the body surface (Beauhaire et al. 1995; Giblin-Davis et al. 2000). Solvent extracts of specific tissues such as the hindguts, abdomen or frass are also common (Budenberg et al. 1993; Giblin-Davis et al. 2000; Lapointe et al. 2012) and aim to target the sites where pheromones are produced or stored (Booth et al. 1983).

Screening samples with EAG and GC-EAD

The presence of a pheromone within samples can be assessed through electrophysiological techniques (Supplementary Table 1.1, Figure 1.5), that determine which compounds are perceived by an antenna. Because the compounds may cause repulsive, attractive or no behavior at all, electroantennography is always accompanied by behavioral studies to confirm the attractive nature of putative pheromone constituents.

Electroantennography (EAG) involves the measurement of the voltage depolarization of an antenna due to the neural perception of a volatile stimulant. Unpurified or semi-purified extracts, may be puffed over an insect antenna during electroantennography (EAG) and those samples that do not give notable responses when compared to suitable blanks can be eliminated as a possible source of a pheromone (Schneider 1957). Further investigation of active samples through EAG coupled to a GC (GC-EAD) can provide evidence for electrophysiological activity of specific chromatographic fractions (Struble and Arn 1984). This technique is particularly difficult for Curculionidae beetles because antennal preparations can give noisy signals. True responses are therefore difficult to detect.

Different antennal preparation methods have been explored to reduce noise in signals. The recording electrode is generally attached to the club-shaped terminal tip of the antenna, and a grounding electrode is attached to the base of the antenna. In some studies, authors pierce the antennal tip with the recording electrode (Chambers

et al. 1996; Giblin-Davis et al. 1997) and in others they simply touch the recording electrode to the tip of the antenna (Van Tol and Visser 2002). Sometimes it is required to remove the distal part of the flagellum to expose the neurons in the antenna to improve the signal to noise ratio (Unelius et al. 2013). The antennae can be cut near the scape (Giblin-Davis et al. 1997) or pedicel (Budenberg et al. 1993) or the grounding electrode can be coupled by inserting it through the ventral surface of the insect head (Chambers et al. 1996; Unelius et al. 2013). Furthermore, signal-to-noise ratios can be enhanced through analysis algorithms (Slone and Sullivan 2007).

From 36 articles that mentioned what screening methodologies were used, 12 screened for pheromone presence with the GC-EAD method (Supplementary Table 1.1, Figure 1.5). Five other articles utilized the insect antenna in other ways (Blight et al. 1984; Chambers et al. 1996; Park et al. 2013; Unelius et al. 2013; Van Tol and Visser 2002). These methods included offline EAG of collected GC fractions, EAG or single sensillum recordings (SSR) of whole samples. The offline EAG method involved semi-purification of synthetic diastereomers with preparative GC before puffing impregnated filter-paper air over individual antennae (Blight et al. 1984; Chambers et al. 1996).

Screening for pheromone presence is not limited to electroantennography, and can also be done through behavioral trials. Screening for attraction behavior was another way in which pheromone presence was determined in seven other articles. These studies required well-described behavior (Jaffé et al. 1993; Kamiya et al. 2015; Marques et al. 2011; Phillips and Burkholder 1981; Ravi and Palaniswami 2002), which could also be determined through explorations in field attraction tests (Blight et al. 1984; Booth et al. 1983). After determining that pheromones are present in a sample, these active components require identification.

Compound identification

Accurate compound identification is of paramount importance to trap weevils successfully in pheromone-baited traps. The compound identifications from previous pheromone studies do not follow a set procedure, but are rather quite varied in approach (Figure 1.6). Tentative identification of candidate pheromone components can be assigned using gas chromatography coupled to mass spectrometry (GC-MS) (Supplementary Table 1.1). Mass spectra associated with those chromatographic

fractions that induce nerve impulses on the antenna can be compared to thousands of known mass spectra on the NIST, WILEY or other databases (Hedin et al. 1997; Romero-Frías et al. 2016). Comparison of retention indexes on different polarity columns together with matching mass spectra from standard reference compounds can be used to assign tentative identities to unknown compounds (Branco et al. 2019; Giblin-Davis et al. 2000; Hedin et al. 1997; Palacio-Cortés et al. 2015; Perez et al. 1997; Szczerbowski et al. 2016; Szendrei et al. 2011). Almost all pheromone identifications of non-Scolytinae Curculionidae weevils included these important methods (Supplementary Table 1.1, Figure 1.6).

It is possible that a compound may be novel, and therefore not present in the mass spectral databases. In these cases a large amount [micro- to milligrams] of the purified compound is needed for structural elucidation through nuclear magnetic resonance (NMR) (Booth et al. 1983; Eller and Bartelt 1996; Eller et al. 1994; Lapointe et al. 2012; Reis et al. 2018; Romero-Frías et al. 2016; Zarbin et al. 2010) (Figure 1.6). Large numbers of beetles may be required to isolate enough material and the active compound needs to be separated from the sample matrix through column chromatography (Tumlinson et al. 1969). This process has its own set of challenges because each collected fraction needs to be screened for the presence of the active compound.

Techniques like high resolution mass spectrometry can be used to assign an empirical formula to unknown compounds if amounts of purified extract material is insufficient for NMR analysis (Lapointe et al. 2012). This requires that the molecular ion is detectable (Collins 1996; Tumlinson et al. 1969; Zarbin et al. 2003). A match of the retention indexes and mass spectra of reference standards to components in extracts can serve as compound identification (Branco et al. 2019; Giblin-Davis et al. 2000; Hedin et al. 1997; Palacio-Cortés et al. 2015; Perez et al. 1997; Szczerbowski et al. 2016; Szendrei et al. 2011) (Supplementary Table 1.1, Figure 1.6). Valuable information on the presence, location and orientation of double bonds can be gained through chemical modification of the target analyte through a series of micro-reactions can, for example (Phillips et al. 1989; Unelius et al. 2013). The presence of certain functional groups such as esters, aldehydes or alcohols can also be determined through modification reactions. If the molecule can be purified sufficiently, infrared

spectroscopy (IR) can be used for the same purpose (Booth et al. 1983; Eller et al. 1994; Reis et al. 2018; Tumlinson et al. 1969) (Supplementary Table 1.1, Figure 1.6).

Confirmation studies for verification of the bioactive component/blend

Electrophysiological confirmation of tentative compound identities

Preliminary confirmation of electrophysiological results with the standard compounds can function as an easy bioassay to eliminate incorrectly assigned chemical structures. Electrophysiology-based confirmation methods include puffing standards over an antenna in the EAG method (Azuara-Domínguez et al. 2013), or confirming responses to separated standards in the GC-EAD method (Branco et al. 2019; Ruiz-Montiel et al. 2008). After these electrophysiologically-based screening procedures, the compound identity can be confirmed based on the same retention index values and mass spectral profiles as was determined from experimental samples (Branco et al. 2019; Phillips et al. 1989; Szczerbowski et al. 2016) (Supplementary Table 1.1, Figure 1.6). Results can also be confirmed on different polarity columns (Eller and Bartelt 1996). A tentative identity is confirmed once results are similar for standards and experimental samples.

Behavior confirmation of tentative compound identities

The behavioral function of the candidate pheromone constituents and the ratios between components require investigation in laboratory bioassays and field trials (Supplementary Table 1.1, Figure 1.7). Ratios of compounds within pheromone blends play an important role to define specificity for species. These ratios can be explored initially through quantitative analysis of GC samples (Szendrei et al. 2011), but the ultimate verification for a pheromone blend is only proven when the correct behavior is observed to a specific blend in field experiments (Supplementary Table 1.1, Figure 1.7). Some pheromones attract, and others repel insects (Ambrogi et al. 2012; Branco et al. 2019) and these bio-assays help assign the possible biological function of the identified pheromone (Supplementary Table 1.1, Figure 1.7).

Laboratory-based bioassays may be preferred before field trials because field trials can be more expensive to implement (Hedin et al. 1979). Uncontrolled variables in the field may also confound results or lead to incorrect conclusions, such as high capture rates being attributed to lure concentration, instead of higher temperatures

affecting lure release rates, subsequently reducing dispenser longevity (Heuskin et al. 2011). Uncontrolled factors may include fluctuations in effectivity of different trap types, and fluctuations in weather and population densities of insect pests that are difficult to predict (Heuskin et al. 2011). A trend was observed from the number of previous studies on non-Scolytinae Curculionidae pheromone implementations, being a focus of research on field confirmations rather than laboratory-based assays (Figure 1.7). Designs of traps can only be optimized after the pheromone was shown to be effective (Abdel-Azim et al. 2017; El-Shafie and Faleiro 2017; Giblin-Davis et al. 1996; Reddy et al. 2011; Reddy et al. 2012).

Conclusions

In this review, it is apparent that the diversity of non-Scolytinae Curculionidae species are reflected in the different approaches taken to identify their pheromones. From 45 previous pheromone identifications, sampling, screening and chemical structure- and behavioral confirmation methods did not follow a specified sequence. Rather, pheromone development approaches for the different species were achieved species-specifically, often in an exploratory manner, especially when a pheromone consisted of more than one constituent.

Where pheromone isolations were not successful initially, the knowledge of the life history of species were vital in understanding how, when and where to sample for pheromones. These life history parameters included knowledge of a species' life cycle (i.e. whether a species undergoes sexual maturation or diapause, if the species is multi- or univoltine, diurnal or nocturnal activity), behavioral tendencies (with different numbers of conspecifics, stages of starvation and sexual orientation) and whether the species shares pheromone constituents with close taxonomic relatives.

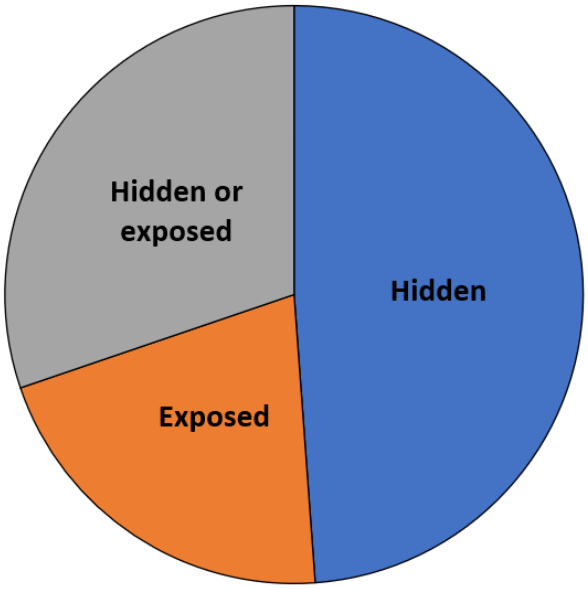
Pheromones are important management tools that enable industries to control pests in an environmentally friendly manner, because they are species-specific and can reduce the use of insecticides in IPM programs (Larsson 2016; Nadel et al. 2012). The number of globally emerging pests are increasing due to global trade (Coulson and Stephen 2006; Hurley et al. 2017; Wingfield et al. 2008), therefore pheromone development is crucial to provide integrable pest management tactics to use in conjunction with existing and novel control measures. This review highlights the necessity of the multifaceted field of chemical ecology in pheromone development and

provides guidelines for identification of pheromones from novel non-Scolytinae Curculionidae pests.

Figure captions

- **Figure 1.1:** The proportion of non-Scolytinae Curculionidae for which pheromones have been described that are exposed during their whole adult life stage, hidden in the soil or their host during their whole adult life stage, or hidden during intervals of the adult life stage in the soil or host with exposure during some periods (n = 43, see references in Table 1.1).
- **Figure 1.2:** The types of damage caused by non-Scolytinae Curculionidae weevils for which pheromones have been described (n = 43, see references in Table 1.3). Borers represent 82% of all the species with known pheromone, and are subdivided into groups including wood-, fruit-, legume-, pseudostem borers and a catch-all term of 'borers' for weevils that feed on miscellaneous host tissues.
- **Figure 1.3:** Development of pheromone-based pest management tactics for non-Scolytinae Curculionidae pests for which pheromones have been described (n = 43, Table 1.3). Integrated pest management (IPM) methods imply that pheromones are used together with insecticide or biocontrol. 'Unknown': studies where pheromones communication has been proven, but pheromones have not been reported as successful attractants in field trials. Mass-trapping implies effective capture and control with only pheromone-baited traps without reported necessity of using other control mechanisms. Studies were classified as 'Research' if studies did not mention current application of pheromone traps in commercial plots.
- **Figure 1.4:** The proportion of non-Scolytinae Curculionidae that were field-collected or reared for pheromone sampling, and their reported mating statuses during sampling (n = 38, Supplementary Table 1.1 and 1.2).
- **Figure 1.5:** Laboratory screening methods used during pheromone identification from non-Scolytinae Curculionidae before testing for attractiveness of pheromones in field conditions (n = 36, Supplementary Table 1.1 and 1.2). Studies were classified under 'None' if synthesized pheromones were tested only in field trials without report of laboratory screening for conspecific attraction.
- **Figure 1.6:** Methods used for verification of pheromone structural identities from non-Scolytinae Curculionidae (n = 35, Supplementary Table 1.1 and 1.2). Numbers represent the number of studies that utilized a specific range of methods and letters a-p refer to the same letters in Supplementary Table 1.1, for respective references.
- **Figure 1.7:** The proportion of pheromone attraction confirmation studies for non-Scolytinae Curculionidae that took place in the field or laboratory or both (n = 38, Supplementary Table 1.1 and 1.2).

Figures



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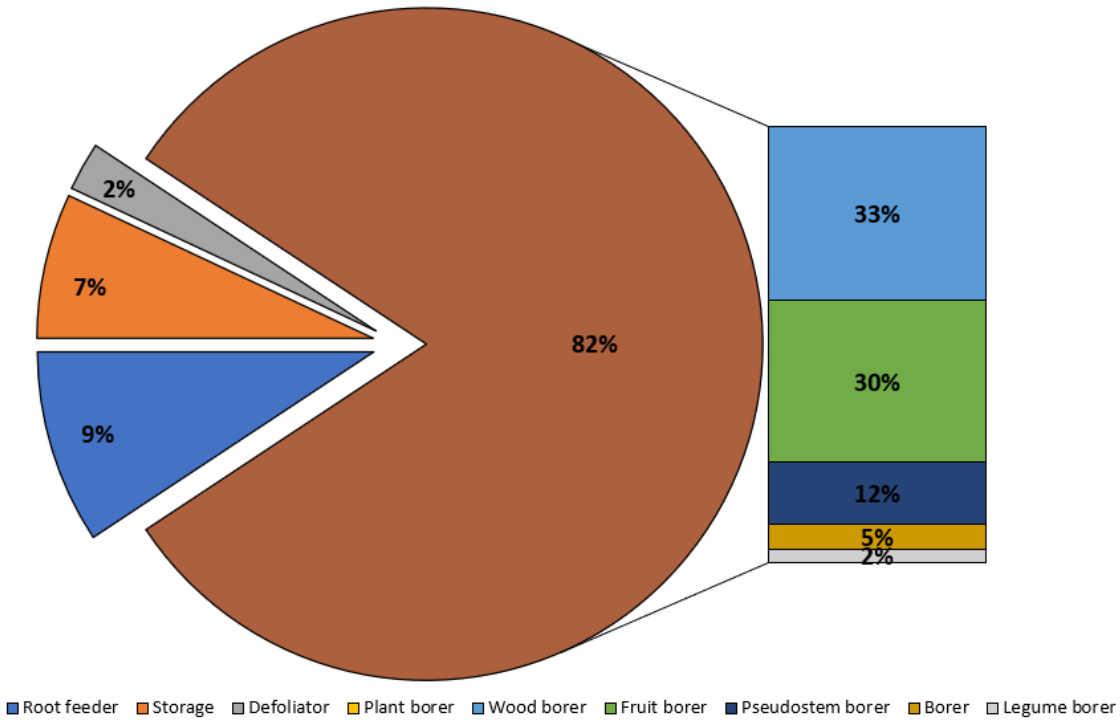

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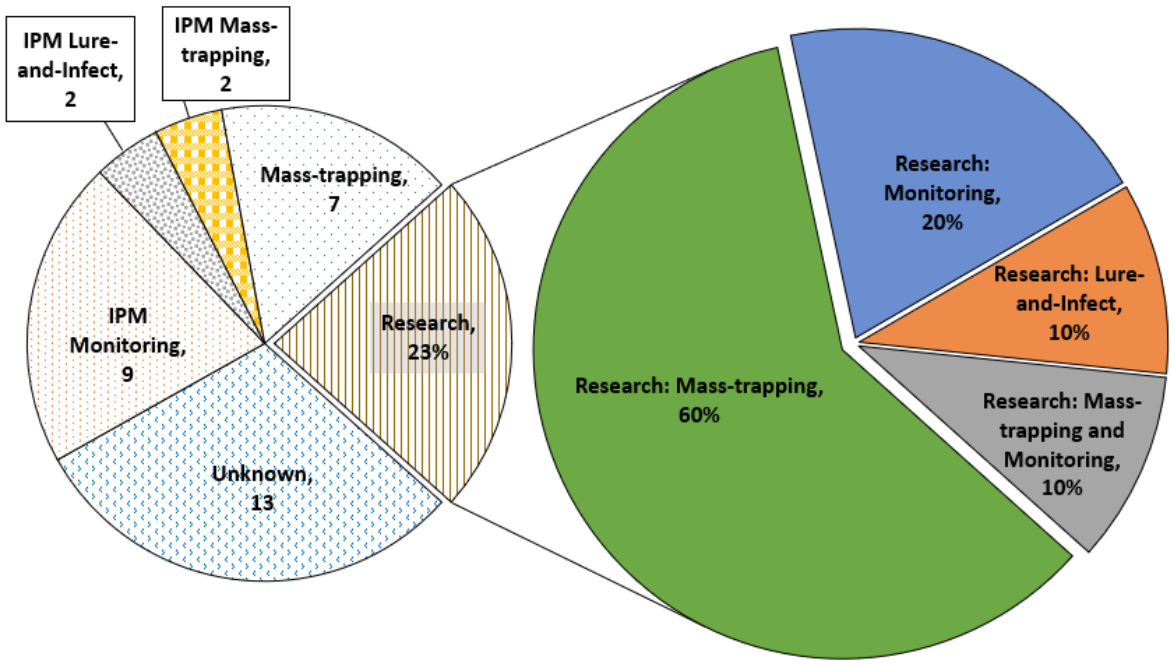

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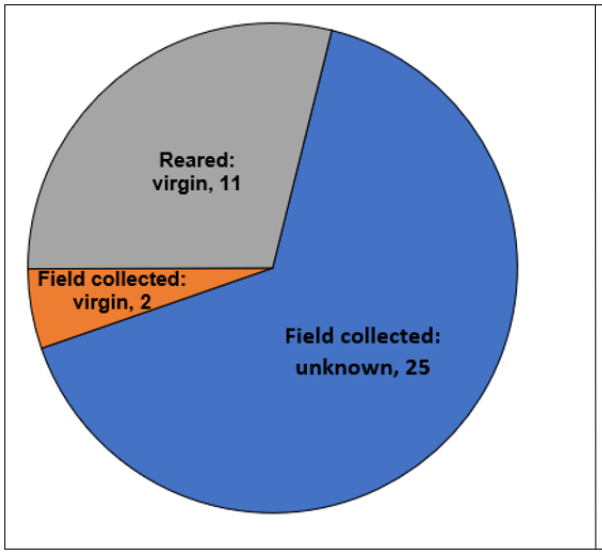

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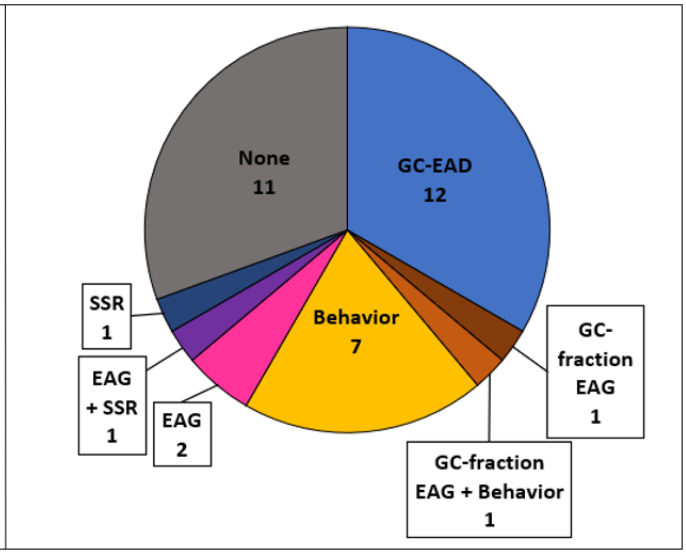

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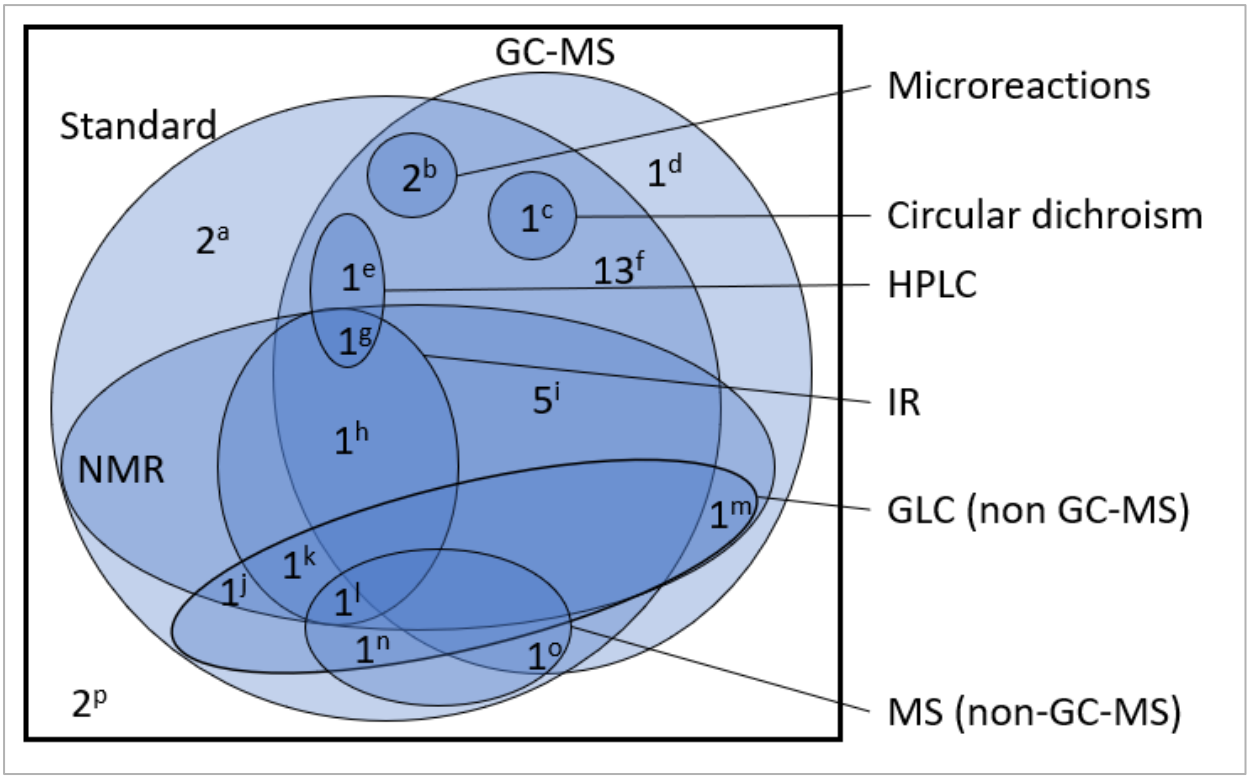

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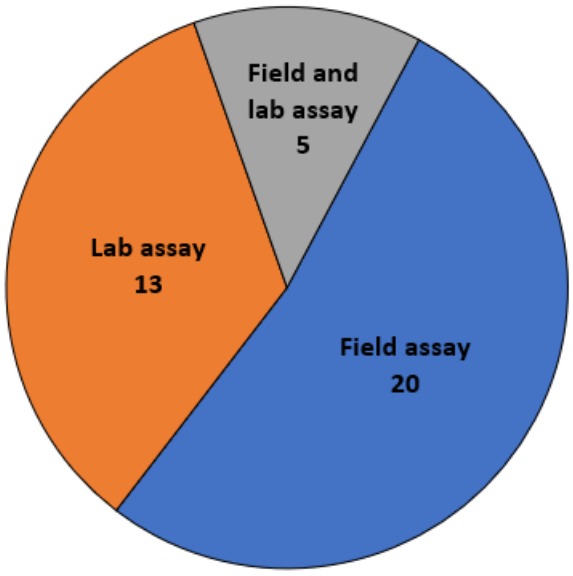

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Table captions

- **Table 1.1:** Differences in life cycles of different non-Scolytinae Curculionidae weevil species.
- **Table 1.2:** Sampling methods and procedures followed for the identification of pheromone components of non-Scolytinae Curculionidae weevils.
- **Table 1.3:** The economic impacts of non-Scolytinae Curculionidae weevil species for which pheromones have been investigated as of 2019.

Supplementary table captions

- **Supplementary Table 1.1:** The range of sampling, screening and identification methods from non-Scolytinae Curculionidae weevils for which pheromones have been identified.
- **Supplementary Table 1.2:** Visualized data from Supplementary Table 1.1 used in Figures 1.4 to 1.7.

Tables

Table 1.1: Differences in life cycles of different non-Scolytinae Curculionidae weevil species. Excel spreadsheet attached in separate document ("Table 1.1").

Curculionid		in/on host/ soil	Host	Industry	Damaging life stages		Life cycle												General notes	References
							Adult				Egg			Larvae						
Latin name	Common name						Size	Mating season	Time after emergence pheromone produced	Feed on	Amount	Where	Time untill hatch	Max size	Larval instars	Feed on	Pupae			
Anthonomus eugenii Cano	Pepper weevil	on	Sweet potato and hot peppers	Fruit	larvae	adults	2-3.5 mm	Overwinter only in Florida, Texas and California	within 2 days after mating; 15 days max, then reduced pheromone production	fruit, also transmits internal pepper mold	200-600 eggs/ life span; 5-7 eggs per day; multivoltine (3-8 generations per year)	in pepper	3-5 days	2.2-5 mm	3	pepper insides	pepper insides	2.5 - 3 weeks to complete life cycle	Eller et al., 1994 & 2014; Capinera et al., 2014
Anthonomus grandis Boheman	Boll weevil	on	Cotton plant/boll plant		larvae	adults	3.2-12.7 mm	-	0-9days after emergence, diminish afterward	Cotton bolls	up to 200 eggs/ life span; multivoltine (6-7 generations per year)	in boll of cotton	2.5-5 days	up to 9.5 mm	3	cotton boll	cotton boll	'overall cycle 16-18 days (up to 3 weeks)	Spurgeon 2003 (look under Agave weevil, Methods summary)
Anthonomus musculus Say	Cranberry weevil	on	Cranberry, Blueberry flowers	Fruit	larvae	adults	1.58-2.54 mm	Spring and summer	Life span: ± 13 months	-	50< eggs/ life span; singly in flower; univoltine	between petioles of unopened, developing flower buds	-	± 2.82 mm	-	flower buds for 7 to 14 days	Under debris and fallen leaves for 4-6 days	-	Szendrei et al., 2011
Anthonomus rubi Herbst	Strawberry blossom weevil	on	Strawberry and raspberry	Fruit	larvae	adults	2-4 mm	-	-	leaves and flower petals for 2 weeks after emergence; then into diapause in July/Aug	up to 50 eggs/ life span, singly in flower bud; univoltine	in unopened flower buds	7 days	-	-	inside bud for up to 2 weeks	develop for 2 weeks	'overall cycle 6- 8 weeks; adults feed 2 weeks then diapausal for winter	Innocenzi et al., 2001
Conotrachelus humeropictus Fiedler	Cocoa borer weevil"	in	Stem and Cocoa fruit	Fruit	larvae	adults (microorganisms)	-	-	-	leaves, floral buds, soft shoots	55-153 eggs/ life span during 80.50 ± 5.58 days; with 1.29 ± 0.03 eggs/day on average	in fruit	4-6 days	-	4	Cocoa fruit: up to 33 larvae per fruit; fourth instar remains in soil for 2-3 months	in soil: period of 20.25 ± 1.50 days overall; 6,07 ± 0,06 days (lava to pre-pupa) and 9,62 ±0,10 days (pre-pupa to pupa); 4,56 ±0,11 days (pupa to	'overall cycle 79 - 151 days	Szczerbowsk et al., 2016; Mendes et al., 1997
Conotrachelus nenuphar Herbst	Plum weevil	in	plums (preferred) stone and Pome fruits/ peaches/ apples/cherries	Fruit	larvae	adults	5-6.35 mm	Spring	-	Plums, peaches, apples	-	Under the fruit skin	7 days	6.35-9.52 mm	4	Fruit for 15-18 days	in soil: 2.5-5 cm under soil surface for 3-5 weeks	'overall cycle 50 - 55 days	Eller et al., 1996
Conotrachelus psidii Marshall	Guava weevil"	in	Gauva fruit	Fruit	larvae	adults	6 mm	-	Life span: 148.0 ± 89.00 days (males and females)	petioles, peducles (stalks), floral buds	539 - 793 eggs/life span; bivoltine	guava fruit	3.9 ± 0.58 days	-	4	guava fruit during 16.0 ± 3.80 days	in soil; burrow after 24 to 48 hours on sand; develop for 16.0 ± 3.80 days (pre-pupa to pupa), and develop further for 16.0 ± 0.90 days (pupa to adult); adult stayed underground for further 34.0 ± 18.00	-	Palacio-Cortez et al., 2015; Bailez al., 2003
Cosmopolites sordidus Germar	Banana weevil (=Rhizome weevil)"	in	Banana plant	Fruit	larvae	-	11-12 mm	-	rarely fly, free living; Life span up to 2 years	Rotting banana tissue	1-3 eggs per week	in rhizome (root) or between leaf sheaths and stems	5-7 days	± 1.85 mm	5-8	rhizome(roots) in plant; 15-20 days development	in banana pseudostem; 6-8 days development	overall cycle 30 - 40 days	Budenberg et al., 1993; Woodruff et al., 2018
Odoiporus longicollis Olivier	Banana pseudostem weevil	in	Banana plant	Fruit	larvae	adults	23-39 mm	-	5 days after females emerge (sex pheromone); 23-28 days before oviposition occurs; Life span: 102.7 ± 17.5 days (males); 139.1 ± 12.4 days	Rotting banana tissue	9 eggs per day	in air chamber of pseudostem	4.5 ± 0.71 days	70.0 ± 7.4 mm	4	pseudostem	pseudostem; prepupal stage lasts 2-3 days with length of 3.2 ± 0.35 cm, prepupal and pupal stages last 10-11 days	adults up to 200 days in fallen/rotten pseudostem; overall 75-80 days (5 months) on average	Ravi et al., 2002; Krishnan et al., 2016; Padmanaban et al., 2001

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Table 1.1: (Continued)

Curculionid		in/on host/ soil	Host	Industry	Damaging life stages		Life cycle														
							Adult				Egg			Larvae			Pupae	General notes	References
Latin name	Common name						Size	Mating season	Time after emergence pheromone produced	Feed on	Amount	Where	Time untill hatch	Max size	Larval instars	Feed on			
Curculio caryae Horn	Pecan weevil	in	Pecan nut	Fruit	larvae	adults	± 9.52 mm	Late summer: July-Sept	2 days after emerge mate; Life span: 15-30 days	Green pecan nuts, prefer large	30-54 eggs/ life span; 2-4 eggs in separate pockets; univoltine	Pecan nut	6-14 days	-	4	Pecan nut interior for 18-22 days	Underground (10-30 cm) in hard cell for extended period; prepupa for 1-2 years, then develops into pharate adult for another year. Pupa development only	Overall 2-3 years	Mody et al 1975; Hedin et al., 1997; Mulder et al., 2018
Cyrtomom luridis Boheman	??	on	Corkwood tree/shrub, Duboisia sp.	Medicine	larvae	adults	-	Spring to mid summer	2 days after emerge mate; Life span: 113.7 ± 15.2 days (females)	Foliage of trees	402 ± 72.9 eggs/ life span; in masses of 42.7 ± 7.9 eggs	-	-	19.0 ± 2.0 mm	-	Roots, externally at depth of 25-45 cm below ground surface; develop for 120-150 days	develop for 6 months	Overall 180 days	Kamiya et al., 2015; Tironi et al., 2005
Diaprepes abbreviatus Linnaeus	Citrus root weevil	on	Neotropical hosts, citrus plants	Fruit	larvae	adults	95-190 mm	Spring and fall (in Florida)	Life span: 4-5 months; 135 days (male), 147 days (female)	Different hosts	5000-29000 eggs /life span, in masses of 30-265 eggs	Wide range of foliage, different hosts prefer broad leaves	7-10 days	up to 25 mm	10-11	Externally on roots; develop for 5-15 months	in soil chamber; develop for 15-30 days	Overall 5-18 months from egg to adult	LaPointe 2012, 2001; Weissling et al., 2002;
Dynamis borassi Fabricius	??	in	Palms, coconut tree, banana	Fruit	?	adults (red ring disease)	23-35 mm	-	-	Star-nuts, coconuts	-	in palm tissue	-	-	-	inner bark, phloem and cambium	pupal chamber in sapwood		Giblin-Davis et al., 1996 & 1997
Metamasius hemipterus Olivier	West Indian Sugarcane borer	in	Sugarcane, banana, coconut, pineapple and ornamental palms	Fruit	larvae	adults (red ring disease)	9-14 mm	live 2-3 months	Lifespan: 60 days	-	± 500 eggs	Replanted stalks	3 days	-	-	terminal bud, leaves	-	Synonymized with *Metamasius hemipterus sericeus*	Alpizar et al., 2002
Metamasius hemipterus sericeus Olivier	West Indian Sugarcane weevil/ Silky cane weevil	in	Sugarcane, banana, pineapple and palms	Fruit	larvae	adults (red ring disease)	9-14 mm	-	Lifespan: 60 days	-	± 500 eggs	Stressed/ damaged replanted stalks of	4 days	-	-	sugarcane pith/ petioles or crown or stem>healthy leaf or stem and rhizome; 40-60 days (7 weeks) in stalk	in stalks for 10 days	Fibrous cocoon; 70-120 days life cycle Pupae live better after dry season due to bacteria and fungi activity.	Alpizar et al., 2002; Weissling et al., 2019
Metamasius spinolae Gyllenhal	Cactus weevil	in/on	Prickly pear/ cactus	Fruit	larvae	adults (not extensive)	23-36 mm	May-Sept emerge	Life span: 12 months; 95-679 days (males); 75-544 days (females)	margins of young pads of cactus	82 eggs/ life span; univoltine	on pads in chewed holes (cactus leaves) near areole	9-32 days (summer vs winter)		-	Prickly pear stem/branch; stay there the entire winter	in cactus pads	Cocoon in (partly) dry prickly pear stem	Tafoya et al., 2003 & 2004 & 2007
Myllocerinus aurolineatus Voss*	Tea weevil (not agg pheromones)	on	Tea foliage	Beverage	?	adults	-	Spring: May	-	young tender tea leaves	-	-	-	-	-	-	-	-	Sun, et al., 2010 & 2017
Paramasius distortus Gemminger & Harold	??	on	banana, pineapple, palms, and sugarcane	Fruit	-	-	± 10.8 mm	-	-	-	-	-	-	-	-	inner bark, damage young trees, kill stressed	pupal chamber in sapwood	-	Perez et al., 1997 (unpublished)
Pissodes approximatus Hopkins	Red Pine weevil	in	Pines, spruces	Wood	larvae	adults	7-8 mm	Spring	-	Sapling tree shoots	univoltine in Florida; clusters of 1-5 in puncture holes	injured trees	7-8 weeks (laid in summer); 22-25 weeks (laid late	-	5	inner bark, damage young trees, kill stressed trees	pupal chamber in sapwood	Synonymized with *Pissodes nemorensis*	Booth et al., 1983
Pissodes castaneus DeGeer	Banded pine weevil	in	Pines trees	Wood	larvae	adults (not extensive)	5-11 mm	Flight season	-	Buds, young shoots and deep bark punctures	>500 eggs/life span, in clusters of 1-8 eggs, univoltine (can take 2 years to develop in Northern		8-10 days	8-10 mm	4	inner bark: bast or phloem	pupal chamber at end of galleries in sapwood	-	Marques et al., 2011
Pissodes nemorensis Germar	Bark/ Deodar weevil	in	Pines, cedar trees	Wood	larvae	adults	6-8 mm	Winter to early Spring (Late March to mid-May)	-	Through bark to phloem	univoltine in Florida; clusters of 1-5 in puncture holes	In weaker spots of bark through feeding holes	7-8 weeks (laid in summer); 22-25 weeks (laid late	-	5	inner bark, phloem and cambium	pupal chamber in sapwood	7-25 weeks for entire life cycle	Phillips et al., 1984; Atkinson et al, 1988
Pissodes strobi Peck	White pine weevil	in	Pines and spruces	Wood	larvae	adults	4-6 mm	Spring (July-September)	-	Pine terminal shoots	clusters of 1-5 per hole; univoltine	under apical bud in feeding	6-14 days	7-10 mm	4	inner bark: phloem; develop 5-7 weeks	pupal chamber in terminal shoots	adults overwinter in forest floor	Booth et al., 1983

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Table 1.1: (Continued)

Curculionid		in/on host/ soil	Host	Industry	Damaging life stages		Life cycle												General notes	References
							Adult				Egg			Larvae			Pupae			
Latin name	Common name						Size	Mating season	Time after emergence pheromone produced	Feed on	Amount	Where	Time untill hatch	Max size	Larval instars	Feed on				
Pseudopiazurus obesus Boheman	Papaya borer weevil"	in/on	Papaya	Fruit	larvae	adults	-	-	-	Papaya stalk	-	-	-	-		papaya stalk meristimatic	papaya stalk		-	Zarbin et al., 2007
Pseudopiazurus papayanus Marshall	Pawpaw borer weevil	in	Papaw	Fruit	-	-	-	-	-	Leaf stems of papaw	-	holes in bark	-	10.83-12.67mm	-	-	-		-	Moreira et al., 2004; De Sousa et al., 2004
Rhabdoscelus obscurus Boisduval	New Guinea Sugarcane weevil" = cane borer weevil"	in	Coconut and ornamental Palms; Bird-of-paradise; sugarcane (softer varieties especially);	Fruit	larvae	-	12-15 mm	-	4 days	-	150 eggs/ life span	in cavities made by female, bark wounds, in leaf bases	5 days	15 mm mature	6	soft pith in stems for 2-3 months	pupate in 20 days in sugarcane galleries. '2-3 months from larvae to adults.		live 6-10 months	Giblin-Davis et al., 2000
Rhynchophorus bilineatus Montrouzier	Black Palm weevil (also called asian palm weevil)	in/on	Coconut Palms	Fruit	larvae	adults (together with Rhinoceros beetles making galleries)	± 40 mm	-	A few days after emerged	leaves	30-400	in palm tissue	-	-	-	terminal bud, leaves	-		-	Oehlschlager 1995; Giblin-Davis et al., 1996; Sukirno et al., 2018
Rhynchophorus cruentatus Fabricius	Palmetto weevil	on	Palm trees	Wood	larvae	adults	24-33 mm	Late spring, early summer; also throughout	-	Crown	207 eggs/ life span	leaf base or wound of dying host	3 days	-	-	crown and stem of palm	in cocoon in palm trunk for 11-45 days		± 84 days for entire life cycle	Weissling 1994 & 2019; Giblin-Davis et al., 1996
Rhynchophorus ferrugineus Olivier	Asian Palm weevil/ Red stripe palm weevil	in/on	coconut, oil palm, sago and aren palms	Fruit	larvae	adults	35-40 mm	mate and lay eggs for up to 8-10 weeks after emergence	Life span 39 days (males); 38 days (females)	Palm tissue	58-531; multivoltine (up to 21 generations per year in Egypt)	in palm tissue	1-6 days	>50 mm; 36.06 ± 3.24 days; followed by nymphal period of 19-26 days	-	soft meristem or leaf bases, develop for 2 months	in cocoon in palm trunk for a few weeks with prepupal and pupal stage		45-139 days for entire life cycle, up to 26 weeks in captivity	Hallet 1993; Giblin-Davis et al., 1996; Sukirno et al., 2018
Rhynchophorus palmarum Linnaeus	American Palm weevil	in/on	coconut, oil palm	Fruit, Range	larvae	adults (red ring disease)	40-50 mm	Possibly during maturation period in pupal cocoon before emergence?	Life span 44.7±17.2 days (males); 40.7±15.5 days (females)	Wounded plant tissue near the crown	718 eggs/life span	Heart of palmito palm	3.2 ± 0.93 days	50-60 mm; 40-60 days development	-	Heart of palmito palm	in cocoon in palm trunk: prepupa for 4-17 days; pupa for 8-23 days; remain in cocoon for 7.8±3.4 days as adult		live 70-120 days	Alpizar 2002, Jaffe 1991, Giblin-Davis et al., 1996
Rhynchophorus phoenicis Fabricius	African Palm weevil	in	Palm trees		larvae	adults	25 mm	-	Life span 68.86 ± 3.3 days (males); 54.71 ± 2.7 days (females)		252.26 ± 3.61 eggs/life span	in palm tissue	4 days	51,52 ± 0,81 mm, 3.24 ± 0.9 days development; followed by nymphal period of 19.3 ±	6	soft palm trunk tissue	10-23 days		108.51 ± 3.6 days for entire life cycle	Giblin-Davis et al., 1996; Ehounou et al., 2018
Rhynchophorus vulneratus Panzer	(red stripe) Asian/Asiatic Palm weevil	in	Palm trees		larvae	adults	19-42 mm	-	sexual maturation in time adults take to emerge from pupal case		250; univoltine (1-1.5 generations annually)	in/near trunk wound	3 days	36-47 mm	-	soft palm trunk tissue	in cocoon in palm trunk		3 weeks pupa to adult; complete life cycle duration: 82 days	Giblin-Davis et al., 1996; Sukirno et al., 2018
Scyphophorus acupunctatus Gyllenhal	Agave weevil*	in	Agave	Beverage	larvae	adults	12-15 mm	Spring: 1 month after emerge at the bottom of leaves	1 month after emerge, males start pheromone production, increases with age.	Agave tissue	20-30 eggs/lifetime in clusters of 1-4	In agave head	5 days	± 12.7 mm	6-8	roots, lower leaves, putrified agave head	in soil, 11-14 days		2-3 months (105-137 days); positive correlation between adult numbers and degree of putrefaction of	Ruiz-Montiel et al., 2003 & 2009; Cuervo-Parra et al., 2019

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Table 1.1: (Continued)

Curculionid		in/on host/ soil	Host	Industry	Damaging life stages		Life cycle											General notes	References
							Adult				Egg			Larvae			Pupae		
Latin name	Common name						Size	Mating season	Time after emergence pheromone produced	Feed on	Amount	Where	Time untill hatch	Max size	Larval instars	Feed on			
Sitona discoideus Gyllenhal	Lucerne weevil"	in/on	Lucerne		larvae	adults	10 mm	Autumn: post-aestivatory flight period. Eastivation for 6-8 weeks starting in	summer aestivation	foliage of lucerne	-	lucerne base	-	8 mm	5	roots, nitrogen fixing root nodules (50 cm below ground surface)	in soil	-	Unelius et al., 2013
Sitona lineatus Linnaeus	Pea and Bean weevil	in/on	Pea and bean	Food	larvae	adults	4-5 mm	Spring	-	Especially young pea or bean plants	-	Around peas/bean plants	±3 weeks	up to 5 mm	5	nitrogen fixing root nodules, roots	5 cm under ground surface, for 2 weeks	-	Blight et al., 1984
Sitophilus granarius Linnaeus	Grain weevil*	in/on	Grain	Grain and cereal	larvae	adults	4-5 mm	same pheromone production in 1st 6 weeks after emergence	unknown maturation period	-	300-400 eggs/ life span	Singly per grain	-	2-3 mm	-	-	-	5-7 weeks overall life cycle completion; life span of 7-8 months	Chambers et al., 1996
Sitophilus oryzae Linnaeus	Rice weevil	in/on	cereal grains	Grain and cereal	larvae	adults	2-3 mm	-	can mate immediately; Life span 3-6 months	Wheat, corn, jaw, barley etc. for up to 7 months	300-550 eggs/ life span; multivoltine	up to 2 in grain cavity	3-4 days (up to 9 in winter)	-	4	rice kernels, other cereal products	19-34 days from grub to prepupa, then pupa within 1-2 days; 3-6 (up to 20) days to develop from pupa to adult	32 days for overall life cycle completion	Walgenbach et al., 1983; Phillips et al., 1981
Sitophilus zeamais Motschulsky	Maize weevil	in/on	corn, wheat, rice, and sorghum	Grain and cereal	larvae	adults	1-17 mm	-	-	Maize, Corn, wheat, rice, sorghum	250-400 eggs/ life span; 4 eggs/day	-	3 days	1.67 ± 0.07 mm	4	grain kernel for 18 days	6-7 days in grain kernel	28 days for single generation. 5 month life span	Walgenbach et al., 1983
Sphenophorus incurrens Gyllenhal	Sugarcane weevil	in	Sugarcane	Sugarcane	larvae	-	-	-	-	-	-	-	-	-	-	rhizome galleries in sugarcane	-	-	Illescas-Riquelme et al., 2016
Sphenophorus levis Vaurie	Sugarcane weevil	in	Sugarcane stalks	Sugarcane	larvae	-	-	-	-	-	-	-	-	-	-	Tunnels in sugarcane stalks	-	-	Zarbin et al., 2003
Sternechus subsignatus Boheman	Soybean stalk weevil"	in/on	Soybean stalks	Food	larvae	adults	-	Feb-March, Jun-Aug	-	Soybean stems	-	-	-	-	-	Medulla in stem during May- Sept	Stalks	-	Ambrogi et al. 2012
Sitona lepidus Gyllenhal	Clover root weevil	in/on	White and red clover	Clover	larvae	-	2.7-3 mm females (excluding antennae and ovipositor	Spring and summer (late summer to autumn in Europe)	-	-	59-121 eggs/ life span; univoltine	on plant material but falls on soil to hatch	-	230 day degrees and moisture needed	5	nodules and roots of white clover	soil	-	Park et al., 2013
Bothynoderes punctiventris Germar	Sugar beet weevil	in/on	Sugar beet plant	Fruit	larvae	adults	10-16 mm	Spring, after winter in soil (50-70 cm beneath surface). First week of May males first, then after 15th week of year, males and females are in equal ratio. Then females dominate	-	beet seedlings	1-10 per female at a time; 94-120 eggs per year; 1.2-1.3 mm long; univoltine	in beet, after hole drilling and backfilling	10-15 days; egg to adult takes 67-148 days.	27-30 mm with 5 life stages	-	beet roots for 143 days	in soil, up to 102 days (between 143th and 245th Julian days, 6 months ave)	adults can overwinter for 2 years in soil	Drmic 2016
Rhinostomus barbirostris Fabricius	Bearded/ bottlebrush weevil"	in	coconut, oil palm	Fruit, Range	larvae	-	15-50 mm	-	-	-	-	on bark	-	up to 5 cm	-	galleries in palms	in soil	Overall 6 months from egg to adult	Reis et al., 2018
Otiorhynchus sulcatus Fabricius	Black vine weevil*"	on	Many, roots	Range	larvae	adults	± 9.52 mm	Spring-summer	3-4 weeks before pheromone production	Greenery of generally toxic plants, strawberries, cranberries, hops	univoltine (up to 2 generations per year in greenhouse)	on soil or plant crevices	3-4 weeks before eggs are laid, allowing insecticidal	± 6.3 mm	6-7	roots, including cambium and phloem. Overwinter in soil	late spring, early summer for 10 days	-	Van Tol et al., 2012
Ceutorhynchus obstrictus Marsham*	Cabbage seed pod weevil	on	Canola	Range	larvae	adults	3-4 mm	Spring emergence (aug); Overwinter as adults	-	Pod and pericarp, nectar and pollen	singly or 2 per pod, univoltine	Pods	6-7 days	50 days	3	in canola pods, seeds for 50 days	in soil; develop for 2 weeks until adults emerge	-	Dosdall et al., 2011

*: Pheromone not described for this species

Table 1.2: Sampling methods and procedures followed for the identification of pheromone components of non-Scolytinae Curculionidae weevils. Excel spreadsheet attached in separate document ("Table 1.2").

Genus	Common name	Type of sampling	Amount of weevils	Container	Additions in chamber	Flow rate (L/min)	Sampling time	Ad/absorbent	Extraction method	Reference
Anthonomus eugenii Cano	Pepper weevil	DHS	Singly or in groups	Pyrex container (20cmx22cm ID)	<5cm Jalapeno fruit/pepper buds/nightshade berries	0.13	1-5 days	Tenax/SuperQ	240uL Hexane for Tenax, or DCM/Hexane for SuperQ. 2500ng internal standard (alpha terpineol) added	Eller *et al* ., 1994
Anthonomus musculus Say	Cranberry weevil	DHS, surface washes	20	Glass vials, 6mL	0.3g blueberry/ cranberry flower buds daily	1	72 hours	SuperQ	150uL DCM, internal std = nonyl acetate (400ng) added	Szendrei *et al.* , 2011
Anthonomus rubi Herbst	Strawberry blossom weevil	DHS	1-3	Glass chamber (10 cm x 3cm ID)	*F. annanasa* plant	1	24 hours	PoropakQ	2 extractions, 1 mL, 0.5 mL DCM	Innocenzi *et al* ., 2001
Conotrachelus humeropictus Fiedler	Cocoa borer weevil	DHS	6	37cm x4cm ID	Sugarcane and moistened cotton	1	12 hours	HayeSep-D	400uL Hexane, conc under Ar to 60uL	Szczerbowski *et al* ., 2016
Conotrachelus psidii Marshall	Guava weevil	DHS	10	Glass chambers (33cmx15cm ID)	12cm guava stem, green guava fruit	?	96 hours	HayeSep-D	200uL Hexane, conc under Ar	Palacio-Cortés *et al.*, 2015
Cosmopolites sordidus Germar	Banana weevil (=Rhizome weevil)	DHS, dissection and surface washes	100/50	Glass containers 5L/15x4cm ID	Damp cotton wool	0.4	24 hours	PoropakQ	15mL DCM, 1 mL DCM, 5 sec in 5mL DCM for surface washes	Budenberg *et al* ., 1993
Dynamis borassi Fabricius	Palm weevil	DHS, dissection	5-10	9L Nalgene polycarbonate dessicator	0-4 day old sectioned apples	0.0015	5 days	PoropakQ	Pentane, conc by distillation	Giblin-Davis *et al.* , 1997
Metamasius hemipterus sericeus Olivier	West Indian Sugarcane borer (Silky cane weevil)	DHS	38	Nalgene dessicator	Sugarcane stalks and moist Kimwipe papers	2	96 hours	PoropakQ	Pentane	Perez et al., 1997
Myllocerinus aurolineatus Voss	Tea weevil	DHS	100 ♂ &♀ together (1:1)	Glass	Living *Longjing* tea plants	1.36	1 hour	SuperQ	500uL DCM, internal standard (50ng Ethyl decanoate) added	Sun, *et al.* , 2010
Pissodes approximatus Hopkins	Red Pine weevil (Red Pine weevil)	DHS, dissection	5-150	Large vacuum dessicator	White pine leaders or red pine branches	?	5-10 days	PoropakQ	5ml/g Pentane, dried ($NaSO_4$), conc to 1-4 ml by distillation. Hindguts/ abdomen crushed in pentane, centrifuged for supernatant	Booth *et al.* , 1983
Pseudopiazurus obesus Boheman	Papaya borer weevil (Papaya weevil)	DHS	30 (20-40 days old)	Glass	Fresh papaya stalk	1	24 hours	SuperQ	Hexane, conc by distillation	Zarbin *et al* ., 2007
Rhabdoscelus obscurus Boisduval	New Guinea Sugarcane weevil**	DHS, dissection	>30	9L Nalgene polycarbonate dessicator	0-4 day old sectioned apples	0.0015	5 days	PoropakQ	Pentane, conc by distillation	Giblin-Davis et al., 2000
Rhinostamus barbirostris Fabricius	Bearded/ bottlebrush weevil	DHS	4	Glass chambers (20cmx7cm ID)	1 piece of sugarcane	1	48 hours	HayeSep-Q	800uL Hexane	Reis *et al.* , 2018
Rhynchophorus bilineatus Montrouzier	Black Palm weevil (Asian palm weevil)	DHS	20-25	Nalgene dessicator	0-4 day old sectioned apples	?, used water aspirator as vacuum puller	6-7 days	PoropakQ	Pentane, conc by distillation	Oehlschlager *et al.* , 1995
Rhynchophorus cruentatus Fabricius	Palmetto weevil	DHS	30-40	9L Nalgene polycarbonate dessicator	500 g sugarcane	0.0015	7 days	PoropakQ	Pentane, conc by distillation	Perez *et al* ., 1994
Rhynchophorus vulneratus Panzer	None	DHS	25	9L Nalgene polycarbonate dessicator	NA	0.0015	5-7 days	PoropakQ	Pentane	Hallet *et al* ., 1993
Sitona discoideus Gyllenhal	Lucerne weevil	DHS	40	1L	8 moist dental rolls	3	24 hours	Tenax cartridge	800uL diethylether, conc to 10uL under Ar	Unelius *et al.* , 2013
Sitophilus granarius Linnaeus	Grain weevil	Static Filter paper extracts, dissections, puffing	1	glass tube, 5 x 2.5cm ID	1 wheat grain into vial, capped	5 min venting with oxygen 3x p/w	7 days	Whatman grade1 filter paper	200mL DCM for 24 hours, Hexane	Chambers *et al.* , 1996
Sternechus subsignatus Boheman	Soybean stalk weevil	DHS	10	Glass container	2 cuttings of soy bean stems	1	24 hours	SuperQ	Hexane, conc under Ar	Ambrogi *et al.* , 2012
Conotrachelus psidii Marshall	Guava weevil	DHS	30	Glass chamber (33 cm x 4cm ID)	3 unripe guava fruit (6-8g)	1	3 days	Hayesep D 80/100	500uL hexane, conc under N2	Romero-Frias *et al.* , 2016
		HS-SPME	10	Glass container (100mL)	NA	0	12 hours accumulation	DVB-CAR-PDMS, 50/30um	NA	Romero-Frias *et al.* , 2016
Scyphophorus acupunctatus Gyllenhal	Agave weevil	SPME	1 male	Glass container (7mL)	With and without 1 g fresh agave or apple	0	1, 3 or 6 days accumulation	PDMS-DVB, 65um	NA	Ruiz-Montiel *et al.*, 2009
Curculio caryae Horn	Pecan weevil	Surface washing	♂&♀ together (1:1)	Glass container (28mL)	NA	NA	NA	NA	1mL DCM or 1mL hexane	Hedin *et al.*, 1997

Table 1.3: The economic impacts of non-Scolytinae Curculionidae weevil species for which pheromones have been investigated as of 2019. Excel spreadsheet attached in separate document ("Table 1.3").

Scientific name	Common name	Type of industry affected	Crop	Specifically in...	Prevalent/native to..	Control now	Stage of pheromone development	Associated microbes/pathogens?	References
Conotrachelus humeropictus Fiedler	Cocoa borer weevil	Fruit borer	Cocoa	Fruit and stem	Brazil	Combined nematode and fungus biocontrol.	Unknown	Also secondary infections/pests	Szczerbowski et al. (2016); Simi et al. (2018)
Conotrachelus nenuphar Herbst	Plum weevil	Fruit borer	Pome and stone fruit	Plums, apples	Eastern and central North America	Pheromone monitoring with insecticide sprays	IPM Monitoring	NA	Eller et al. (1996); Collins (1996); Piñero et al. (2001 & 2003); Foshee et al. (2018)
Conotrachelus psidii Marshall	Guava weevil	Fruit borer	Guava	Fruit	Colombia	Insecticides on adults	Unknown	NA	Palacio-Cortés et al. (2015); Romero-Frías et al. (2016)
Pseudopiazurus obesus Boheman	Papaya borer weevil/Papaya weevil	Fruit borer	Papaya	Stalks	Prevalent in Northeastern Brazil	Insecticides on adults	Unknown	NA	Zarbin et al. (2007 & 2010)
Pissodes approximatus Hopkins	Red Pine weevil	Wood borer	(Stressed) trees	Spruce, Pine	Northeastern North America	Insecticides on adults. Research on aggregation-pheromone traps.	Research: mass-trapping	NA	Booth et al. (1974); Booth et al. (1978); Booth et al. (1983)
Pissodes castaneus DeGeer	Banded pine weevil	Wood borer	(Stressed) trees	Pine	Siberia, North Africa, Canary Islands, Madeira, South America, Brazil,	Insecticides on adults. Research on aggregation-pheromone traps.	Research: mass-trapping	NA	Marques et al. (2011)
Pissodes nemorensis Germar; = *P. approximatus*	Bark/ deodar weevil	Wood borer	(stressed or reforested or seedling) trees	Spruce, Pine	Southeastern US, Ivory coast	Insecticides on adults. Research on sex-pheromone traps.	Research: Monitoring	Procerum root disease, Pitch canker: *Fusarium moniliforme* var. *subglutinans*	Hibbard et al. (1993); Booth et al. (1974)
Pissodes strobi Peck	White pine weevil/ Sitka spruce weevil	Wood borer	Trees (plantations 5-35 years old)	White Pine, Engelmann and Sitka Spruce	Northeastern America	Insecticides on adults. Research on fungi biocontrol, pheromone traps.	Research: Lure-and-Infect	Secondary decay fungi	Booth et al. (1983)
Curculio caryae Horn	Pecan weevil	Fruit borer	Nuts	Pecan, walnuts	Native to Southern US	Pheromone monitoring with insecticide sprays.	IPM Monitoring	NA	Tedders et al. (1994); Collins (1996); Tedders et al. (1996); Hedin et al. (1997)
Anthonomus eugenii Cano	Pepper weevil	Fruit borer	Peppers	Sweet and hot peppers	Southern United States, Mexico, and Central America	Pheromone monitoring with insecticide sprays.	IPM Monitoring	Internal pepper mold	Eller et al. (1994 & 2014)
Anthonomus grandis Boheman	Boll weevil	Fruit borer	Cotton	Cotton buds	Southern United States, Mexico, and Central America	Pheromone mass trapping	Mass-trapping	NA	Tumlinson et al. (1969); Rebeiro et al. (2017); Hardee et al. (1974)
Anthonomus musculus Say	Cranberry weevil	Fruit borer	Cranberry and blueberry	Flowers and flower buds	Northeastern US	Insecticides on adults (resistance to broad spectrum); Research on pheromone monitoring	Research: Monitoring	NA	Szendrei et al. (2011); Silva et al. (2018)
Anthonomus rubi Herbst	Strawberry blossom weevil	Fruit borer	Berries	Strawberry, raspberry	UK and continental Europe	Insecticides on adults	Unknown	NA	Innocenzi et al. (2011)
Rhynchophorus bilineatus Montrouzier	Black Palm weevil (Asian palm weevil)	Wood borer	Palm trees	Coconut palms	New Guinea, Central and South America	Pheromone mass trapping	Mass-trapping	Red ring disease	Oehlschlager et al. (1995); Giblin-Davis et al. (1996)
Rhynchophorus cruentatus Fabricius	Palmetto weevil	Wood borer	(Stressed) palm trees	Crown, stem	Southeastern US, Ivory coast	Insecticides on adults. Research on aggregation-pheromone traps.	Research: mass-trapping	Red ring disease	Weissling et al. (1994); Perez et al. (1994)
Rhynchophorus ferrugineus Olivier	Asian palm weevil	Wood borer	(Stressed) palm trees	Coconut, oil, sago, aren palms	Pakistan, India, Egypt, Indonesia, United Arab Emirates, South and southeast Asia	Insecticides on adults. Research on pheromone mass traps.	Research: mass-trapping	Red ring disease	Hallett et al. (1993); El-Shafie et al. (2017); Abdel-Azim et al. (2018)
Rhynchophorus palmarum Linnaeus	American Palm weevil	Wood borer	Palm trees	Coconut, oil palms	Central and South America, Venezuela, Mexico, Brazil, and the Caribbean	Mass trapping (Metamasius genus pheromone)	Mass-trapping	Red ring disease	Jaffé et al. (1993); Chinchilla et al. (1996); Alpizar et al. (2002); Oehlschlager et al. (2002)
Rhynchophorus phoenicis Fabricius	African Palm weevil	Wood borer	Palm trees	Oil palms	Ivory coast, Senegal, Ethiopia, South Africa	?	Unknown	Red ring disease	Perez et al. (1994)
Rhynchophorus vulneratus Panzer	Red stripe Palm weevil	Wood borer	Palm trees	Coconut, oil, sago, aren palms	Indonesia, south and southeast Asia	?	Unknown	Red ring disease	Hallett et al. (1993)
Sphenophorus incurrens Gyllenhal	Sugarcane weevil	Pseudostem borer	Grass, pseudostem	sugarcane, rice and kikuyu grass	Neotropical regions from Mexico to Panama	Research on aggregation-pheromone traps.	Research: mass-trapping	Oppertunistic pathogen entry via larval feeding	Illescas-Riquelme et al. (2016)
Sphenophorus levis Vaurie	Sugarcane weevil	Pseudostem borer	Sugarcane	Stalks/ pseudostem	South America, including Northern Argentina, Paraguay, and Brazil	Research on aggregation-pheromone traps.	Research: mass-trapping	NA	Zarbin et al. (2003)
Sitophilus granarius Linnaeus	Grain weevil	Storage	Grain	Wheat, barley, maize, various other cereals, and cereal products	Worldwide, especially temperate regions in Northern hemisphere	Pheromone monitoring with insecticide sprays	IPM Monitoring	NA	Chambers et al. (1996); Faustini et al. (1982); Phillips et al. (1989)
Sitophilus oryzae Linnaeus	Rice weevil	Storage	Rice	Wheat, rice, barley, corn, peas, pasta	Worldwide, especially temperate regions in Northern hemisphere	Pheromone monitoring with insecticide sprays	IPM Monitoring	Also secondary infections/pests	Phillips et al. (1981); Phillips et al. (1989); Trematerra et al. (1989)
Sitophilus zeamais Motschulsky	Maize weevil	Storage	Corn, wheat, rice, sorghum	Corn, wheat, rice, and sorghum.	?	Insecticide sprays, fumigation of storage rooms, facilitated by pheromone monitoring	IPM Monitoring	NA	Walgenbach et al. (1986); Walgenbach et al. (1983)
Sitona discoideus Gyllenhal	Lucerne weevil	Root feeder	Lucerne	Leaves and roots	New Zealand, Australia, Mediterranean	Pheromone monitoring for insecticide spray, also biocontrol	IPM Monitoring	NA	Unelius et al. (2013)

(Cont.)

Table 1.3: (Continued)

Sitona lineatus Linnaeus	Pea and Bean weevil	Fruit borer	Leguminous crops like peas, beans and vetches	Leaves and roots	Britain, central Europe and north-western USA	Pheromone monitoring; Insecticide spray (resistance developed); EPN control under investigation	IPM Monitoring	NA	Bardner et al. (1983); Blight et al. (1984 & 1987)
Diaprepes abbreviatus Linnaeus	Neotropical or Citrus root weevil	Root feeder	Citrus	Wide host range	Carribean, Tropical US states	Lure predators with synomonal sex pheromone, and IPM with EPN-filled pheromone lures	IPM Lure-and-Infect	Phytophothora secondary pathogen infection	Tafoya et al. (2004); LaPointe et al. (2012)
Sternechus subsignatus Boheman	Soybean stalk weevil	Legume borer	Soybean	Stalks/ legumes	Brazil (native)	Mass trapping	Mass-trapping	NA	Ambrogi et al. (2012)
Cosmopolites sordidus Germar	Banana weevil (=Rhizome weevil)	Fruit borer	Banana and plantain	Stalks/pseudostem	Uganda, Costa Rica, East Africa and South Africa	EPN/Pheromone lure and infect IPM	IPM Lure-and-Infect	NA	Budenberg et al. (1993); Padilla-Cubas et al. (2010)
Metamasius hemipterus Olivier	West Indian Sugarcane borer/ Silky cane weevil	Wood borer	Sugarcane and ornamental palms	Sugarcane, banana, pineapple, coconut, oil and ornametnal palms	Florida, Caribbean, Mexico, Central and South America	Pheromone mass trapping	Mass-trapping	Red ring disease	Chinchilla et al. (1996); Ramirez-Lucas et al. (1996); Alpizar et al. (1999 & 2002)
Metamasius hemipterus sericeus Olivier	West Indian Sugarcane borer/ Silky cane weevil	Wood borer	(Stressed/ damaged) sugarcane and ornamental palms	Banana, pineapple, palms, and sugarcane	Central and South America, the Caribbean and Africa	Pheromone mass traps, insecticides on adults. Research on entomopathogens as biocontrol agents.	Mass-trapping	Red ring disease	Perez et al. (1997)
Metamasius spinolae Gyllenhal	Cactus weevil	Borer	Prickly pear	Stems	Mexico, South Africa	Insecticides on adults, biocontrol fungi.	Unknown	NA	Tafoya et al. (2003 & 2004 & 2007)
Scyphophorus acupunctatus Gyllenhal	Agave weevil	Borer	Agave	Stems, roots and bottom leaves	Southern United States to Brazil, Hawaii, Borneo, Java, Australia, and eastern Africa	Insecticides on adults.	Unknown	Putrefication by a gram positive bacteria, *Pseudomona fluosescens* biotype I	Ruiz-Montiel et al. (2003 & 2008 & 2009); Azuara-Domínguez et al. (2013)
Rhinostomus barbirostris Fabricius	Bearded/ bottlebrush weevil	Wood borer	Palm trees	Coconut trees, oil palms	Brazil	Manual removal/incineration of infected tree. Research in pheromone mass trapping/ monitoring.	Research: mass-trapping and monitoring	Resinosis and red ring disease	Reis et al. (2018)
Rhabdoscelus obscurus Boisduval	New Guinea Sugarcane weevil**	Pseudostem borer	Sugarcane, banana, bird-of-paradise, coconut and palms	Crown, stalks, sheaths and stems	Native to Austromalayan area, introduced in Christmas Island, Marquesas Islands, Queensland, Hawaii, Micronesia, Okinawa, and the Bonin Islands of southern Japan	Insecticidal control, biocontrol with *Lixophaga sphenophori* (Villeneuve) (Diptera: Tachinidae), pheromone mass-trapping.	Mass-trapping	NA	Giblin-Davis et al. (2000); Reddy et al. (2011 & 2012)
Dynamis borassi Fabricius	Palm weevil	Wood borer	Coconut	Inlfuoresence and stem	Central and South America	Pesticide treatments and palm weevil pheromone mass trapping	IPM Mass-trapping	Red ring disease	Giblin-Davis et al. (1996 & 1997)
Odoiporus longicollis Olivier	Banana pseudostem weevil	Pseudostem borer	Banana	Pseudostem	Southeast Asia, New Guinea	Pheromone monitoring with insecticide sprays	IPM Monitoring	NA	Palanichamy et al. (2011)
Cyrtomon luridis Boheman	??	Defoliator	*Duboisia* sp.	Terminal leaders, larvae feed on roots or defoliate	Native to South America, prevalent in Neotropical regions like Brazil	NA	Unknown	NA	Tironi et al. (2005)
Otiorhynchus sulcatus Fabricius	Black vine weevil	Root feeder	Ornamental plants and fruit crops eg within taxa incl Taxus, Rhododendron, and Euonymus	Cranberry, Hops, Strawberry	Endemic to temperate Europe, moved to Canada, US, and Northern Europe	Insecticides and EPN applications	Unknown	NA	Van Tol et al. (2002)
Bothynoderes punctiventris	Sugar beet weevil	Fruit borer	Beet tuber	Fruit	Prevalent in Brazil	Mass trapping	IPM Mass-trapping	NA	Drmić et al. (2017)
Sitona lepidus Gyllenhal	Clover root weevil	Root feeder	White and red clover	Fruit	Native to Europe, invaded North America and New Zealand	Due to univoltine parthenogenetic reproduction, manual population counts followed by insecticide spays are used	Unknown	NA	Park et al. (2013)
Paramasius distortus Gemminger & Harold	New Guinea Sugarcane weevil	Pseudostem borer	Sugarcane?				Unknown		
Pseudopiazurus papayanus Marshall; = *Pseudopiazurus obesus*	Papaya borer weevil	Fruit borer					Unknown		

For ease of visualization, separate field descriptors were used (Figure 2). These were 'borers', 'root-feeders', 'defoliators' and 'storage-feeders'. The 'borers'-term was used as an umbrella term with subfields including 'fruit-feeders', 'wood-borers', 'pseudostem-borers', 'legume-borers' and a more general 'borer'-term, to include species such as the agave and prickly pear weevils, that are known to bore into the host's stems (not wood or pseudostems), fruit and/or roots (Ruiz-Montiel et al., 2003; Tafoya et al., 2003).

IPM methods imply the use of pheromones together with insecticide or other effective control method (Figure 3). Unknown development phases include those cases where pheromones have been found, but have not been reported as successful in field trials. Mass-trapping implies effective capture and control with only pheromone-baited traps without reported necessity of using other control mechanisms.

Supplementary Table 1.1:The range of sampling, screening and identification methods from non-Scolytinae Curculionidae weevils for which pheromones have been identified. Excel spreadsheet attached in separate document ("Supplementary Table 1.1").

Genus	Common name	Type of sampling	mating status of sampled weevils	nocturnal/diurnal	Screening method	Identification method	Confirmations	Reference
Anthonomus eugenii Cano	Pepper weevil	DHS	reared, virgin	?	None	GC-MS, EI-MS, FTIR, HPLC, H1-NMR, Purchased/synthesized standards[h]	Field	Eller *et al*., 1994
Anthonomus musculus Say	Cranberry weevil	DHS, surface washes	field collected	?	None	GC-MS, EI/CI-MS, Purchased standards[f]	Field	Szendrei *et al*., 2011
Anthonomus rubi Herbst	Strawberry blossom weevil	DHS	field collected	?	None	GC-FID, EI/CI-MS, GC-MS, Purchased/ obtained standards[f]	Field	Innocenzi *et al*., 2001
Conotrachelus humeropictus Fiedler	Cocoa borer weevil	DHS	field collected	nocturnal	Unknown	GC-MS, purchased standards[f]	None	Szczerbowski *et al*., 2016
Conotrachelus psidii Marshall	Guava weevil	DHS	field collected	nocturnal	GC-EAD	GC-MS, purchased/obtained standards[f]	Lab assays (standards and extracts)	Palacio-Cortés *et al*., 2015
Cosmopolites sordidus Germar	Banana weevil (=Rhizome weevil)	DHS, dissection and surface washes	field collected	nocturnal	EAG, surface washes	None[p]	Lab assays (extracts and live)	Budenberg *et al*., 1993
Dynamis borassi Fabricius	Palm weevil	DHS, dissection	field collected	?	GC-EAD	HPLC, GC-MS, Obtained/ synthesized standards[e]	Field	Giblin-Davis *et al*., 1997
Metamasius hemipterus sericeus Olivier and *M. hemipterus* Olivier	West Indian Sugarcane borer (Silky cane weevil)	DHS	field collected	?	GC-EAD	GC-MS, synthesized/ purchased standard[f]	Field	Perez et al., 1997
Myllocerinus aurolineatus Voss	Tea weevil	DHS	field collected	?	GC-EAD	GC-MS[d]	Lab assays (live plants, plant+adult, adult, frass, standards)	Sun, *et al*., 2010
Pissodes approximatus Hopkins	Red Pine weevil (Red Pine weevil)	DHS, dissection	field collected	?	None	GC, GLC, H-NMR, IR, Synthesized and purchased standards[k]	Field	Booth *et al*., 1983
Pseudopiazurus obesus Boheman	Papaya borer weevil (Papaya weevil)	DHS	reared, virgin	nocturnal	None	GC-MS, GC-MS-MS, purchased/synthesized standard[o]	Lab assays (extracts)	Zarbin *et al*., 2007
Rhabdoscelus obscurus Boisduval	New Guinea Sugarcane weevil**	DHS, dissection	reared, virgin	?	GC-EAD	GC-MS, EI/CI-MS, Obtained standards[f]	Field	Giblin-Davis et al., 2000
Rhinostomus barbirostris Fabricius	Bearded/ bottlebrush weevil	DHS	reared, virgin	diurnal	GC-EAD	GC-MS, GC-FTIR, NMR, synthesized standards[l]	Lab assays (extracts, standards), Field	Reis *et al*., 2018
Rhynchophorus bilineatus Montrouzier	Black Palm weevil (Asian palm weevil)	DHS	field collected	?	GC-EAD	GC-MS, EI/CI-MS, SIM mode, synthesized standards[f]	Field	Oehlschlager *et al*., 1995
Rhynchophorus cruentatus Fabricius	Palmetto weevil	DHS	field collected	diurnal	GC-EAD	GC-MS, SIM mode, CI-MS, GC-FID, H/C-NMR, synthesized standards[i]	Field	Perez *et al*., 1994
Rhynchophorus vulneratus Panzer	None	DHS						Hallet *et al*., 1993
Sitona discoideus Gyllenhal	Lucerne weevil	DHS	field collected	?	EAG, SSR	GC-MS, EI-MS, Purchased/ synthesized standards[f]	Lab assays (live)	Unelius *et al*., 2013
Sitophilus granarius Linnaeus	Grain weevil	Static Filter paper extracts, dissections	reared, virgin	?	GC-EAG	GC-FID, EI/CI-MS, GC-MS, prep-GC, Circular dichroism spectroscopy, Purchased/ synthesized/ obtained standards, H-NMR[c]	Lab assay (extracts, standards)	Chambers *et al*., 1996
Sternechus subsignatus Boheman	Soybean stalk weevil	DHS	field collected	?	GC-EAD	GC-MS, EI-MS, purchased/ synthesized / obtained standard, microreactions[b]	Lab assay (extracts)	Ambrogi *et al*., 2012
Conotrachelus psidii Marshall	Guava weevil	DHS and HS-SPME	field collected	crepuscular	None	GC-MS, EI/CI-MS, H/C-NMR, purchased/obtained/ synthesized standards[i]	Lab assay (extracts, standards)	Romero-Frias *et al*., 2016
Scyphophorus acupunctatus Gyllenhal	Agave weevil	SPME	reared, virgin	diurnal	None	GC-MS, obtained standards[f]	Lab-assay (live, standards), field	Ruiz-Montiel *et al*., 2009
Curculio caryae Horn	Pecan weevil	Surface washing	field collected	diurnal	None	GLC-MS, EI-MS, purchased standards[n]	Lab assay (live, standards), Field	Hedin *et al*., 1997
Anthonomus grandis Boheman	Boll weevil	Dissection, extracts	reared, virgin	diurnal	None	GLC, MS, H-NMR, IR, synthesized standards[g]	Lab assay (standards/purified extracts)	Tumlinson *et al*., 1969
Bothynoderes punctiventris Germar	Sugar beet weevil							Tóth et al., 2007
Conotrachelus nenuphar Herbst	Plum weevil	DHS?	reared, virgin	?	None	GC-FID, GLC, EI-MS, H/C-NMR, synthesized/purchased standards[j]	Field	Eller et al., 1996
Cyrtomom luridis Boheman	?	None	field collected, virgin	?	Behavior		Lab assay (live)	Kamiya et al., 2015; Tironi et al., 2005
Diaprepes abbreviatus Linnaeus	Neotropical root weevil	DHS	reared, virgin and field collected	diurnal	GC-EAD	GC-MS, EI/CI-MS, SIM-mode, purchased/ synthesized standards, COSY, NOESY, H/C-NMR[j]	Lab assay (standard)	Lapointe et al., 2012; Rivera et al., 2017
Metamasius spinolae Gyllenhal	Cactus weevil	DHS	field collected	diurnal	None	GC-MS, EI-MS, purchased or synthesized standards[f]	Field	Tafoya et al., 2007
Odoiporus longicollis Olivier	Banana pseudostem weevil	None	reared, virgin	crepuscular	Behavior	None[p]	Lab assay (live)	Palanichamy et el., 2011; Ravi et al., 2002
Otiorhynchus sulcatus Fabricius	Black vine weevil	None	field collected	?	EAG	Purchased standards[a]		Van Tol et al., 2002
Pissodes castaneus DeGeer	Banded pine weevil	DHS	field collected	?	Behavior	GC, GC-MS, Purchased/ obtained standards, microreaction[b]	Lab assay (live)	Marques et al., 2011
Pissodes nemorensis Germar and *P. strobi* Peck	Bark/deodar weevil	DHS, dissections	reared, virgin	?	Behavior	GC, GC-MS, Purchased/ obtained standards[f]	Field	Booth et al., 1983; Eller et al., 1996; Phillips et al., 1984; Hibbard et al., 1993
Rhynchophorus ferrugineus Olivier	Asian Palm weevil/ Red stripe palm weevil		field collected					Hallet et al., 1993
Rhynchophorus palmarum Linnaeus	American Palm weevil	None	field collected	diurnal	Behavior	GC-MS, purchased standards[f]	Lab assay (plant, live, standard), Field	Jaffé et al., 1993; Perez et al., 1994
Rhynchophorus phoenicis Fabricius	African Palm weevil	Unknown	field collected	diurnal	GC-EAD	GC-MS, SIM mode, CI-MS, GC-FID, H/C-NMR, synthesized standards[i]	Field	Perez *et al*., 1994
Sitona lepidus Gyllenhal	Clover root weevil	None	field collected	?	SSR	synthesized/ purchased standards[a]	None	Park et al., 2013
Sitona lineatus Linnaeus	Pea and Bean weevil	None	field collected	?	GC-EAG, Behavio	GC-MS, synthesized standard, H-NMR[l]	Field	Blight et al., 1984 & 1987
Sitophilus oryzae Linnaeus and *S. zeamais* Motschulsky	Rice weevil	DHS (filter disks)	reared, virgin	?	Behavior	GLC, GC-MS, H/C-NMR[m]	Field	Walgenbach et al., 1983
Sphenophorus incurrens Gyllenhal	Sugarcane weevil	DHS	field collected	?	GC-EAD	GC-FID, GC-MS, purchased/ synthesized standards[f]	Lab assay (live, plants), Field	Illescas-Riquelme et al., 2016

Supplementary Table 1.2: Visualized data from Supplementary Table 1.1 used in Figures 1.4 to 1.7. Excel spreadsheet attached in separate document ("Supplementary Table 1.2").

Mating status		**Screening methods**		**Identification methods**		**Confirmation studies**	
Articles with data:	38	Articles with data:	36	Articles with data:	35	Articles with data:	33
Field collected: unknown (FU)	25	GC-EAD (gD)	12	GC-MS	1	Field assays (F)	20
Field collected and virgin (FV)	2	GC-EAG (gG)	1	GC-MS, Std	13	Laboratory assays (L)	13
Reared: virgin (V)	11	GC-EAG, Behavior (gGB)	1	Std	2	Field and laboratory assays (F+L)	5
		Behavior (B)	7	GC-MS, Std, NMR	5		
		EAG (G)	2	GC-MS, Std, HPLC	1		
		EAG and SSR (GS)	1	GC-MS, Std, MS	1		
		SSR (S)	1	GC-MS, Std, Microreactions	2		
		None (N)	11	GC-MS, Std, NMR, CD	1		
				GC-MS, Std, NMR, IR	1		
				GC-MS, Std, NMR, IR, HPLC	1		
				GC-MS, NMR, GLC	1		
				Std, NMR, GLC	1		
				Std, NMR, GLC, IR	1		
				Std, NMR, GLC, IR, MS	1		
				Std, GLC, MS	1		
				None	2		

GC-MS: Gas chromatography coupled with mass spectrometry detector
Std: Purchased, synthesized or obtained standard
NMR: Nuclear magnetic resonance with either H^1-NMR, C^{13}-NMR, COSY/NOESY
HPLC: High performance liquid chromatography
MS: Mass spectrometry
Microreactions: derivatization reactions for identification with GC-MS
SSR: Single sensilla recording

CD: Circular dichroism spectroscopy and preparatory GC
IR: Infrared spectroscopy or Fourier Transform IR (FTIR)
GLC: Gas-liquid chromatography
GC-EAD: gas chromatography coupled with electroantennographic detection
GC-EAG: EAG puffing experiments using semi purified preparative GC

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Chapter 2

Trends and opportunities in the use of pheromones for management of non-Scolytinae Curculionidae

Abstract

Pheromones have become popular pest management tools for sustainable control measures against weevil pests in agriculture. Traditionally, pheromone development requires valuable, and often unavailable time for optimization. In this study, the current knowledge on aggregation- and sex pheromones from non-Scolytinae Curculionidae weevils is synthesized, with particular focus on trends in molecular structures of pheromone constituents and key factors considered during the development and optimization of pheromones post-identification. Furthermore, recently discovered non-Scolytinae Curculionidae pheromones are reviewed and weevils for which evidence of pheromone communication has been reported, are highlighted. Together, this review identifies key considerations for pheromone development and implementation as pest management tactics for emerging unstudied non-Scolytinae Curculionidae pest species.

Introduction

Beetles from the Curculionidae order, also known as “true” or “snout” weevils, are among the most diverse lineages of known insects. There are approximately 83 000 described species throughout the world (GBIF: The Global Biodiversity Information Facility 2019), and many of them are regarded as important phytophagous pests (Jones et al. 2019). Scolytinae weevils are not considered here, because they have specific behaviors and pheromones that are subfamily-specific and have been reviewed elsewhere (Borden 1989; Gitau et al. 2013; Tunset et al. 1988; Vité et al. 1972).

Most non-Scolytinae weevil species synthesize pheromone compounds *de novo* (Chambers et al. 1996; Innocenzi et al. 2001; Taban et al. 2006) through the polyketide (Phillips et al. 1989; Schmuff et al. 1984) or isoprenoid pathways (Francke and Dettner 2005; Hibbard and Webster 1993; Mitlin and Hedin 1974). These reactions are catalyzed by enzymes in micro-organisms often found in the insect gut. The biosynthesis mechanism of pheromone production in beetles are fundamental tools that aid in understanding how beetles use pheromones, but will also not be reviewed here (Blomquist et al. 2010; Blomquist and Vogt 2003; Jurenka 2004).

There is a level of plasticity in attraction to pheromones of a few related weevil species. This can be explained by patterns in pheromone structures, which are also mirrored in their phylogeny (Ambrogi et al. 2009). Understanding how these signals evolved into species-specific pheromones is of importance for exploring the diversity of pheromones found in the non-Scolytinae Curculionidae weevils. Specificity exists through the presence of unique major pheromone compounds, unique minor compounds, differences in pheromone blend ratios and chirality in pheromone components (Booth et al. 1983; Eller et al. 1994; Hibbard and Webster 1993; Illescas-Riquelme et al. 2016). The level of specificity in pheromones is also an important factor to consider when developing effective pheromone-based management strategies especially when specificity is desired (Dickens and Wiygul 1987; Oehlschlager et al. 1995). Cross-attraction of Curculionidae species with known pheromones has been observed and can be used to guide new pheromone identification studies (Alpizar et al. 2002; Eller et al. 1994; Ramirez-Lucas et al. 1996).

Application of pheromones in trapping interventions are intricate. Besides designing traps for retaining the targeted weevils (Reddy et al. 2011), effectiveness and specificity of traps are dependent on the presence of the correct pheromone blend (Eller et al. 1994), presence of synergistic host kairomones (Trematerra and Girgenti 1989), population density and various environmental and biological factors that vary in time and space (Perez et al. 1997; Piñero et al. 2001; Walgenbach and Burkholder 1986). Dispensers have to mimic natural blend ratios and release rates that are biologically active for their target species (Hibbard and Webster 1993; Unelius et al. 2013). Placement and timing of pheromone-baited traps is crucial for optimal effectivity (Drmić et al. 2017; Hallett et al. 1993b), because weevils must be sexually mature and responsive to pheromone signals during implementations (Booth et al. 1983). These elements are complex and species-specific, necessitating optimization of the application of pheromone blends to each non-Scolytinae Curculionidae weevil.

In this review, the patterns of pheromone structures are compared amongst species and opportunities for the development of new pheromone-based pest management methods for non-Scolytinae Curculionidae are introduced. Factors to consider for successful pheromone-based trapping are also described. Finally, previous successes and failures of pheromone-based management methods are reviewed for non-Scolytinae Curculionidae weevils.

Pheromone specificity

The specificity of pheromone signals is important for species isolation. The specificity referred to here, is how species maintain a level of uniqueness in their pheromone signals in order to prevent cross-attraction. There are four primary end products in which pheromone signal specificity has resulted due to evolutionary pressures in non-Scolytinae Curculionidae (Figure 2.1).

Specificity due to unique compounds

The presence of unique compounds in a species' pheromone can result in signal specificity. For example, banana weevils have different molecular structures in their pheromone blends than any other Curculionidae for which pheromones have been described (Fletcher et al. 1997; Jayaraman et al. 1997). The recent pheromone identification of *Homalinotus depressus* has also been described as the first of its kind in Curculionidae (Vidal et al. 2019). Boll weevils, *Anthonomus grandis*, and cranberry

weevils, *A. musculus* are part of the same genus, but have different major compounds and a number of similar minor compounds in their pheromone blends. The boll weevil, *A. grandis* has Grandlure I (cis-2-isopropyl-1-methylcyclobutane ethanol) as the major compound (Tumlinson et al. 1969) and the cranberry weevil, *A. musculus* has Z-Grandlure II ((Z)-3,3-dimethylcyclohexane-Δ1,ß-ethanol) as the major compound (Szendrei et al. 2011).

Specificity due to unique minor constituents in pheromone blends

Secondly, specificity results from the presence of unique minor compounds in blends where the major compound is the same between species. For example, there are three minor pheromone compounds present in the pepper weevil, *A. eugenii* pheromone blend that were not found in the boll weevil, *A. grandis* pheromone blend (Eller et al. 1994). Both these species use (2)-2-(3,3-dimethylcyclohexylidene)-ethanol as a major pheromone constituent, however geranic acid - another male-specific constituent, significantly increased trap efficacy when added to the pheromone blend of the pepper weevil, *A. eugenii* (Eller et al. 1994). *Sitona discoideus* and *S. lineatus* use the same major pheromone component, 4-methyl-heptan-3,5-dione, but cross-attraction may be avoided by the production of 4S5R-sitophinone by *S. discoideus* (Blight and Wadhams 1987; Unelius et al. 2013) (Figure 2.1). This may hint at the presence of antennal receptors that have evolved in some species to specifically detect antagonists produced by related species (Unelius et al. 2013), but this would have to be confirmed experimentally.

Specificity due to pheromone blend ratio differences

Thirdly, specificity can result due to the use of the same pheromone compounds in different blend ratios. For example, *Pissodes approximatus* and *P. strobi* have been shown to synthesize different blends of the enantiomers of grandisol and grandisal. These two species respond differently to different blend ratios of these compounds (Booth et al. 1983) (Figure 2.1).

Specificity gained from chiral centers in pheromone compounds

Finally, chirality in pheromone molecules also play an important role in specificity of pheromone signals. Chiral centers may bind specifically to olfactory receptors on antennae due to the different possible orientations of the four carbon constituents in

three-dimensional space. Some pheromones within the weevil group have more than one chiral carbon. For example, the banana weevil, *Cosmopolites sordidus*, utilizes all four diastereomers of sordidin, a molecule with two chiral centers (Fletcher et al. 1997). The pheromone isolated from the sugarcane weevil, *Sphenophorus levis*, 2-methyl-4-octanol, has also been identified as a molecule with a single chiral center, and that natural isolates only contain the S-enantiomer (Zarbin et al. 2003). In contrast, its close relative, *S. incurrens,* has been shown to be attracted by a racemic mixture of the same component in its pheromone (Illescas-Riquelme et al. 2016), indicating a possible mechanism of cross-attraction prevention. Even though species-specificity can also be gained by geographic or timing isolation (Illescas-Riquelme et al. 2016; Oehlschlager et al. 1995), these are secondary means, and thus not included for the purpose of this review.

Degrees of specificity due to pheromone compounds that have similar chemical structures

There is a degree of plasticity around the perception of pheromone signals for certain weevil species, rendering the pheromone blend attractive to other, often taxonomically related, species. This may be explained by structural similarities in their pheromone components (Figure 2.1 and 2.2). The similar constituents (Ambrogi et al. 2009) are hypothesized to be able to bind to similar olfactory receptors and result in the same attraction behavior (Unelius et al. 2013) among species from the same genus and also between different genera (Dickens and Mori 1989; Oehlschlager et al. 1995; Perez et al. 1994; Unelius et al. 2013; Zarbin et al. 2003). For example, studies on weevils in the *Pissodes, Sitophilus, Rhynchophorus,* and *Anthonomus* genera have shown that different species within these genera can be lured through similar pheromone blends (Alpizar et al. 2002; Eller et al. 1994; Hallett et al. 1993a; Walgenbach et al. 1983). Weevils of different genera can also be lured with the same pheromone compounds. For example, the commercial boll weevil, *A. grandis*, pheromone was attractive to pecan weevils, *Curculio caryae* (Collins 1996; Eller et al. 1994). There are few biological similarities between these species (Collins 1996), but both species are part of the Curculioninae subfamily and have similar pheromone components (Figure 2.3).

Different species of weevils may be lured to traps with combination lures. Weevils from the *Rhyncophorus* - and *Metamasius* genera could be lured by placing two lures

with two different pheromones, or a combination lure with two pheromones on one trap (Alpizar et al. 2002). The American palm weevil, *R. palmarum,* and West Indian sugarcane borer, *M. hemipterus*, were caught in the same trap using Combolure, consisting of a 1:1 mixture of commercial pheromone compounds, Metalure and Rhyncolure. One of the two compounds from Metalure was structurally related to the single constituent of Rhyncolure (Figure 2.2). There is also evidence that these two structurally similar compounds act synergistically for *R. palmarum*, but not for *M. hemipterus*. These weevils share the same host plant, sugarcane, which synergized trapping (Alpizar et al. 2002). It is possible that these weevils use the same host compounds to produce their respective pheromones.

Pheromone structure patterns mirrored in the weevil taxonomy

The pheromones that have been identified from non-Scolytinae Curculionidae exhibit structural trends (Figure 2.3) that appear to be mirrored in their taxonomy (Marvaldi et al. 2002; Ambrogi et al. 2009; Hundsdoerfer et al. 2009). The distribution of the functional groups in all described pheromone constituents give one example of trends from molecular structures of pheromones (Figure 2.4). This section describes these trends for different subfamilies of Curculionidae.

Pheromone molecular structure trends in the Entiminae subfamily

The recurrence of polar pheromone components is demonstrated in species within the Entiminae subfamily and in the *Sitophilus* genus (Curculionidae: Dryophothorinae) (Figure 2.3). In the Entiminae subfamily, *S. discoideus* has both enantiomers of 4S5R/S-sitophinone that have alcohol and ketone functional groups and *S. lineatus* has a diketone, an ester and an alcohol in its three pheromone compounds (Blight and Wadhams 1987; Park et al. 2013; Unelius et al. 2013). *Diaprepes abbreviatus* has ester functionalities combined with alcohol groups in the two components of the pheromone (Lapointe et al. 2012).

The pheromone of *S. granarius, S. zeamais* and *S. oryzae* has similar chemical structures in their constituents with variation occurring in the orientation of the methyl and hydroxyl moieties (Phillips et al. 1989). The difference between the pheromones from *S. granarius* and the others, include that 2S3R-sitophilate, contains an ester functional moiety bound to 1-ethylpropyl in its structure, as opposed to the ketone

functionality in 4S,5R-sitophinone that is connected to an ethyl group. The differences in these chemical structures are enough to prevent cross-attraction between these species (Walgenbach et al. 1983). Because these species in the Entiminae subfamily exhibit very similar functional moieties in their pheromone constituents, the unidentified pheromones of *Cyrtomon luridis* (Kamiya et al. 2015) and *Otiorhynchus sulcatus* (Van Tol and Visser 2002) are thus expected to have similar highly polar pheromone constituents, because they belong to this subfamily.

The trend of oxidative levels

Trends were observed regarding the oxidative levels in the pheromone structures of the different families of non-Scolytinae Curculionidae weevils (Figure 2.3). All described aggregation- and sex pheromones in this subfamily appear to be oxygenated except ß-caryophyllene (Supplementary Table 2.1, Figure 2.3). This compound was reported to be the sex pheromone produced by female *A. grandis*, but the structure is also a known plant and microbe volatile (Hedin et al. 1979; Kanchiswamy et al. 2015; Machado et al. 2018). Another trend includes that species within the Dryophthorinae subfamily tend to produce major pheromone constituents that have alcohol moieties. This trend seems to hold for all species except *Scyphophorus acupunctatus* (Ruiz-Montiel et al. 2008) and *Sitophilus* species (Chambers et al. 1996; Walgenbach et al. 1983) (Figure 2.3). The members of the Molytinae subfamily produce pheromone compounds with different levels of oxidation. These include alcohol major components from *Conotrachelus psidii* (Romero-Frías et al. 2016) and *P. castaneus* (Booth et al. 1983), aldehyde major constituents from *P. strobi* and *P. nemorensis* (Booth et al. 1983), and carboxylic acid major components from *C. nenuphar* (Eller and Bartelt 1996) *and C. humeropictus* (Szczerbowski et al. 2016).

Minor constituents have similar backbones than major constituents

Minor pheromone compounds generally have similar chemical structures when compared major pheromone compounds (Figure 2.3, major components precede minor components). Some species appear to have the oxidized form of the same pheromone structure in its minor pheromone component to form the blend specific to that species. Examples include *Rhynchophorus vulneratus* (Hallett et al. 1993a), *R.*

ferrugineus (El-Shafie and Faleiro 2017)*, C. humeropictus (Szczerbowski et al. 2016)*, and *C. caryae* (Hedin et al. 1997). The components with alcohol moieties within pheromone blends tend to be the major components and the corresponding aldehydes form the minor components in the pheromone blends. This was seen in weevils including *C. psidii* (Romero-Frías et al. 2016) and *P. castaneus* (Marques et al. 2011), among many others (Supplementary Table 2.1, Figure 2.3). In contrast, other species have reduced functional groups in the minor components of their pheromone blend, such as *S. acupunctatus* (Ruiz-Montiel et al. 2008) and *S. discoideus* (Unelius et al. 2013).

Additional components that mask attraction

Closely related species often avoid cross-attraction by a phenomenon that was termed odor camouflaging by Szendrei et al. (2011). It is the effect of the addition of one or more extra volatile component(s) that masks the attraction of an attractive blend. For example, *S. discoideus* produces a minor pheromone constituent, 4S5R-sitophinone, which has been hypothesized to prevent cross-attraction of *S. lineatus*, that utilizes the same major pheromone component alone (Unelius et al. 2013) (Figure 2.1 and 2.3). Similarly, the pheromones of *A. eugenii* and *A. musculus* share identical compounds, except for the addition of geranic acid and the alcohol derivative of Grandlure II, 1R2S-grandisol in *A. eugenii* (Szendrei et al. 2011).

Enantiomeric composition and recognition

Some species have enantiomeric blends and others have the pure enantiomers as pheromones. For example, *R. phoenicus* detects only the S-enantiomer of rhynchopherol with olfactory and behavioral responses, but *Rhabdoscelus obscurus* detects both R and S-enantiomers (reviewed in Giblin-Davis et al. 1996) (Figure 2.3). *Metamasius hemipterus sericeus* detects 4S5S-ferrugineol and only the R-enantiomers of both 2-methylheptan-4-ol and 2-methyloctan-4-ol (Perez et al. 1997). In contrast, *M. hemipterus* detects all enantiomers of these pheromone components (Perez et al. 1997). Other Curculionidae species with enantiomers in their pheromone constituents include *D. abbreviatus (Lapointe et al. 2012)*, *R. bilineatus* (Oehlschlager et al. 1995)*, R. phoenicis* and *R. cruentatus* (Perez et al. 1994). The appropriate receptor proteins need to be expressed on each species' antennae for such differential detection to occur (Unelius et al. 2013).

Unnecessary constituents for attraction

Weevils may produce volatiles that are not necessary for attraction (Figure 2.5). These compounds also appear to be very similar between related species (Figure 2.3). For example, *S. acupunctatus* produces two ketone components, 2-methylheptan-4-one and 2-methyloctan-4-one as the attractive pheromone blend. The alcohol minor constituents, 2-methylheptan-4-ol, 2-methyloctan-4-ol were found to be unnecessary for attraction (Ruiz-Montiel et al. 2008). The reason for production of these volatiles are not yet understood in weevils (Giblin-Davis et al. 1996). Similarly, *M. hemipterus sericeus* only needs ferrugineol and 2-methylheptan-4-ol for attraction of both sexes to traps, but eight EAD-active compounds were found from aerations of mixed sex beetles (Perez et al. 1997) (Supplementary Table 2.1, Figure 2.3). Four of these were the oxidized ketones corresponding to four alcohol compounds. The ketones were found to decrease trap efficacy and it is thus possible that these ketones have a different function for this species or they may just be artefacts from the analysis procedures (Perez et al. 1997).

Kairomones structures related to the pheromone structures?

There are numerous examples where host volatiles synergize the attractiveness of pheromone blends of Curculionidae weevil species (Tewari et al. 2014). The molecular structures of these host volatiles are hypothesized to resemble at least one pheromone component, or a pheromone precursor in the biosynthetic pathway (Supplementary Table 2.1). Hexanal, ethanol, pentane and ethyl acetate (Jaffé et al. 1993; Van Tol et al. 2012) were described as short-range attractants for *R. palmarum* (Weissling et al. 1994). These host volatile metabolites may be necessary for the biosynthesis of this palm weevil's major aggregation pheromone, rhynchophorol. Lavandulol, has been found to synergize the aggregation pheromone of *A. rubi* (Innocenzi et al. 2001), similar to linalool that augments pheromone attraction for *S. lineatus* (Blight et al. 1984). Receptors on the antennae of these species probably recognize the functional moieties on these kairomones or pheromone precursors, which are similar to the pheromone compound structures of these weevil species (Park et al. 2013).

Recent pheromone identifications

Pheromones have been identified for at least five non-Scolytinae Curculionidae species up until 2019, since the last review on Curculionidae pheromones (Tewari et al. 2014). These species include the bearded weevil, *Rhinostomus barbirostris* (Reis et al. 2018), the cocoa borer weevils, *C. humeropictus* (Szczerbowski et al. 2016) and the sugarcane weevil, *S. incurrens* (Illescas-Riquelme et al. 2016) (Supplementary Table 2.1). The pheromones are not effective for management of these species because understanding of the underlying chemical ecology for these species is still lacking.

Rhinostomus barbirostris (Curculionidae: Dryophthorinae) infests Arecaceae hosts such as oil palms and coconut trees in Brazil and they transmit the red ring disease (Reis et al. 2018). This weevil belongs to the same subfamily as *S. oryzae* and *S. zeamais* (Schmuff et al. 1984), and produces the same major pheromone component, (4S-5R)-sitophinone. The minor component was identified as the mirror image of the minor compound of rice and maize weevils, (4S-5S)-sitophinone (Reis et al. 2018). This pheromone blend is yet to be utilized commercially for this pest.

Nocturnal cocoa borer weevils, *C. humeropictus* (Curculionidae: Molytinae), oviposit eggs into plant stems of cocoa and cupuacu fruit-palms, causing crop losses of up to 100% in northern Brazil (Lopes and Silva 1998; Szczerbowski et al. 2016). The pheromone has been described recently as a mixture of (1R,2S)-grandisol, (1R,2S)-grandisoic acid, and grandisolide (Szczerbowski et al. 2016). These components are similar to the pheromone constituents of the *Pissodes* (Booth et al. 1983; Marques et al. 2011; Phillips et al. 1984) and *Conotrachelus spp.* (Eller and Bartelt 1996; Palacio-Cortés et al. 2015) in the Molytinae subfamily (Figure 2.3). Biological activity is yet to be confirmed in the field.

A pheromone was recently identified for the sugarcane weevil, *S. incurrens* (Curculionidae: Rhynchophorinae), of which a single racemic compound, 2-methyl-4-octanol was found to elicit aggregation behavior of both males and females (Illescas-Riquelme et al. 2016). This pheromone induces similar behavioral responses when compared to related species including *S. levis* (Zarbin et al. 2003) and many others in the Rhyncophorinae subfamily. Current research is aimed at lure optimization by identifying host volatiles that may act synergistically with the pheromone (Illescas-Riquelme et al. 2016).

The male-specific aggregation pheromones from *H. validus* and *H. depressus* (Curculionidae: Molytinae) have recently been identified (Vidal et al. 2017; Vidal et al. 2019). *Homalinotus validus* produces (1R,2S)-grandisol and (1R,2S)-grandisyl acetate and *H. depressus* produces a mixture of 1R2R6S-homalinol, 1S6S-epoxyisophorone, isophorone and 2-hydroxyisophorone. This four-component pheromone is equally attractive in field assays as a mixture compared to the use of only 1R2R6S-homalinol (Vidal et al. 2019). Blend ratio and trap optimization trials are underway.

Potential new pheromones for some weevil species.

Opportunities exist for the identification of pheromones for many weevil species (Table 2.1). Some behavioral or physiological evidence for the existence of pheromones has been reported for *Phlyctinus callosus* (Curculionidae: Entiminae) (Bredenhand et al. 2010) and *Paramasius distortus* (Curculionidae: Rhynchophorinae) (Giblin-Davis et al. 1996). Male and female *P. callosus* weevils are attracted to male frass (Barnes and Capatos 1989), and *P. distortus* weevils were unintentionally trapped in Rhynchophorol-baited traps (Giblin-Davis et al. 1996). Other studies have identified candidate pheromone components but field confirmation is lacking (Branco et al. 2019; Park et al. 2013). There are also many emerging non-Scolytinae Curculionidae pests for which no pheromone studies have been reported (Haseeb et al. 2019; Jones et al. 2019).

Control applications

The type of pheromone that is used for pest management of non-Scolytinae Curculionidae usually dictates what strategy is applied in the field. Short-range or contact pheromones are known for some Curculionidae (Ravi and Palaniswami 2002; Rivera et al. 2017; Tumlinson et al. 1969), but they have not been described as useful for pest management because of the limited range of luring capability. There are some examples where oviposition-deterring pheromones have been used in control measures, but these are few (Buntin and Raymer 1994; Ferguson et al. 1999). Long-range pheromones are more useful for trapping purposes than short-range pheromones (Tewari et al. 2014). Here, we discuss factors that influence the success of pheromones in field applications, including trap design, proper spacing of traps, pheromone release rates from lures and timing of trapping interventions. Examples of

monitoring, mass-trapping and mating-disruption are given with a focus on previous successes and failures (Baker 2008; El-Shafie and Faleiro 2017; Foshee et al. 2008; Gold et al. 2001).

Trap design

Trap design depends on the target species and how they move around in their environment (Laurent and Frérot 2007; Piñero et al. 2011; Silva et al. 2018). Knowing the behavior of the target pest can often help with the design of more effective traps. For example, a trap was developed to exploit the rice weevil's, *S. oryzae*, tendency to hide or crawl into cracks in the floor or in corners of enclosed spaces. This behavior was exploited by placing traps in the corner of the grain storage rooms which improved trap efficiency (Trematerra and Girgenti 1989). A floor trap was made of a corrugated cardboard with a center hole that was designed so that beetles would crawl into the hole containing host-synergized pheromone lures (Trematerra and Girgenti 1989). An added advantage for this trap design was that captured weevils signaled the presence of a food source within the trap and enhanced the trap efficiency (Trematerra and Girgenti 1989). Extensive research has gone into the optimal design of the plum curculio, *C. nenuphar* trap (Akotsen-Mensah et al. 2010; Leskey and Wright 2004), showing the importance of trap design for optimal sensitivity in field trapping implementations.

Trap design studies have shown that trap color and shapes may also have significant effects on the capture rate of weevils. For example, black and green traps of pyramidal shape consistently captured more plum weevils (Leskey 2006). Other weevils like the pecan weevil, *C. caryae*, was trapped more successfully with brown pyramidal traps (Tedders and Wood 1994) and cranberry weevils, *A. musculus*, with yellow sticky traps (Silva et al. 2018).

Trap placement and timing

Trap density has proven crucially important for enhanced capture rates of different weevil species. Confusion, or the onset of uncharacteristic behavior, such as sudden aggression or periods of no movement, is known to occur when weevils are exposed to high doses of pheromone (Ravi and Palaniswami 2002). Plum weevils, *C. nenuphar*, were captured in traps 1 m apart (Piñero et al. 2001), but 10 meter spacings proved even more effective (Piñero and Prokopy 2003). The behavior of the weevils near traps

1 m apart were observed and it was seen that beetles avoided each other possibly due to confusion (Piñero and Prokopy 2003), or avoidance of other beetles (Phillips et al. 1989). Trap densities of the New Guinea sugarcane weevil, *R. obscurus*, have also been investigated, where it was determined that 9 traps/ ha was optimal for effective control (Reddy et al. 2012).

The location of traps with the same lure types may cause trap interference, subsequently causing bias capture rates. It is thus necessary to randomize lures between traps as in the plum curculio case to avoid such biased results (Piñero and Prokopy 2003; Piñero et al. 2001). This minimizes the effect of environmental gradients that may be present, such as gradients of plot altitude, tree density, ground nutrition or weevil density in plantations. Having traps on plot edges rather than deeper into the plot can increase sensitivity of monitoring for weevil presence, especially when the source of weevils come from outside the plots (Eller and Bartelt 1996). Horizontal and vertical orientation of traps have also been proven to affect capture rates of the strawberry blossom weevil, *A. rubi* (Innocenzi et al. 2001).

Timing of trap interventions is crucial. Different species have different times of peak activity, and this affects when most weevils would be captured. Some species are active during the scotophase like the guava- and papaya borer weevils, *C. psidii* and *Pseudopiazurus papayanus* (Palacio-Cortés et al. 2015; Zarbin et al. 2007), whereas others are active during the photophase like the cactus weevil, *M. spinolae*, and West Indian sugarcane borer, *M. hemipterus* (Ramirez-Lucas et al. 1996; Tafoya et al. 2003). Understanding when lures will attract their target beetles optimally guide foresters to count captures at appropriate times.

Effective timing is also related to the physiological state of beetles. For instance, female maize weevils, *S. zeamais*, showed a significant reduction in responsiveness toward male-specific pheromones once mated. This could result in decreased trapping efficiency when more mated females are in the environment later in the season (Walgenbach and Burkholder 1986; Walgenbach et al. 1983). Another example was shown for the strawberry blossom weevil, *A. musculus*, where different sex ratios were captured during different seasons of the year (Innocenzi et al. 2001). These results may have reflected either the difference in behavioral responses of diapausal weevils or scattered emergence times between the sexes through the year. This knowledge is vital to interpret capture data, and may inform threshold counts before application of

population management measures. In contrast, plum curculio weevil, *C. nenuphar* lures stay attractive throughout host fruit development, and is not affected significantly by mating status and female sexual maturity (Piñero and Prokopy 2003; Piñero et al. 2001).

Release rates of pheromones from lures

Pheromone component release rates also need to mimic their target species for effective trapping (Hardee et al. 1974). Different ways have been utilized to manipulate pheromone release rates from traps. Dilution of pheromones with mineral oil that is loaded in lures can be used to lower lure release rates (Piñero et al. (2001). Similarly, an increase of lure release rates can be obtained by using more than one dispenser on a single trap. Doubling the number of lures on traps can double the release rates from traps (Piñero and Prokopy 2003). However, increasing pheromone release rates can result in a repellent behavior if the release rates become too high (Piñero and Prokopy 2003; Ravi and Palaniswami 2002). Innocenzi et al. (2001) has also indicated that the strawberry blossom weevil, *A. rubi*, flight might be arrested when conspecifics are exposed to high concentrations of the attractive pheromone in trapping experiments.

Pheromone concentrations in the environment must be as low as possible when weevils must pinpoint pheromone-baited trap locations. Therefore, pheromones should not be released into the environment in an uncontrolled manner. Sealed polymer materials are often used to release pheromone constituents from sealed dispensers. In pursuit of the best mimicry of natural release of pheromones, i.e. pheromone release per individual, the most effective polymer can be determined by investigating if the polymer consistently releases pheromone at rates near the natural release rate. Different polymer materials have different porosities and intrinsic polarities that influence the interaction of the pheromone constituents with the dispenser membrane. An effective polymer does not interact too strongly or weakly with the pheromone constituents' molecular structures so as to release the correct attractive blend for an optimal time.

Different polymers have been used for pheromone releases of non-Scolytinae Curculionidae. For example, Giblin-Davis et al. (2000) used high density polyethylene in black buckets. These lures released 3 mg of pheromone per day. Ruiz-Montiel et

al. (2008) used polypropylene microcentrifuge tubes with two or three pinholes in the caps and they released pheromone at a rate of 2.6 to 3 mg/day. It is important to know what the effective release rates are for specific species and applications.

Specificity in pheromone ratio blends

Pheromone release rate differences between species are less important than differences in their respective blend compositions. Pepper weevil males, *A. eugenii*, release approximately three times more pheromone than boll weevil males, *A. grandis* (Eller et al. 1994; Tumlinson et al. 1969), but their similar blend compositions causes interspecific attraction to the same pheromone blend (Eller et al. 1994). A similar trend is also seen in *Sitophilus* species, where rice and maize weevils, *S. oryzae* and *S. zeamais*, are attracted to the same pheromone components in very similar blend ratios (Walgenbach et al. 1983). Pheromones from species that have significantly different compound blend ratios normally do not function for other species that use similar compounds (Innocenzi et al. 2001).

Natural pheromone component ratios should be mirrored in a pheromone blend. If synthesized pheromone component ratios are not similar to the natural extracts, skewed sex ratios or failure of trapping interventions may occur (Ramirez-Lucas et al., 1996). The grain weevil, *S. granarius*, showed differential responses to pure synthesized 2S,3R-sitophilate compared to natural extracts from conspecifics (Faustini et al., 1982). This was due to the absence of another pheromone component still to be identified (Phillips et al., 1989; Chambers et al., 1996). The degree of skewness of the sex ratio of captured weevils with synthetic pheromone lures can be determined by comparing results to calling conspecifics as comparative controls in field trials (Hallett et al. 1993a; Mitchell and Hardee 1974).

Pheromone degradation may occur

Some environmental elements may degrade pheromones if they are sensitive to them. Researchers developed several strategies to combat these effects. For example, inverted green plastic cups kept out UV radiation and rain from grandisoic acid pheromone lures of plum weevils, *C. nenuphar* (Piñero and Prokopy 2003). Similarly, the use of white, rather than colorless low density polyethylene vials as lure dispensers for trapping of plum curculios, *C. nenuphar,* have been reported to minimize the UV radiation and polymerization of benzaldehyde (Piñero and Prokopy

2003; Piñero et al. 2001). Antioxidant UV absorbers like Tinuvin™ and Songsorb™ are also used in commercial insect traps to combat these effects, although these, or similar products have not been used in traps to capture non-Scolytinae Curculionidae. Pheromone components within a blend may also interfere with the stability of other components. An example of this was described for the strawberry blossom weevil, *A. rubi* pheromone, which contains aldehyde components that cause formation of alcohol breakdown products that can interfere with the alcohol-based pheromone blend (Innocenzi et al. 2001).

Strategies for trapping non-Scolytinae Curculionidae pests

Mass-trapping

Mass-trapping is one of the strategies to control weevil pests with pheromones. The aim is to capture as many individuals as possible, in order to reduce total population levels. Capture of females or both sexes are preferred for maximal effect. Mass-trapping has been used in individual weevil infested plots (Alpizar et al. 2002; Oehlschlager et al. 2002). However, area-wide efforts are preferred for maximal effect (Drmić et al. 2017; Haney et al. 2009; Reddy et al. 2012) because it will minimize the risk of re-infestation from nearby crops where trapping was not implemented. Mass-trapping can be improved when synergistic kairomones are used in conjunction with pheromone lures (Marques et al. 2011; Perez et al. 1997; Vera et al. 2016). When the pheromone lures only attract males into traps, there is a risk that the population levels might not be reduced significantly, especially when females mate more than once and lay large clutches of eggs (Innocenzi et al. 2001; Kamiya et al. 2015). For example, the potent female sex pheromone of the sweet potato weevil, *Cylas formicarius,* can attract many males (Yasuda 1999), but it is not effective enough to reduce progeny population numbers (Yasuda 1995). In such cases, pheromone-trapping would be more successful as monitoring strategy as a means to time the application of other pest management measures.

Mating-disruption

Mating-disruption is a pest management strategy that requires many pheromone point-sources distributed throughout the target environment. The aim is to release high concentrations of pheromones in order to interfere with the normal attraction between

males and females (Brown et al. 1992). This effectively disrupts mating over generations and reduces population growth (Baker 2008). The method has been proposed to be an effective control method for rhizome weevils, *Odoiporus longicollis,* because high doses of the sex pheromone in the environment confuses males when they search for females (Ravi and Palaniswami 2002). The pheromones of the *S. oryzae* (Trematerra and Girgenti 1989), *A. musculus* (Szendrei et al. 2011) and *A. eugenii* weevils (Eller et al. 1994) have been found useful in mating-disruption strategies, mostly for timing of control strategies that use insecticides.

Monitoring

Monitoring strategies are mostly used to detect the presence and relative abundance of weevils and is often used as a guide for timing of other management options (Baker 2008). The plum curculio, *C. nenuphar*, has been monitored in apple orchards (Piñero and Prokopy 2003), and peach orchards (Akotsen-Mensah et al. 2010; Johnson et al. 2002; Leskey and Wright 2004). Monitoring is used to estimate when population levels escalate to an unacceptable level (Reddy et al. 2012). Optimal sensitivity is necessary especially due to the high risk of large infestations from even a small number of emerged adults. For example, the pepper weevil, *E. eugenii*, can lay up to 300 eggs in their lifetime of up to 3.5 weeks (Capinera 2014), or a citrus root weevil female*, D. abbreviatus*, that can lay up to 29 000 eggs in its lifetime of three to four months (Mannion et al. 2003).

Pheromones in IPM

Pheromones are often used in conjunction with other pest management strategies. For example, if pheromone-baited monitoring traps indicate high weevil population densities, mass-trapping can be initiated in an attempt to lower population levels. This has been done for *R. obscurus* (Reddy et al. 2011; Reddy et al. 2012). Lure-and-release infection traps have been developed for banana weevils, *C. sordidus* (Tinzaara et al. 2005). In these traps, banana weevils are lured with a species-specific pheromone into infection traps, which are laced with an entomopathogenic fungus like *Beauveria bassiana*. Infected *C. sordidus* weevils are allowed to escape from specialized traps in order to spread the entomopathogen among other banana weevils (Tinzaara et al. 2007).

Lure-and-kill traps can be laced with insecticides as in studies on *R. ferrugineus* and *R. palmarum* (El-Shafie and Faleiro 2017; Oehlschlager et al. 2002). Biocontrol agents can also be used in this strategy. Lure-and-kill traps were developed for banana weevils, *C. sordidus* (Tinzaara et al. 2007), sweet potato weevils, *C. formicarius* (Yasuda 1999) and citrus root weevils, *D. abbreviatus* (Rivera et al. 2017). Comprehensive reviews have been written on the use of entomopathogenic nematodes and other biocontrol agents (Van Zyl and Malan 2014). Insecticides are mostly used as separate control measures especially if control through other methods is not achieved (Drmić et al. 2017; Hoffmann et al. 2009).

Conclusions

The family of Curculionidae is a diverse group of weevils and many are significant pests in agriculture and forestry. Since the discovery of the first pheromone from a non-Scolytinae Curculionidae species in 1969 from the boll weevil (Tumlinson et al. 1969), many pheromones have been identified from this group and are in various stages of development. Findings from these previous studies provide valuable tools for future studies, in the form of trends.

The molecular structures of the pheromone components previously identified from these weevils reveal four main end products by which evolutionary pressures have resulted in pheromones becoming species-specific. Similar chemical structures between related weevils also clarify why interspecific attraction is sometimes observed between species of even different genera during pheromone implementations in field trials. When this plasticity within species-specificity is applied responsibly, it can cut costs of pheromone-based lures without sacrificing the integrity of natural pheromones. For instance, when two related weevils produce pheromone constituents with adequately similar chemical characteristics, the same pheromone lure can be used to capture co-occurring pests, eliminating the need for a second lure. However, this system is dependent on the natural interspecific attraction between these species.

Patterns were apparent between weevils in close taxonomic relation and their corresponding pheromone constituents' molecular structures. Even though these trends do not ease the analysis and elucidation of novel pheromone components, the patterns can be used to predict whether a pheromone of a non-described weevil would follow a molecular structure trend specific to a certain subfamily.

This review also showed that optimizations of pheromone component blend ratios, trap design, trap placement and timing of trapping interventions are species-specific and have to be determined on a case-to-case basis. Even though innovative technology and new knowledge may facilitate pest management in the agriculture and forestry sectors, the use of good cultural practices such as ploughing (Tooke 1953) and soil rotation (Drmić 2016) cannot be replaced by these methods. Such practices should be implemented universally and integrated with pheromone-based tactics, such as monitoring, mass-trapping or mating-disruption for maximal effect.

In addition to the identification of trends, this review highlighted recent pheromone and putative pheromone identifications from non-Scolytinae Curculionidae species up until 2019. In light of these successes, opportunities for further identifications were summarized together with preliminary evidence of the use of pheromone communication in species for which pheromones have not been described.

This review outlined key factors to consider in future pheromone development studies specifically aimed for implementation of pheromone-based management options for non-Scolytinae Curculionidae species. With the use of trends from literature on pheromone chemical structures and implementation optimizations presented in this review, the time-frame needed for future pheromone development can hopefully be shortened.

Figure captions

- **Figure 2.1:** An overview of the main four solutions shaped by natural selection to maintain species-specificity of pheromones in terms of pheromone structures of non-Scolytinae Curculionidae weevil pheromones.
- **Figure 2.2:** The structural similarities of pheromone components in Metalure and Rhyncolure from *Metamasius hemipterus* and *Rhynchophorus palmarum*, respectively.
- **Figure 2.3:** Aggregation- and sex pheromone chemical structures for non-Scolytinae Curculionidae weevils in a hypothetical phylogenetic tree based on molecular work by Hundsdoerfer et al. (2009) and Marvaldi et al. (2002). Major pheromone constituents are drawn first. Species that have candidate pheromone structures are shown in brackets. The subfamilies are indicated with a key. Data from Supplementary Table 2.1.
- **Figure 2.4:** Functional moieties in the pheromone structures described to be necessary for field attraction in non-Scolytinae Curculionidae weevils. Data from Supplementary Table 2.1.
- .**Figure 2.5:** Weevil-specific volatile constituents that are either necessary or unnecessary for effective luring of non-Scolytinae Curculionidae weevils to pheromone-baited traps in field assays. Unneccessary constituents were identified as weevil-specific volatiles, but were proven to not have significant attraction capability toward the weevil under scrutiny. Data from Figure 2.2 and Supplementary Table 2.1.

Figures

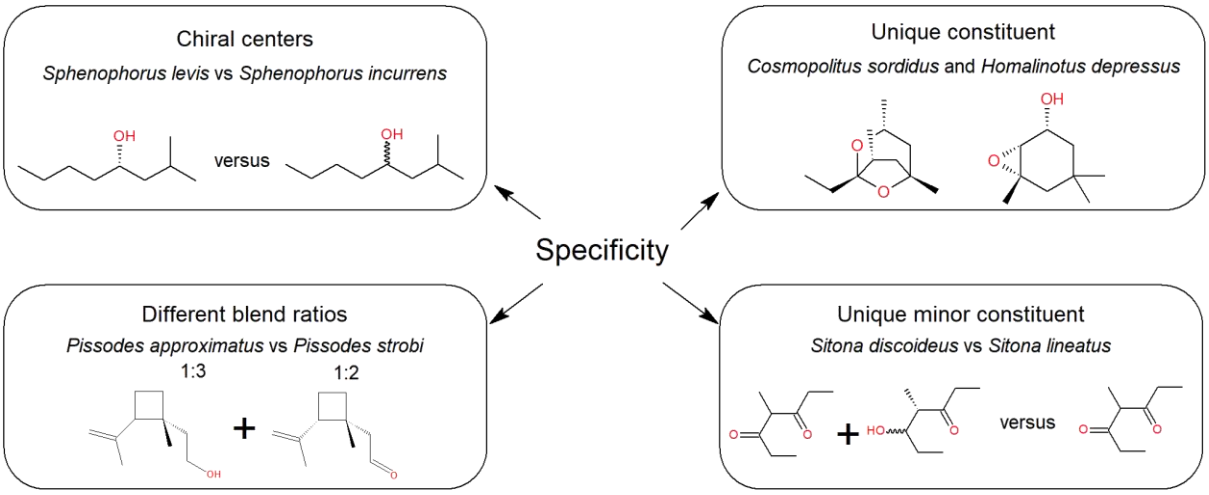


Figure 2.1: An overview of the main four solutions shaped by natural selection to maintain species-specificity of pheromones in terms of pheromone structures of non-Scolytinae Curculionidae weevil pheromones.

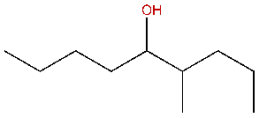
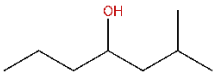
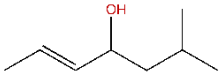
Metalure		Rhyncholure
		
Ferrugineol	2-methyl-4-heptanol	Rhynchopherol

Figure 2.2: The structural similarities of pheromone components in Metalure and Rhyncolure from *Metamasius hemipterus* and *Rhynchophorus palmarum*, respectively.

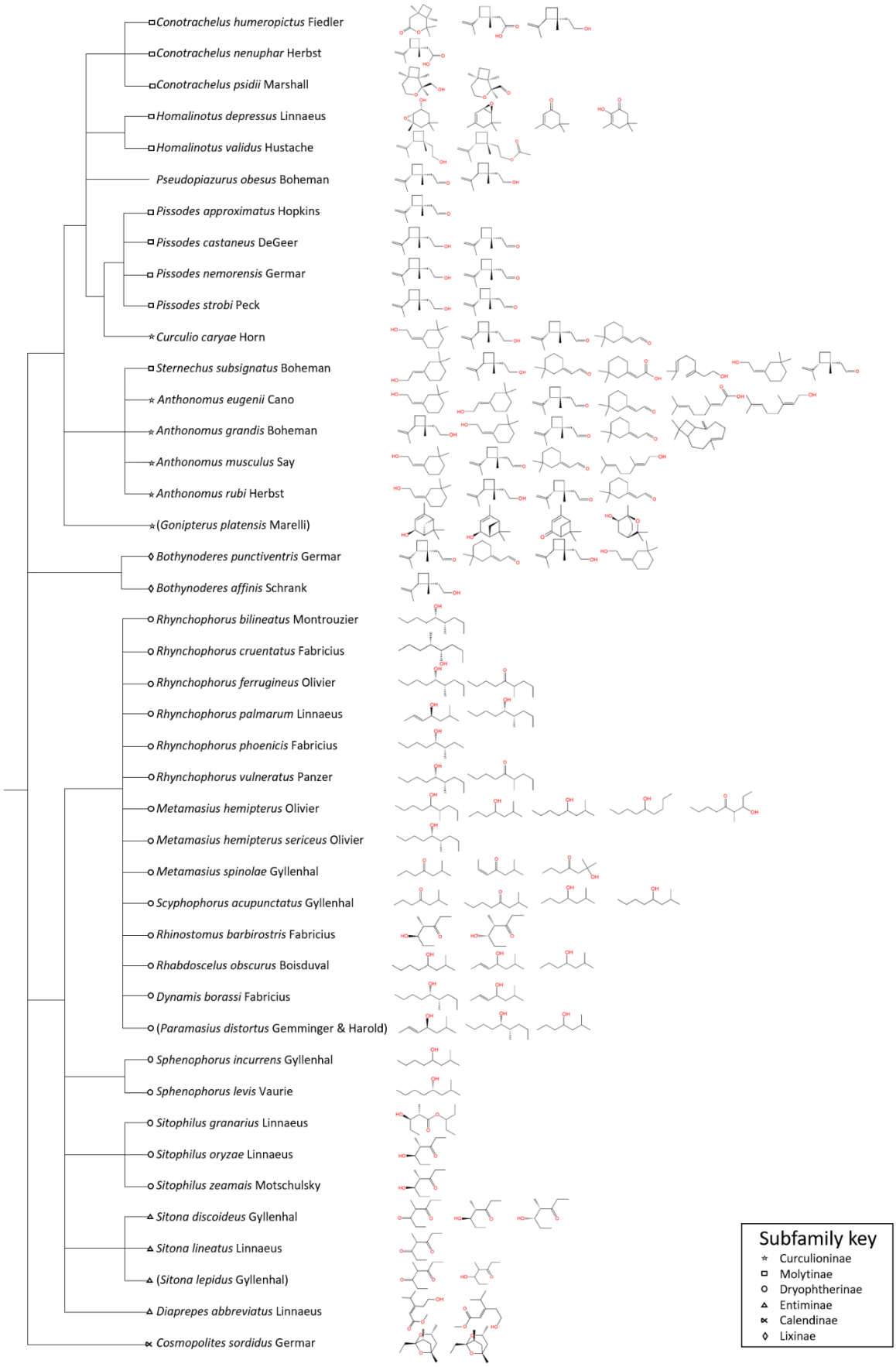


Figure 2.3: Aggregation- and sex pheromone chemical structures for non-Scolytinae Curculionidae weevils in a hypothetical phylogenetic tree based on molecular work by Hundsdoerfer et al. (2009) and Marvaldi et al. (2002). Major pheromone constituents precede minor constituents. Species that have candidate pheromone structures are shown in brackets. The subfamilies are indicated with a key. Data from Supplementary Table 2.1.

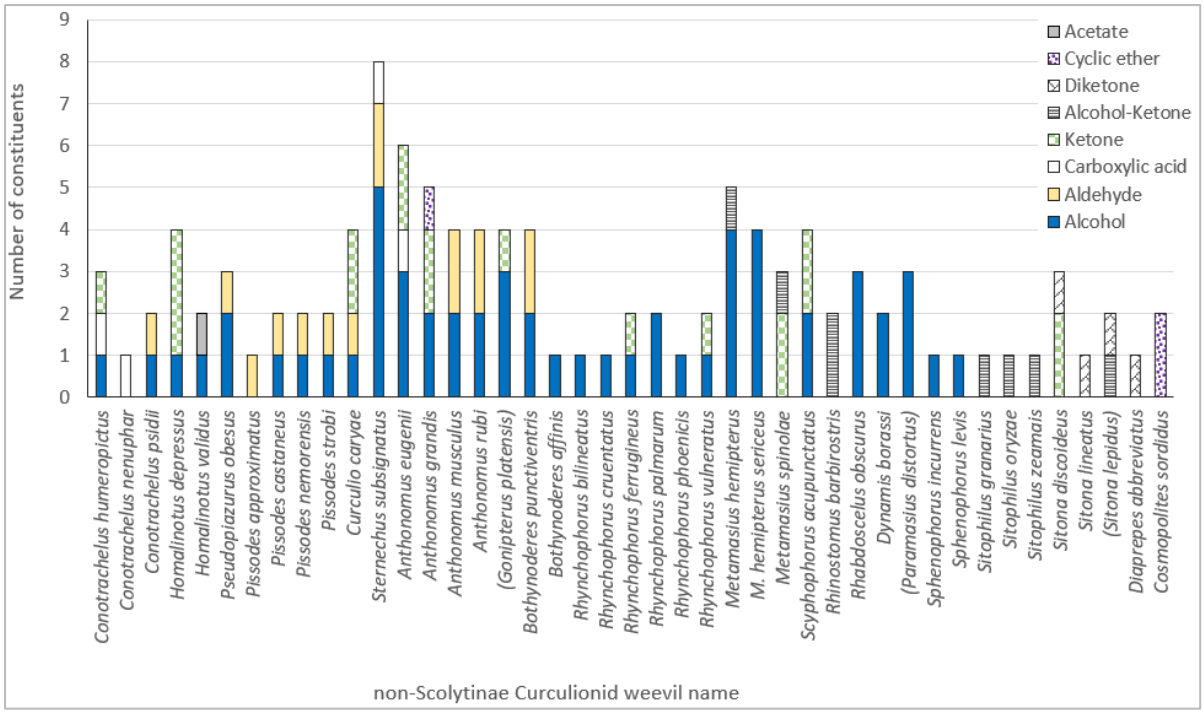


Figure 2.4: Functional moieties in the pheromone structures described to be necessary for field attraction in non-Scolytinae Curculionidae weevils. Data from Figure 2.2 and Supplementary Table 2.1.

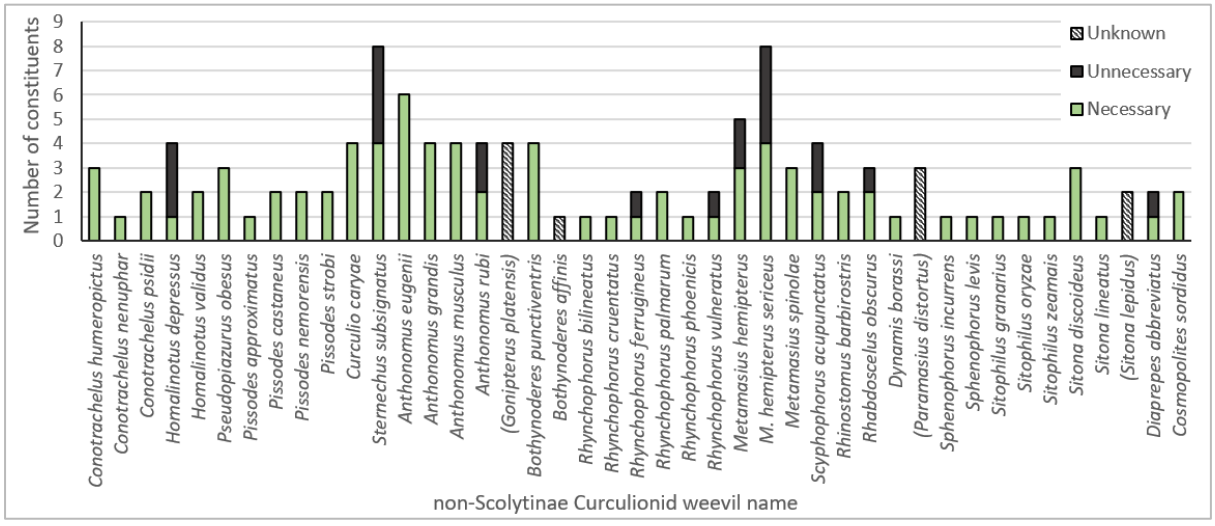


Figure 2.5: Weevil-specific volatile constituents that are either necessary or unnecessary for effective luring of non-Scolytinae Curculionidae weevils to pheromone-baited traps in field assays. Unneccessary constituents were identified as weevil-specific volatiles, but were proven to not have significant attraction capability toward the weevil under scrutiny. Data from Supplementary Table 2.1.

Table captions

- **Table 2.1:** Proposed weevils to consider for pheromone identifications and their known occurrence and behavior.

Supplementary table captios

- **Supplementary Table 2.1:** Pheromone components and attractants associated with non-Scolytinae Curculionidae.

Table and Supplementary table

Table 2.1: Proposed weevils to consider for pheromone identifications and their known occurrence and behavior.

Subfamily	Scientific name	Common name	Industry affected	Crop	Prevalent/native to..	Pheromone development	Previous observations	References
Baridinae	*Apinocis angustus* Casey	?	Pseudostem	Sugarcane	Mexico	Not started	None	Jones et al., 2019
Conoderinae	*Capturus aquacatae* Kessinger	Avocado twig borer/ avocado branch weevil	Fruit	Avocado	Mexico	Not started	None	Jones et al., 2019
Cossoninae	*Caulophilus oryzae* Gyllenhal	Broad-nosed grain weevil	Storage	Grain	Southern US, California, Baja, Central America, Cuba, Jamaica, Puerto Rico, Hawaii, Madeira, Europe	Not started	None	Haseeb et al., 2019
Cryptorhynchinae	*Euscepes postfasciatus* Fairmaire	Scarabee weevil	Fruit	Sweet potato	South America, Greater and Lesser Antilles, California, Hawaii, Tahiti	Not started	None	Haseeb et al., 2019
Curculioninae	*Smicronyx fulvus* LeConte	Red sunflower weevil	Flower/ oil	Sunflower	Unknown	Not started	Synergism between host and male volatiles to females	Roseland, 1990
Curculioninae	*Gonipterus platensis* Marelli	*Eucalyptus* snout weevil	*Paper and pulp*	*Eucalyptus* trees	Tasmania, Brazil, Western Australia, New Zealand, Argentina, Chile, the Iberian Peninsula, Canary Islands, Hawaii and California	Candidate pheromones identified	None	Branco et al., 2019
Entiminae	*Phlyctinus callosus* Schoenherr	Banded fuit weevil/ garden weevil	Fruit	Blueberries, apples, nectarines, grape vines/ root vegetables	South Africa, New Zealand, Australia	Not started	Both sexes aggregate to conspecific male frass	Bredenhand et al., 2010
Entiminae	*Myllocerinus aurolineatus* Voss	Tea weevil	Tea	Tea leaves	China	Not started	Attraction to host when males feed	Sun, 2010 & 2017
Entiminae	*Sitona lepidus* Gyllenhal	Clover root weevil	Legumes	Clover	Europe, North America, New Zealand	Candidate pheromones identified	Olfactory receptor neurons are sensitive to previously identified pheromones	Park et al., 2013
Entiminae	*Epicaerus operculatus* Say	?	Fruit	Garlic	Mexico	Not started	None	Jones et al., 2019
Entiminae	*Epicaerus aurifer* Boheman	?	Legumes, Sugarcane	Alfalfa	Mexico	Not started	None	Jones et al., 2019
Entiminae	*Amphidees latifrons* Sharp	?	Fruit	Apples	Northern Coahuila (Mexico)	Not started	None	Jones et al., 2019
Entiminae	*Diaprepes comma* Boheman	Citrus root weevil	Fruit	Citrus	South America, Carribean	Not started	None	Haseeb et al., 2019
Entiminae	*Exophthalmus vittatus* Linnaeus	Jamaican weevil	Fruit	Citrus	Jamaica	Not started	None	Haseeb et al., 2019
Entiminae	*Naupactus xanthographus* Germar	Grape weevil	Fruit	Grapes, avocado, citrus	Uruguay, Paraguay, Chile, Brazil, Argentina	Not started	Starvation promotes attraction to host volatiles	Vera et al., 2016
Molytinae	*Conotrachelus aguacatae* Barber	Mexican avocado seed borer/ small seed weevil	Fruit	Avocado	Mexico, Guatemala	Not started	None	Jones et al., 2019
Molytinae	*Heilipus lauri* Boheman	Avocado seed weevil/ large seed weevil	Fruit	Avocado	Mexico, Central America and Colombia	Not started	None	Jones et al., 2019
Molytinae	*Conotrachelus dimidiatus* Champion	?	Fruit	Guava	Mexico, Honduras, Guatemala	Not started	None	Jones et al., 2019
Molytinae	*Rhyssomatus nigerrimus* Fåhraeus	?	Fruit	Soybeans	Mexico, Panama, Lesser Antilles, Honduras, Guatamala and Belize	Not started	None	Jones et al., 2019
Molytinae	Pissodes schwarzi	?	Palm trees	Palm trees	Unknown	Not started	None	Marques et al., 2011
Rhynchophorinae	*Paramasius distortus* Gemminger & Harold	?	Palm trees	Palm trees	Unknown	Not started	Attraction to Rhynchophorus pheromone in field assays	Giblin-Davis et al., 1996

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Supplementary Table 2.1 :Pheromone components and attractants associated with non-Scolytinae Curculionidae.

Subfamily	Scientific name	Common name	Chemical structures	Producer	Pheromone type	#components	Major functionality	Minor functionality	# Unnecessary	Crops affected	Control mechanism	Synergism with host volatiles?	References
Molytinae	*Conotrachelus humeropictus* Fiedler	Cocoa borer weevil	1R6R-grandisolide, 1R2S-grandisoic acid, Grandlure I	Male	Aggregation (prediction)	3	Ketone	Carboxylic acid (1); Alcohol (1)		Cocoa and cupuaçu cultures	Under investigation	N	Szczerbowski et al., 2016
Molytinae	*Conotrachelus nenuphar* Herbst	Plum weevil	1R2S-grandisoic acid, benzaldehyde(h), ethyl-isovalerate(h), limonene(h)	Male	Aggregation	1	Carboxylic acid			Stone and pome fruit	Monitoring and insecticide applications	Benzaldehyde	Piñero et al., 2001 & 2003 & 2011; Eller et al., 1996; Johnson et al., 2002; Akotsen-Mensah et al., 2010
Molytinae	*Conotrachelus psidii* Marshall	Guava weevil	1R2S6R-papayanol, 1R2S6R-papayanal, limonene (h), Beta-caryophyllene (h)	Male	Aggregation	2	Alcohol	Aldehyde		Guava	Monitoring, mass-trapping and insecticide applications	Guava host volatiles	Palacio-Cortés et al., 2015; Romero-Frías et al., 2016
Molytinae	*Homalinotus depressus* Linnaeus	?	1R2R6S-homalinol, 1S6S-epoxyisophorone(u), isophorone(u), 2-hydroxyisophorone(u)	Male	Aggregation	4	Alcohol	Ketone (3)	3	Coconut trees	Research: mass trapping and monitoring	Unknown	Vidal et al., 2019
Molytinae	*Homalinotus validus* Hustache	?	(1R,2S)-grandisol, (1R,2S)-grandisyl acetate	Male	Aggregation	2	Alcohol	Acetate		Babassu palm	Research: mass trapping and monitoring	Unknown	Vidal et al., 2017
Unknown	*Pseudopiazurus obesus* Boheman	Papaya borer weevil/Papaya weevil	1R2S-grandisal, Grandlure I, papayanol	Male	Aggregation	3	Aldehyde	Alcohol (2)		Papaya	Insecticide applications. Pheromone traps under investigation	Fresh papaya stalk	Zarbin et al., 2007 & 2010
Molytinae	*Pissodes approximatus* Hopkins	Red Pine weevil	1R2S-grandisal	Male	Aggregation	1	Aldehyde			Stressed or transplanted pine trees, Spruce trees	Insecticide applications, pheromone lure and EPN mortality (not commercial)	Red pine branches	Booth et al., 1974; Booth et al., 1983
Molytinae	*Pissodes castaneus* DeGeer	Banded pine weevil	Grandlure I, 1R2S-grandisal	Male	Sex pheromone	2	Alcohol	Aldehyde		Pine trees	Unknown	N	Marques et al., 2011
Molytinae	*Pissodes nemorensis* Germar	Bark/ deodar weevil	Grandlure I, 1S2R-grandisal	Male	Aggregation	2	Alcohol	Aldehyde		Pine trees, Cedar trees	Monitoring under investigation	*Pinus elliotti* Engelmann bolts	Booth et al., 1983; Eller et al., 1996; Phillips et al., 1984; Hibbard et al., 1993
Molytinae	*Pissodes strobi* Peck	White pine weevil/ Sitka spruce weevil	Grandlure I, 1R2S-grandisal	Male	Aggregation	2	Alcohol	Aldehyde		Pine trees, Spruce trees	Insecticide applications, pheromone lure and EPN mortality (not commercial)	White pine bolts	Booth et al., 1983; Trudel et al., 2007
Curculioninae	*Curculio caryae* Horn	Pecan weevil	Grandlure II, E-(±)-Grandlure I, Grandlure III, Grandlure IV	Male	Aggregation	4	Aldehyde	Alcohol (1), Ketone (2)		Pecan nuts	Monitoring and insecticide applications	N	Collins, 1996; Hedin et al., 1997; Mody et al., 1975; Johnson et al., 2002
Molytinae	*Sternechus subsignatus* Boheman	Soybean stalk weevil	E-Grandlure II, Grandisol, Grandlure I, Grandlure IV, (Z)-3,3-dimethylcyclohexane-▲1,α-acetic acid, Z-Grandlure II, Grandlure III	Male	Aggregation	8	Alcohol	Alcohol (4), Aldehyde (2), Carboxylic acid (1)	4	Legumes, like soybean	Mass trapping	Soybean volatiles	Ambrogi et al., 2012
Curculioninae	*Anthonomus eugenii* Cano	Pepper weevil	Z-Grandlure II, E-Grandlure II, Grandlure III, Grandlure IV, Geranic acid, Geraniol, E-ß-ocimene(h)	Male	Aggregation	6	Alcohol	Alcohol (2), Ketone (2), Carboxylic acid (1)		*Capsicum* spp.	Monitoring and insecticide applications	E-ß-ocimene	Eller et al., 1994 & 2014; Patrock, 1986
Curculioninae	*Anthonomus grandis* Boheman	Boll weevil	(+)-Grandlure I, Grandlure II, Grandlure III, Grandlure IV, ß-caryophyllene, α-pinene(h), myrcene(h), limonene(h)	Male/Female	Aggregation/Sex pheromone	4	Alcohol	Alcohol (1), Ketone (2), other (1)		Cotton/boll plant	Mass trapping/ Monitoring	α-pinene, myrcene, and l-limonene	Tumlinson et al., 1969; Dickens et al., 1989
Curculioninae	*Anthonomus musculus* Say	Cranberry weevil	Grandlure II, Grandlure III, Grandlure IV, Geraniol	Male	Aggregation	4	Alcohol	Aldehyde (2), Alcohol (1)		Blueberry and cranberry flowers and flowerbuds	Monitoring	N	Szendrei et al., 2011; Silva, et al., 2018
Curculioninae	*Anthonomus rubi* Herbst	Strawberry blossom weevil	Grandlure II, Grandlure I, Grandlure III(u), Grandlure IV(u), Lavandulol(h), Germacrene-D(h)	Male	Aggregation	4	Alcohol	Alcohol (1), Aldehyde (2)	2	Strawberries and raspberries	Under investigation	*F. ananassa* volatiles	Innocenzi et al., 2001
Curculioninae	*(Gonipterus platensis* Marelli)	Eucalyptus snout weevil	Cis verbenol(?), trans-verbenol(?), verbenone(?), 2-α-hydroxy-1,8-cineole(?), myrtenol(h), α-pinene(h), ß-pinene(h), eucalyptol(h)	Unknown	Unknown	4	Alcohol	Alcohol (2), Ketone (1)		Eucalyptus plantations	Under investigation	Unkown, suspected.	Branco et al., 2019
Lixinae	*Bothynoderes punctiventris* Germar	Sugar beet weevil	Grandlure III, Grandlure IV, Grandlure I(u), Grandlure II(u)	Male	Aggregation	4	Aldehyde	Aldehyde (1), Alcohol (2)		Sugar beet	Mass-trapping	Unknown	Tóth et al., 2007
Lixinae	*Bothynoderes affinis* Schrank	Sugar beet weevil	Grandisol(?)	Male	Aggregation	1	Alcohol			Sugar beet	Monitoring	Unknown	Toshova et al., 2019
Dryophthorinae	*Rhynchophorus bilineatus* Montrouzier	Black Palm weevil (Asian palm weevil)	4S5S-ferrugineol	Male	Aggregation	1	Alcohol			Coconut palm trees	Mass trapping	Sugarcane	Oehlschlager et al., 1995; Giblin-Davis et al., 1996
Dryophthorinae	*Rhynchophorus cruentatus* Fabricius	Palmetto weevil	4S5S-cruentol	Male	Aggregation	1	Alcohol			Stressed or transplanted palms	Mass trapping	Palm tissue	Weissling, et al. 1994; Perez, et al. 1994
Dryophthorinae	*Rhynchophorus ferrugineus* Olivier	Asian palm weevil	4S5S-ferrugineol, 4S5S-ferrugineone(u)	Male	Aggregation	2	Alcohol	Ketone	1	Date palms	Monitoring, mass-trapping and insecticide applications	Palm tissue	El-Shafie et al., 2017; Giblin-Davis et al., 1996
Dryophthorinae	*Rhynchophorus palmarum* Linnaeus	American Palm weevil	5-rhynchophorol, 4S5S-ferrugineol, ethyl acetate(h), ethyl alcohol(h), isopentyl acetate(h)	Male	Aggregation	2	Alcohol	Alcohol		Commercial palms	Mass trapping together with removal of infected host material	Plant tissue and/or ethyl acetate; or sugarcane	Jaffé et al., 1993; Chinchilla et al., 1996; Alpizar et al., 2002; Oehlschlager et al., 2002; Perez et al., 1994
Dryophthorinae	*Rhynchophorus phoenicis* Fabricius	African Palm weevil	3S4S-phoenicol	Male	Aggregation	1	Alcohol			Oil palms	Monitoring and/or mass trapping	Oil palm tissue	Perez, et al. 1994; Giblin-Davis et al., 1996
Dryophthorinae	*Rhynchophorus vulneratus* Panzer	?	4S5S-ferrugineol, 4S5S-ferrugineone(u)	Male	Aggregation	2	Alcohol	Ketone	1	Palms	Unknown	Unknown	Oehlschlager et al., 1995; Giblin-Davis et al., 1996
Dryophthorinae	*Metamasius hemipterus* Olivier	West Indian Sugarcane borer/ Silky cane weevil	Ferrugineol, 2-methyl-heptan-4-ol, 2-methyl-octan-4-ol(u), nonan-5-ol(u), 3-hydroxy-4-methyl-nonan-5-one(u)	Male	Aggregation	5	Alcohol	Alcohol (3), Alcohol-Ketone	2	Sugarcane, banana, pineapple, various palm species including coconut, oil and ornamental palm trees	Mass trapping together with *Rhynchophorus palmarum*	Sugarcane	Ramirez-Lucas et al., 1996; Alpizar et al., 2002; Giblin-Davis et al., 1996
Dryophthorinae	*Metamasius hemipterus sericeus* Olivier	West Indian Sugarcane borer/ Silky cane weevil	4S5S-ferrugineol, (±)-2-methyl-heptan-(S/R)-4-ol, R-2-methyl-octan-4-ol(u), pentan-3-ol(u), 4S5S-ferrugineone(d), (±)-2-methyl-heptan-(S/R)-4-one(d), R-2-methyl-octan-4-one(d), pentan-3-one(d), ethyl acetate(h), ethyl butyrate(h), ethyl propionate(h)	Male	Aggregation	8	Alcohol	Alcohol (3)	4	Banana, pineapple, palms, and sugarcane	Mass trapping and entomopathogen infections	Sugarcane and ethyl acetate	Perez et al., 1997
Dryophthorinae	*Metamasius spinolae* Gyllenhal	Cactus weevil	2-methyl-heptan-4-one, 6-methyl-delta2-hepten-4-one, 2-hydroxy-2-methyl-heptan-4-one	Male	Aggregation	3	Ketone	Ketone (1), Alcohol-Ketone (1)		Prickly pear	Pheromone monitoring under investigation	Prickly pear host volatiles	Tafoya et al., 2003 & 2004 & 2007
Dryophthorinae	*Scyphophorus acupunctatus* Gyllenhal	Agave weevil	2-methyl-heptan-4-one, 2-methyl-octan-4-one, 2-methyl-heptan-4-ol(u), 2-methyl-octan-4-ol(u)	Male	Aggregation	4	Ketone	Ketone (1), Alcohol (2)	2	Agave	Mass trapping	Feshly cut agave foliage	Ruiz-Montiel et al., 2003 & 2008 & 2009; Azuara-Domínguez et al., 2013
Dryophthorinae	*Rhinostomus barbirostris* Fabricius	Bearded/ bottlebrush weevil	4S5R-sitophinone, 4S5S-sitophinone	Male	Aggregation	2	Alcohol-Ketone	Alcohol-Ketone		Coconut trees, oil palms; other *Arecaceae* species	Pheromone monitoring under investigation	Sugarcane volatiles	Reis et al., 2018
Dryophthorinae	*Rhabdoscelus obscurus* Boisduval	New Guinea Sugarcane weevil	2-methyl-octan-4-ol, rhynchophorol, 2-methyl-heptan-4-ol(u)	Male/Female	Aggregation	3	Alcohol	Alcohol (2)	1	Ornamental palms and palm plantations, sugarcane	Pheromone monitoring and mass trapping under investigation	Sugarcane and ethyl acetate	Chang et al., 1972; Giblin-Davis et al., 2000; Reddy et al., 2011 & 2012
Dryophthorinae	*Dynamis borassi* Fabricius	Palm weevil	4S5S-ferrugineol, rhynchopherol(a), cruentol(a)	Male	Aggregation	1	Alcohol	Alcohol		Coconut tree and star-nut palm	Pheromone monitoring and mass trapping under investigation	Sugarcane	Giblin-Davis et al., 1996 & 1997
Dryophthorinae	*(Paramasius distortus* Gemminger & Harold)	New Guinea Sugarcane weevil	5-rhynchophorol(a), 4S5S-ferrugineol(a), 2-methylheptan-4-ol(a)	Male	Aggregation	0	Alcohol	Alcohol (2)		?? No information given in article	?? No information given in article	?? No information given in article	Giblin-Davis et al., 1996
Dryophthorinae	*Sphenophorus incurrens* Gyllenhal	Sugarcane weevil	2-methyl-octan-4-ol	Male	Aggregation	1	Alcohol			Plants within the Poaceae family, also sugarcane	Monitoring under investigation	Sugarcane	Illescas-Riquelme et al., 2016
Dryophthorinae	*Sphenophorus levis* Vaurie	Sugarcane weevil	5-2-methyl-octan-4-ol	Male	Aggregation	1	Alcohol			Sugarcane	Monitoring under investigation	Unknown	Zarbin et al., 2003
Dryophthorinae	*Sitophilus granarius* Linnaeus	Grain weevil	2S3R-sitophilate	Male	Aggregation	1	Alcohol-Ketone			Wheat, barley, maize, cereal products	Monitoring	Unknown	Chambers et al., 1996; Faustini et al., 1982; Phillips et al., 1989
Dryophthorinae	*Sitophilus oryzae* Linnaeus	Rice weevil	4S5R-sitophinone	Male	Aggregation	1	Alcohol-Ketone			Wheat, rice, barley, corn, peas, pasta	Monitoring	Cracked corn, rice kernels, corn germ oil	Trematerra et al., 1989; Walgenbach et al., 1987

(Cont.)

Supplementary Table 2.1: (Continued)

Subfamily	Scientific name	Common name	Chemical structures	Producer	Pheromone type	#components	Major functionality	Minor functionality	# Unnecessary	Crops affected	Control mechanism	Synergism with host volatiles?	References
Dryophthorinae	*Sitophilus zeamais* Motschulsky	Maize weevil	4SSR-sitophinone	Male	Aggregation	1	Alcohol-Ketone			Corn, wheat, rice, sorghum	Monitoring	3-pentanone	Walgenbach et al., 1983 & 1986; Schmuff et al., 1984
Entiminae	*Sitona discoideus* Gyllenhal	Lucerne weevil	4-methyl-3,5-heptanedione, 4SSR-sitophinone, 4SSS-sitophinone	Male	Sex	3	Diketone	Ketone (2)		Lucerne	Monitoring under investigation	Unknown	Unelius 2013
Entiminae	*Sitona lineatus* Linnaeus	Pea and Bean weevil	4-methyl-3,5-heptanedione, (Z)-3-hexen-1-ol(h), (Z)-3-hexen-1-yl acetate(h), linalool(h)	Male	Aggregation	1	Diketone			Leguminous crops	Monitoring and/ or mass trapping under investigation	Z-3-hexen-1-ol, Z-3-hexen-1-yl acetate, linalool	Blight et al., 1984 & 1987
Entiminae	(*Sitona lepidus* Gyllenhal)	Clover root weevil	4-methyl-3,5-heptanedione(?), sitophinone(?)	Unknown	Unknown					Clover	Not implemented	Proposed (E)-2-hexenol, (Z)-2-hexenol, (Z)-3-hexenol, (E)-2-hexenal, (±)-linalool and (±)-a-terpineol	Park et al., 2013
Entiminae	*Diaprepes abbreviatus* Linnaeus	Neotropical or Citrus root weevil	Methyl (E)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate, Methyl (Z)-3-(2-hydroxyethyl)-4-methyl-2-pentenoate(u)	Male	Sex pheromone and synomone	2	Alcohol-Ketone	Alcohol-Ketone	1	Citrus, sugarcane, vegetables, potatoes, strawberries, woody ornamentals, sweet potatoes, papaya, guava, mahogany, containerized ornamentals, and non-cultivated wild plants.	Pheromone/EPN lure&infect	Citrus leaves	Lapointe et al., 2012; Rivera et al., 2017
Calendinae	*Cosmopolites sordidus* Germar	Banana weevil (=Rhizome weevil)	1S3R5R7S-sordidin, 7-episordidin	Male	Aggregation	2	Special	Special		Banana/plantain	Pheromone/EPN lure&infect	Unknown	Gold et al., 2001; Tinzaara et al., 2007, De Graaf et al., 2008, Budenberg et al., 1993
	Odoiporus longicollis Olivier	Banana pseudostem weevil	?	Male/Female	Unknown					Banana/plantain		Banana pseudostem extract	Palanichamy et el., 2011; Ravi et al., 2002
	Cyrtomon luridis Boheman	?	?	Male	Aggregation					*Duboisia spp.*	Pheromone lure (not commercial)	*Duboisia* host plant	Kamiya et al., 2015; Tironi et al., 2005
	Otiorhynchus sulcatus Fabricius	Black vine weevil	?		(Kairomones)					Ornamental plants and fruit crops eg within taxa incl Taxus, Rhododendron, and Euonymus	Pheromone monitoring under investigation	Unknown	Van Tol et al., 2002

() = putative pheromone identifications

(u) = attractive weevil volatile unneccessary for optimal attraction
(a) = attractive to this weevil in the field, but not produced by this weevil
(d) = dispersive weevil volatiles
(?) = candidate pheromones, not tested in field studies/structures not confirmed with standards
(h) = host volatiles

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Chapter 3

Characterization of the pheromone of the Eucalyptus snout weevil, *Gonipterus* sp. 2 (Coleoptera: Curculionidae)

Abstract

Gonipterus species 2 is a defoliator pest of *Eucalyptus* plantations in Africa, Italy and France. Recent economic loss due to this weevil suggests decreased effectiveness of biological control with *Anaphes nitens* in South Africa. Pheromone-based pest management strategies provide a sustainable and species-specific solution that can be used in integrated approaches with existing pest management strategies. In this study, the semiochemical profiles from *Gonipterus* sp. 2 headspace samples are explored with GC-EAD and GC-MS methods to find weevil-specific volatiles that may be pheromone components. We show that *Gonipterus* sp. 2 headspace volatiles contain cis- and trans-verbenol. These are two of eleven components that were recently identified as pheromone candidate components of *G. platensis*. This finding suggests that *Gonipterus* sp. 2 and *G. platensis* may utilize similar pheromone components, possibly in different blends. Treatment with Juvenile Hormone III (JHIII) did not increase putative pheromone production for *Gonipterus* sp. 2. Electroantennographic response signals differed between male and female antennae, suggesting the possibility of a dimorphism in the olfactory receptor neurons on their antennae. Findings from this study provide a good foundation for further development of the pheromone of *Gonipterus* sp. 2.

Introduction

The *Gonipterus* species complex is native to southeast Australia, Queensland, Victoria and Tasmania and comprises of eight cryptic species (Mapondera et al. 2012; Tooke 1953). *Gonipterus* species feed exclusively on *Eucalyptus* trees as larvae and adults (Loch and Matsuki 2010; Newete et al. 2011; Richardson and Meakins 1986; Tooke 1953). Some *Gonipterus* species have been introduced in areas where exotic *Eucalyptus* trees are grown and have become pests in these areas (Garnas et al. 2012; Tooke 1953; Wingfield et al. 2008). The first record of accidental introduction of this pest was in New Zealand in 1890 (Broun 1893; Garnas et al. 2012) and it has since spread into Africa (Mally 1924; Tooke 1953), non-native regions of Australia, parts of North America, South America and Europe (Garcia et al. 2019; Schröder et al. 2019). *Gonipterus* species preferentially feed on *Eucalyptus* flush, and can cause stunted growth of plantation trees in severe cases (Loch and Matsuki 2010; Richardson and Meakins 1986).

Yellow slug-like larvae were first observed in South African *Eucalyptus* plantations near Cape Town in 1916 (Tooke 1953). Entomologists from Australia could not identify the species initially (Tooke 1953), but a consensus was reached to name the weevil *Gonipterus scutellatus*. Recent genetic studies confirm that these beetles are part of cryptic species complex that are hardly distinguishable based on differences of their morphology (Mapondera et al. 2012). From only three invasive species in the *Gonipterus* sp. complex, *Gonipterus* sp. 2 is the only known species in South Africa (Garcia et al. 2019; Schröder et al. 2019) (Figure D.L). This cryptic species has also been reported to occur in France and Italy (Garcia et al. 2019; Mapondera et al. 2012; Schröder et al. 2019). *Gonipterus platensis* is known in New Zealand, Argentina, Uruguay, Brazil, Spain, parts of the USA, Portugal and Chile and *G. pulverulentus* has been reported from Argentina, Brazil and Uruguay (Garcia et al. 2019; Mapondera et al. 2012; Schröder et al. 2019).

Economic loss caused by this weevil species was initially devastating in South Africa because the preferred hosts of the weevil accounted for 80% of all *Eucalyptus* species planted in 1925 (Mally 1924). Foresters were advised not to plant 23 preferred species of *Eucalyptus*, of which *E. puncatata*, *E. maideni*, *E. viminalis* and *E. globulus* were described as being the most susceptible (Mally 1924; Tooke 1953). Insecticide application and ploughing were investigated as possible control measures

(Richardson and Meakins 1986; Tooke 1953). These methods were impractical especially in hard to reach areas and due to unintended wind dispersal of aerially applied insecticides (Richardson and Meakins 1986; Tooke 1953). Insecticides were not effective over long time periods and this meant that reapplication contributed to costs for control (Sarmento 2015; Tooke 1953; Valente et al. 2018). *Anaphes nitens,* an egg parasitoid of *Gonipterus* sp. 2, was discovered in southeastern Australia and subsequently introduced as biocontrol agent in South Africa in 1926 (Barratt et al. 2018; Tooke 1953). This control method was so successful in South Africa that it was regarded as a model example for biological control (Barratt et al. 2018; Garcia et al. 2019; Londt 1996).

Outbreaks of *Gonipterus* populations have occurred recently and it suggests that biological control with *A. nitens* is inadequate in South Africa (Barratt et al. 2018; Lawson et al. 2012). It is known that *A. nitens* is less effective as a control agent in drier areas of higher altitude such as those occurring in the highveld regions of Northwest, Limpopo, Mpumalanga and Gauteng (Sarmento 2015; Tooke 1953; Tribe 2005). It is also possible that the recent dry conditions in South Africa (Jury 2018) are contributing to the decreased effectivity of *A. nitens* as a control agent for *Gonipterus* sp. 2.

Pheromone-based control strategies are a great alternative to conventional methods and a number of pheromones have been identified from related weevil species (Gitau et al. 2013; Tewari et al. 2014). Some behavioral attributes of *Gonipterus* beetles can potentially be linked to the production of pheromones. A recent study has indicated that *G. platensis* adults reach sexual maturity roughly 21 days after emergence, and that peak reproductive activity is reached after 60 days (Branco et al. 2019). *Gonipterus* males display peculiar and prolonged (10 min to 24 hours) courtship behaviors that are followed by copulation (Santolamazza-Carbone and Cordero Rivera 1998). It is possible that this behavior is mediated by contact pheromones.

Gonipterus weevils can only be found sparsely in their native regions (Tooke 1953) and the existence of an aggregation pheromone to mediate aggregation and mate finding for *Gonipterus* sp. 2 is thus plausible. A recent behavioral assay of *G. platensis* showed that virgin females were significantly more attracted to extracts from virgin males (Branco et al. 2019). Eleven pheromone candidate components were identified

from samples of *G. platensis* weevils (Figure 3.1), and the identity of seven of these were confirmed with synthetic standards (Branco et al. 2019). The eleven compounds included verbenene, cis-verbenol, trans-verbenol, verbenone, 2-oxo-1,8-cineole, 2-α-hydroxy-1,8-cineole, 2-ß-hydroxy-1,8-cineole, 7-hydroxy-1,8-cineole, 9-hydroxy-1,8-cineole and 3-α-hydroxy-1,8-cineole. The authors suggest that *G. platensis* produce sex-pheromones (Branco et al. 2019), rather than aggregation pheromones (Ambrogi et al. 2009; Tewari et al. 2014). (S)-cis-verbenol, (+)-trans-verbenol and verbenene were identified as potential sex pheromones for *G. platensis* (Branco et al. 2019). Behavioral assays with these standards gave mixed results. Male and female virgins were attracted to cis-verbenol, whereas only virgin females were attracted to trans-verbenol (Branco et al. 2019). Mated females were repelled by verbenone and no significant responses were observed from mated males (Branco et al. 2019). These findings suggest that *Gonipterus* pheromones may be more complex than initially thought.

Topical application of Juvenile hormone III (JHIII) has been shown to increase pheromone production in other Curculionidae species including *Anthonomus grandis* males and *Ips confusus* (Borden et al. 1969; Hedin et al. 1982). The use of this hormone on *Gonipterus* sp. 2 is based on the assumption that this species follows either the polyketide or isoprenoid pheromone biosynthesis pathways (Vanderwel 1994). These metabolic pathways are used in most species in the Curculionidae family (Blomquist and Vogt 2003; Francke and Dettner 2005; Hibbard and Webster 1993; Phillips et al. 1989; Schmuff et al. 1984). Exploring whether this technique works for pheromone production changes in *Gonipterus* sp. 2 may be a way to overcome the production of small amounts of pheromone constituents per individual.

As discussed above, there is mounting evidence that *Gonipterus* weevils use pheromones as adults. Currently, however, there is no known effective pheromone for the control of *Gonipterus* sp. 2. The aim of this research is, therefore, to sample, identify and compare potential pheromone candidates of *Gonipterus* sp. 2 to those identified by Branco et al. (2019). Results will aid in the development of new pheromone formulations for *Gonipterus* sp. 2. Pheromone-based control strategies can potentially provide a sustainable pest management system for the *Gonipterus* species.

Materials and Methods

Insects

Adult *Gonipterus* sp. 2 weevils were collected from Jessievale (-26.221804, 30.468118), Bulwer (-29.8049195, 29.7553241) and the Die Wilgers (-25.765418, 28.310263) suburb in Pretoria, South Africa. Virgin individuals were obtained by rearing larvae in an insectarium at the FABI biocontrol facility on the UP experimental farm. Fourth-instar larvae were separated and placed into individual wells to pupate (Figure D.M and N). This ensured that they emerged and stayed virgin. Males and females were kept separated in plastic containers (lock&lock, 23 cm x 17 cm x 14 cm). These containers were placed in environmentally controlled incubators (Memmert, IPP260 Plus) that were programed with a 12-hour light regime and temperature of 22 ± 2 °C. Larvae and adults were fed *Eucalyptus dunnii* foliage.

Volatile profile characterization

Sampling methods

i. Static headspace sampling

The SPME sampling method was used to explore the method as a potential pheromone isolation protocol for *Gonipterus* weevils. Samples were taken from groups of reared or field collected weevils. The clean, dried (110°C) glass chambers consisted of a 40 mm female ground joint with a corresponding male 40/38 ground joint adapter. The male adapter had an open screw top that was sealed with a N12 1.3 mm PTFE lined septum (Machery-Nagel, #702292). One of two different conditioned SPME fibers (PDMS, #57300-U and 50/30 DVB/CAR/PDMS, #57328-U) were used for each experiment. A single fiber was sequentially exposed to the sealed environment of a blank or sample glass chamber for 15 minutes at a time, unless stated otherwise. After sampling, the fiber was directly desorbed in the injection port of a GC-EAD or GC-MS system.

ii. Dynamic headspace sampling (DHS)

Dynamic headspace sampling of groups of weevils (up to 75) was done by placing the insects of the same sex in 1-liter glass CONSOL jars, modified as part of a dynamic headspace sampling facility (Figure D.J). These jars were cleaned with soap and water and dried overnight in a drying oven (110°C). Blank jars were prepared in the same

way as the sampling chambers, just without any beetles. During sampling events, bottled air was filtered through a hydrocarbon trap (Supelco Superpure HC, #2-2445-U) and the sampling flow rates (5.34 ± 0.11 ml/min, mean ± standard deviation (SD)) were regulated with a manually adjustable regulator, specifically a glass bubble flowmeter. Teflon tubing (1.8" OD, Supelco, #20532) was used to pass clean air through bulkhead union fittings that were attached to custom modified lids of each Consol jar. PoropakQ (Supelco ORBO 1103, 50/80, 150/75 mg) filled glass air sampling filters were connected to the 6.35 mm (¼ inch) bulkhead union fitting at the outlet of the glass sampling chamber. The PoropakQ adsorbent was eluted with double distilled n-hexane (3 x 0.5 ml, 60 minutes). These extracted samples were stored at 4°C in a fridge before analysis.

iii. Frass solvent extracts

Frass (0.13 g from males (n = 1) and 0.32 g females (n = 1)) was collected over the period of one week from the rearing jars of between 20 to 50 separated male and female beetles. The frass was directly extracted with double distilled n-hexane (1.00 ml) for 1 hour.

Electroantennography

i. Antennal preparation

Antennae from field collected individuals were prepared by removal of the flagellum from the pedicel with a minora blade under a light microscope. The tip of the club end was also removed. Each antenna was oriented with the tip connected to the recording electrode of the EAD. The antennal preparation was moved to within 5 mm of the stimulus delivery system of the EAD. Micro-electrodes were made from pulled glass capillaries (Hirschmann, 120 mm) and Ag/AgCl wire electrodes. The capillaries were filled with Beadle-Ephrussi Ringer electrolyte solution (129 millimolar NaCl, 4.7 millimolar KCl, and 1.9 millimolar CaCl) and connected to the EAD (Figure D.I).

ii. GC-EAD

An Agilent 6890N gas chromatography system (GC) (Chemetrix, Midrand, South Africa) was coupled to an electroantennographic detection system (EAD) (Syntech, Hilversaum, The Netherlands). Two different temperature programs were used for the two columns that were installed in the GC-EAD system. For the ZBWax column (30 m

x 0.25 mm ID, 0.25 µm, 7HG-G007-11, Zebron™), the oven was held at 50°C for 1 minute and ramped to 250°C at a rate of 20°C/min. For the semi-nonpolar HP5 column (30 m x 0.32 mm ID, 0.25 µm, 19091J-413, Agilent™) the temperature was held at 50°C for 2 minutes and ramped to 300°C at a rate of 20°C/min, and held for 5 minutes. A volume of either 1 or 2 µL of each extracted sample was injected in splitless mode (vent time = 2 minutes, flow = 50 ml/min) under constant column head pressure (16 psi, He). SPME samples were directly injected into the inlet of the GC-EAD with the same parameters. Resultant deflections observed in the electroantennographic data upon blowing stimulus over antennae were regarded as true responses only when two or more of the replicated GC-EAD runs showed responses with the same retention index and when the responses were the same width as the chromatographic peaks.

GC-MS screening

A gas chromatography system (Agilent 7890B) was coupled to a mass spectrometer (5877B MSD). A volume of 1 to 2 µl of the dynamic headspace or solvent extracts were injected in spitless mode (vent time = 50 seconds, flow = 100 ml/min) into the GC-MS inlet (SPME injection liner, 0.75 mm ID, Supelco). SPME samples were directly desorbed into the same inlet. The temperature was held at 50°C for 2 minutes and ramped (10°C/min) to 250°C where it was held for up to 12 minutes. Separations were done on a ZBWax column (30 m x 0.25 mm ID, 0.25 µm, 7HG-G007-11, Zebron™) or ZB5 column (30 m x 0.32 ID, 025 µm, 7HM-G002-11, Zebron™).

Treatments

i. Males sampled with *E. dunni* leaf material

Three field collected male weevils were placed in a custom-made glass chamber (100 ml) together with a single *E. dunni* leaf. This treatment was sampled (n = 3) with a SPME fiber and analyzed on the GC-EAD. The blank chamber (n = 1) for this experiment, contained a single *E. dunni* leaf that was partially fed on by male weevils. Samples with similar contents were also sampled in large Consol jars with DHS and 1 µl of these extracts were injected into the GC-MS only.

ii. Virgin weevils

In the first experiment, twenty reared virgin male (n = 2) and female weevils (n = 2) were transferred separately into 1-liter glass CONSOL jars. These chambers contained strips of paper towel that provided a holding grip for the beetles. The headspace of each chamber was sampled with a SPME fiber and screened on male (n = 2) and female antennae (n = 2) with the GC-EAD. Seventy reared virgin males (n = 1) and females (n = 1) were also sampled with a SPME fiber, but these samples were desorbed into the GC-MS only.

In a second experiment, six virgin males and seven virgin females were sampled with dynamic headspace sampling. A volume of 1 µl of these extracts was injected into both the gas chromatography-flame ionization detector (GC-FID) and GC-MS. Blank chambers were prepared with paper towels, similar to sample chambers in each experiment.

iii. Field collected weevils
    1. Juvenile hormone III (JHIII) treatment

Juvenile hormone III (Sigma Aldrich, #J2000) was applied to *Gonipterus* sp. 2 beetles in an attempt to force pheromone production. A small volume of JHIII solution (between 1 and 2 µl, 5 µg/ml, acetone) was applied to 75 males (n = 1), and 75 female weevils (n = 1). This was done by touching the ventral abdomen with a loaded glass capillary (Hirschmann, 120 mm). This enabled absorption of the hormone through the gut (Dickens and Mori 1989). A similar set of 75 male (n = 1), and 75 female weevils (n = 1) were treated only with the acetone solvent as control treatments (hereafter referred to as untreated). Beetles were rested and fed for 24 hours after the treatment. Beetles were removed from their food source 30 minutes before dynamic headspace sampling commenced in the dynamic headspace facility.

Dynamic headspace sampling took place over four days without feeding, with the same sampling and extraction parameters that were described in the dynamic headspace sampling procedure. A volume of 2 µl of these extracts was screened on the GC-EAD. The untreated female sample was screened with six male antennae and four female antennae. The untreated male sample was screened with six male antennae and seven female antennae. All JHIII-treated samples were screened with five male and five female antennae. A volume of 1 µl of each of the extracts was also injected into the GC-MS.

The same 150 treated, and 150 untreated weevils were also sampled with a SPME fiber and solvent rinsing of the sampling chambers. SPME samples were prepared by following the same procedures as previously described. Chamber rinse samples were prepared by washing the surface of sampling chambers with double distilled n-hexane (4 x 0.25 ml) after removal of weevils from the sampling chambers. Both the SPME and solvent rinse samples (2 µl) were analyzed on the GC-MS. The surface wash samples were stored in GC vials at 4°C until analysis.

iv. Frass samples

The headspaces of male (0.13 g, n = 1) and female frass (0.32 g, n = 1) were sampled with a SPME fiber for a period 30 minutes in custom made glass chambers (100 ml). The same chamber contents were also directly extracted (n = 1) with n-hexane. The frass extracts (1 µl) were screened on male antennae (n = 1), and the SPME samples were screened on female antennae (n = 2). The chambers were washed and dried (100°C) before use. During sampling, a sealed chamber contained either frass or no frass as a control.

Confirmation of compound identities

i. Standards

Runs of a 1 ppm n-alkane (C7-C30, Sigma Aldrich, #49451-U) standard were used for the calculation of Kovat's retention indexes (I_K) for every experiment. The S-cis-verbenol (Sigma Aldrich, 95 %, #247065-5G) and S-(-)-verbenone (Sigma Aldrich, 94 %, #218251-10G) standards were purchased from Sigma Aldrich. A blend of *Eucalyptus* green leaf volatile reference standards was used to confirm the identity of some compounds in the samples (Addendum A).

ii. Electrophysiological confirmations

Male and female antennal responses (n = 5) were recorded on the GC-EAD system toward 100 ppm S-(-)-verbenone and S-cis-verbenol purchased standards. The same GC method parameters were used as for extract analyses performed on the ZBWax column.

iii. GC-MS confirmations

A volume of 1 µl of the S-cis-verbenol, verbenone (both 100 ppm in double distilled n-hexane) or *Eucalyptus* green leaf volatile (unknown concentrations) reference

standards were injected into the inlet port of the GC-MS system for comparison of mass spectra and retention index characteristics with sample compounds. The same instrumental parameters were used for reference standards as for samples.

Results

Males sampled with *E. dunni* leaf material

The headspace samples of males (n = 3) in the presence of *Eucalyptus* leaf material did not result in clearly distinguishable and repeatable electroantennographic responses from male or female antennae (Figure 3.2). Small responses toward chromatographic peaks included those with retention times of 6.60 min and 6.93 min, corresponding to retention indexes of I_K = 1664 and 1715 (DBWax) (Figure 3.2, Supplementary Table 3.1).

Analysis of DHS of single male or female weevils with *E. dunni* leaf material (n = 1 for each of the six treatments) on the GC-MS revealed that the chromatographic peaks associated with the antennal responses were not cis-verbenol or verbenone. The respective mass spectra were more similar to γ-gurjunene (I_K = 1661) and ß-longipinene (I_K = 1716) (Figure 3.3, ZBWax column). Trans-verbenol (12.038 min, I_K = 1681) was detected only in DHS of males irrespective of the presence of host material (Figure 3.3, Table 3.3).

Virgin weevils

SPME

No clear EAD responses were detected from female antennae (n = 2) toward SPME samples of 20 virgin female beetles (n = 4 of the same treatment, Figure 3.4). Barely detectable, repeatable signals from male antennae (n = 2) to the female headspace samples included those at 3.33 min (I_K = 1209), 3.447 min (I_K = 1224), 4.86 min (I_K = 1409), 6.76 min (I_K = 1688) and 6.86 min (I_K = 1703), each corresponding to chromatographic peaks with the same retention characteristics (DBWax, Figure 3.4, Supplementary Table 3.1).

Male antennae did not respond with the same intensity to the same chromatographic peaks from male headspace samples (n = 5, Figure 3.4). The concentration of many chromatographic peaks was higher in samples obtained from female beetles. Some of these included peaks with retention characteristics of 3.72

min (I_K = 1259), 5.32 min (I_K = 1474), 6.23 min (I_K = 1606), 6.63 min (I_K = 1668), 6.76 min (I_K = 1688), 7.15 min (I_K = 1751) and 8.72 min (I_K = 2022), among others (Figure 3.4).

There were repetitive gradual deflections in the baseline of all responses throughout the GC-EAD screening procedure for this experiment, but they did not interfere with the identification of antennal responses. These deflections could have been caused by background noise that could have come from disturbances in the environment or were of instrumental origin. EAD signals from female antennae did not result in clear observable responses when compared to responses seen for male antennae. Barely detectable potential deflections of individual female antenna sometimes occurred but they were not repeatably observed in multiple recordings (Figure 3.4, Supplementary Table 3.1). Some of the notable deflections, that did not meet criteria to be considered as responses, had retention indexes that included I_K = 1206, 1310, 1688, 1704 and 1875 (Supplementary Table 3.1).

Chromatographic elution profiles showed that most peaks had similar retention characteristics when comparing samples obtained from 20 and 70 virgin males (Figure 3.4 and 3.5). The same was true when comparing chromatograms from samples of 20 and 70 virgin females (Figure 3.4 and 3.5). Two chromatographic peaks that gave antennal responses (I_K = 1688 and 1704) were present in both male and female headspace samples. These peaks had retention indexes of I_K = 1683 and 1714 on the GC-MS system (Figure 3.5, ZBWax column). Mass fragmentation patterns and elution times from the samples of 70 virgin weevils were similar to those obtained for the trans-verbenol and S-(-)-verbenone reference standards (Figure 3.5 and Supplementary Figure 3.1, Table 3.3). However, both compounds were also present in the sample blank in high abundance. It is possible that either the blank or sample injection syringes were not sufficiently clean when injected into the system.

DHS

Chromatographic peaks with retention indexes of I_K = 1659, 1683 and 1713 were detected in dynamic headspace samples of small groups (6-7) of weevils. These compounds were not detected in blank samples (Supplementary Table 3.2). The presence of trans-verbenol was confirmed via analysis on the GC-MS (peak 9 in Figure 3.6). The presence of cis-verbenol and verbenone could not be confirmed,

however a tentative identification was made for allo-aromadendrene (I_{Klit} = 1650) (peak 8 in Figure 3.6) and n-dodecanal (I_{Klit} = 1711) (peak 10 in Figure 3.6, Table 3.4).

Field collected weevils

JHIII-treatment

More compounds were detected in dynamic headspace samples from groups of untreated females when compared to groups of untreated males (Supplementary Table 3.3). GC-EAD screening of the same (untreated) samples on multiple male and female antennae, revealed that male and female antennae responded with larger, more frequent responses to chromatographic peaks in untreated female samples than untreated male samples (Figure 3.7A and 3.7B). Female antennae also responded to more chromatographic peaks than male antennae (Table 3.5).

Both male and female antennae responded repeatably to the untreated female extract sample (n = 1). Female antennae (n = 4) responded at I_K = 1202, 1396 and 1723 and male antennae (n = 6) responded at I_K = 1402, 1614 and 1772 (Figure 3.7A, Table 3.5). Male and female antennae also responded to the untreated male extract (n = 1). Female antennae (n = 7) responded at I_K = 1684, 1521 and 1971 and male antennae (n = 6) at I_K = 1283, 1617 and 1947 (Figure 3.7B, Table 3.5). Most deflections were generally below our measurement limit of the EAD software (20 µV).

Analysis of JHIII-treated samples also revealed multiple, barely detectable electroantennographic responses. Female antennae responded to the tentatively identified α-terpineol (6.85 min, I_K = 1702) in both treated male (n = 5) and female samples (Table 3.6, Figure 3.7C (not shown), n = 5). Other responses were also seen, including those with retention indexes I_K = 1298, 1664 and 1684 (Figure 3.7C). Male antennae responded at 6.6 min (I_K = 1664) and 6.8 min (I_K = 1694) only in treated male samples (Figure 3.7D, n = 5). These retention indexes correspond to cis-verbenol and tentatively identified 2-oxo-1,8-cineole, respectively (Table 3.6 and Supplementary Table 3.2). Responses with retention indexes I_K = 1322, 1609, 1842 and 1929 were also observed (Figure 3.7C). The signal of male antennae generally resulted in a more stable baseline after data processing than female antenna signals toward JHIII-treated samples. In general, more responses were seen toward untreated or treated samples that contained more volatiles, especially in larger concentrations (Figure 3.7A-D).

Certain chromatographic peaks were only detected via SPME sampling and not DHS, irrespective of the samples analyzed. These included peaks with retention indexes of I_K =1121, 1695, 1699 and 1717. These peaks were tentatively identified as dehydrosabinene, 2-oxo-1,8-cineole, ß-longipinene and verbenone, respectively (Figure 3.8 and Supplementary Figure 3.4, Table 7 and Supplementary Table 3.2). Verbenone, specifically, was only identified in the JHIII-treated male sample. Solvent-wash samples of the sampling chambers revealed mostly semi-volatile compounds, that were not present in DHS or SPME samples (Supplementary Figure 3.4, Table 3.4). Volatiles previously detected in SPME and DHS samples, were present in low to undetectable quantities in the chromatograms of the solvent-wash samples (Supplementary Figure 3.4, Table 3.4).

Tentative identification of some chromatographic peaks, that potentially resulted in antennal responses, included 4-methyl-1-pentanol (7.319 min, I_K = 1316, I_{Klit} = 1315), 3-pentan-2-ol (8.025 min, I_K = 1366, I_{Klit} = 1366), α- terpineol (12.265 min, I_K = 1702, I_{Klit} = 1697), bicyclogermacren (12.714 min, I_K = 1742, I_{Klit} = 1735), 2-hydroxy-1,8-cineole (14.071 min, I_K = 1866, I_{Klit} = 1845), globulol (16.287 min, I_K = 2085, I_{Klit} = 2086) and viridiflorol (16.378 min, I_K = 2094, I_{Klit} = 2095) (Table 3.6 and Supplementary Table 3.2). These peak identities require confirmation with reference standards.

Compound identities that were verified with reference standards, included dehydrosabinene (4.553 min, I_K = 1121, I_{Klit} = 1124), limonene (5.631 min, I_K = 1198, I_{Klit} = 1200), cis-verbenol (11.798 min, I_K = 1663, I_{Klit} = 1663), trans-verbenol (12.055 min, I_K = 1684, I_{Klit} = 1683), verbenone (12.428 min, I_K = 1717, I_{Klit} = 1725) and myrtenol (13.365 min, I_K = 1800, I_{Klit} = 1796) (Table 3.6 and Supplementary Table 3.2) in this experiment.

Frass samples

Headspace samples of male and female frass showed the same elution profile when sampled with the solvent extraction method (Figure 3.9A and 3.9B). The electroantennographic responses of a female antennae (n = 1) to male frass extracts (n = 1) resulted in antennal responses occurring at 3.28 min (I_K = 1202), 6.80 min (I_K = 1710) and 8.82 min (I_K = 2060) (Figure 3.9A) and these responses were also observed to female frass samples (n = 1) with another female's antenna (n = 1) (Figure 3.9B, Supplementary Table 3.3).

Confirmation of compound identities

Reference standards of S-cis-verbenol, trans-verbenol and S-(-)-verbenone eluted at I_K = 1659, 1683 and 1713, respectively on the ZBWax column (1,2 and 3 in Figure 10), and at I_K = 1151, 1152 and 1224 on the ZB5 column (Supplementary Figure 3.5, Table 3.4). Female antennae responded repeatably to S-cis-verbenol, trans-verbenol and S-(-)-verbenone (Figure 3.10). Male antennae responded repeatably to trans-verbenol, but responses to S-cis-verbenol and S-(-)-verbenone were not repeatable, and mostly unclear in recordings (Figure 3.10). The intensity of all the responses from male and female antennae were below the detection limit of our EAD software (20 µV), even when standards were screened at a high concentration of 1000 ppm (Figure 3.10).

The standard blend of *Eucalyptus* host volatiles contained (-)-ß-pinene, dehydrosabinene, limonene, eucalyptol, p-cymene, cis-3-hexenyl acetate, cis-3-hexenol, α-terpinyl acetate, benzyl acetate, myrtenol, ß-phenethyl alcohol and phenethyl alcohol (Addendum A). From these, only eight host volatiles were found in our samples, including (-)-ß-pinene, dehydrosabinene, limonene, eucalyptol, p-cymene, cis-3-hexenol, myrtenol and phenethyl alcohol (Table 3.4).

Discussion

Gonipterus sp. 2 is a notorious defoliator in *Eucalyptus* plantations in Africa, Italy, France and Western Australia (Mapondera et al. 2012; Schröder et al. 2019). Identifying a pheromone could potentially improve current pest management tools for it. In this study, we confirmed the presence of three candidate pheromone components for *Gonipterus* sp. 2. The compounds include trans-verbenol, cis-verbenol and a tentative identification for 2-oxo-1,8-cineole. This finding confirms that the candidate pheromone of *Gonipterus* sp. 2 is in part similar to the candidate pheromone of the cryptic *Gonipterus platensis* species (Mapondera et al. 2012; Schröder et al. 2019; Tooke 1953). These compounds can now be tested for behavioral activity in *Gonipterus* sp. 2.

We found cis-verbenol in female samples and trans-verbenol in both male and female samples. Our results differ from those in Branco et al. (2019), because they found that both cis- and trans-verbenol were male-specific for *G. platensis* (Branco et al. 2019). Branco et al. (2019) also determined that the reference standards of cis-

and trans-verbenol were attractive toward virgin *G. platensis* weevils (Branco et al. 2019). *Gonipterus* sp. 2 is closely related to *G. platensis* (Mapondera et al. 2012) and it is possible that these compounds are also attractive for *Gonipterus* sp. 2. Blend composition between these compounds may differ between the two species. Production of different blends of the same components is often the mechanism of preventing interspecific attraction in the Curculionidae family, such as for species in the *Pissodes*, *Anthonomus* and *Sitophilus* genera (Booth et al. 1983; Schmuff et al. 1984; Szendrei et al. 2011). Blend ratio differences between these two species still require investigation.

Electroantennographic responses from *Gonipterus* sp. 2 antennae were particularly difficult to detect. Some repeatable antennal deflections were observed, but they were mostly below the detection limit (20 µV) of the EAD software. Noisy EAD signals appear to be a common observation for this group of insects and success may be very dependent on the quality of the antennal preparation. Noisy signals were recorded for *G. platensis* (Branco et al. 2019), however antennal preparations similar to ours have resulted in better signals in *Sitona discoideus* (Unelius et al. 2013). Antennal preparations in other Curculionidae studies were performed by piercing antennal tips or weevil heads with either the recording electrode or grounding electrode (Chambers et al. 1996; Unelius et al. 2013). Different methods for connecting electrodes in *Gonipterus* sp. 2 should be explored and could potentially resolve some of these difficulties.

Measurable electroantennographic responses were typically seen when larger concentrations of compounds (>10 ng) eluted from the capillary column. Smaller concentrations (<10 ng) often did not result in measurable responses. It is likely that *Gonipterus* weevil antennae are not as sensitive to some of the compounds in our samples as expected. The concentration of the compounds in the samples may need to be increased to detect the small responses from the antenna. Branco et al. (2019) used large concentrations of reference standards for behavioral assays of *G. platensis*, and it is thus possible that these weevils only respond behaviorally when the compounds reach a certain threshold concentration. This behavior may be related to how sensitive their antennae are for certain compounds. In comparison, moth antennae are generally more sensitive to pheromones and recordings often show clear responses to minute amounts of pheromone (Millar et al. 2010). From our results, it

appears that larger concentrations (>50 ng) often give better results on the EAD with the weevil antennae.

Female antennae respond with larger intensity and sharper depolarizations when large concentrations of active components are blown over it. Similar findings were reported for *Cosmopolites sordidus* and *Sphenophorus incurrens*, where female antennae were also more sensitive than male antennae (Budenberg et al. 1993; Illescas-Riquelme et al. 2016). This dimorphism has been shown to be due to a difference in the male and female olfactory receptor neurons on weevil antennae, such as for *Sitona lepidus* and *S. discoideus* (Park et al. 2013; Unelius et al. 2013). It is possible that female weevils are more sensitive to host plant volatiles and these volatiles inevitably end up in aeration samples of weevils.

Juvenile hormone III did not induce or increase pheromone production in *Gonipterus* sp. 2. However, the successful upregulation of pheromone production with JHIII has been shown to be dependent on presence of sufficient pheromone precursors and/or removal of insect antennae to prevent autodetection with subsequent downregulation of pheromone production (Vanderwel 1994). These sampling conditions have to be investigated in future studies. Juvenile hormone III regulates the synthesis of certain enzymes including the cytochrome P450, which is responsible for pheromone biosynthesis in *Leptinotarsa decemlineata* and species in the *Pityokteines, Dendroctonus* and *Ips* genera (Dickens et al. 2002; Harring 1978; Vanderwel 1994).

The putative pheromones of *G. platensis* were previously proposed to be derivatives of terpineol and α-pinene (Branco et al. 2019; Wheeler et al. 2002) and it is known that pheromone production can occur through metabolism of plant precursors like terpineol, myrcene and α-pinene with JHIII (Vanderwel 1994). The possibility therefore still exists that JHIII may be successful in pheromone production upregulation if the correct sampling conditions are used. Due to the close relation of *G. platensis* and *Gonipterus* sp. 2, these cryptic species might follow the same pheromone biosynthesis pathway. This study provides initial results in the first step to test this hypothesis. Determining whether JHIII plays a role in *Gonipterus* sp. 2's pheromone production will aid in understanding the mechanism of pheromone biosynthesis and the chemical ecology of the *Gonipterus* species complex as a whole.

This study provides a good foundation for the elucidation of a pheromone for *Gonipterus* sp. 2. The presence of cis- and trans-verbenol in both *G. platensis* and *Gonipterus* sp. 2 suggests that these cryptic species may share at least two compounds in their pheromone blends. Further studies can now explore the function of these respective components through bioassays and quantification of these components. Field-based trapping experiments with different blend ratios of these compounds can be done to determine optimal blend composition and if these compounds are indeed used as long range pheromones in *Gonipterus* sp. 2. Other, yet unidentified compounds may also be identified and are possibly necessary for behavioral activity (Eller et al. 1994).

It is possible that *Gonipterus* species rely on short-range sexual orientation cues (Hedin et al. 1979; Lapointe et al. 2004; Santolamazza-Carbone and Cordero Rivera 1998). Long copulation periods have been observed for *Gonipterus* species (Santolamazza-Carbone and Cordero Rivera 1998) and short-range orientation cues are known to exist in the related weevils such as *Cylas formicarius elegantulus* (Heath et al. 1986). Direct solvent extraction of the beetles would be an alternative sampling method to reveal cuticular hydrocarbons that may function as short-range sexual orientation cues for *Gonipterus* sp. 2. The use of such short-range signals may be limited for pest control. Knowing that *Gonipterus* sp. 2 follows a similar pheromone communication system as *G. platensis* provides valuable insight in the development of a pheromone-based trapping tool for *Gonipterus* sp. 2.

Figure captions

- **Figure 3.1**: Eleven recently identified candidate pheromone components of *Gonipterus platensis* (Branco et al. 2019). The seven chemical structures that were confirmed by comparison with synthetic standards (Branco et al. 2019) are indicated with asterisks.
- **Figure 3.2:** Overlaid chromatographic elution profiles (black, ZBWax column) of a blank (below) and two replicates (middle and top) of a SPME-sampled chamber containing three *Gonipterus* sp. 2 males and a *E. dunni* leaf (n = 2). Small but repetitive electroantennographic responses from female *Gonipterus* sp. 2 antennae (red, n = 5, 3 shown) included those at 6.60 min (I_K = 1664) and 6.93 min (I_K = 1715), as shown with arrows on the figure. Antennal deflections were below detection limits of the EAD software.
- **Figure 3.3:** Top: TIC elution profiles (ZBWax column) of DHS samples of single male and female *Gonipterus* sp. 2 weevils sampled without leaf material, overlaid on top of individuals sampled with a *E. dunni* leaf. Bottom left: The mass fragmentation pattern match of the sample peak (11.799 min, I_K = 1661) to γ-gurjunene on the NIST database. Middle bottom: A representative sample overlaid with the reference standard of cis-verbenol (left black peak), which also contained trans-verbenol (middle black peak) and verbenone (right black peak). Peak 2 corresponds to the same retention index and mass spectrum as the trans-verbenol standard, confirming this peak identity (see also Table 3.3).. Bottom right: The sample peak with the same retention index as verbenone (12.443 min, I_K = 1716), was tentatively identified as ß-longipinene. This was based on a sample peak mass spectrum match to ß-longipinene on the NIST database.
- **Figure 3.4:** Sequential GC-EAD screenings of SPME headspace samples of 20 virgin *Gonipterus* sp. 2 males or females, sampled for 15 minutes. Typical FID traces (black, n = 2, ZBWax column) from males (top, n = 2) and females (bottom, n = 2) are overlaid to simplify chromatographic peak and electroantennographic response comparisons between these samples (indicated with arrows). EAD traces from male antennae (red, n = 2) are overlaid with EAD traces from female antennae (purple, n = 2) and corresponding FID traces (black) are shown. Small deflections were seen at 1: 3.33 min (I_K = 1209), 2: 3.447 min (I_K = 1224), 3: 4.86 min (I_K = 1409), 4: 6.76 min (I_K = 1688) and 5: 6.86 min (I_K = 1703). Deflection sizes were below detection limits of the EAD software.
- **Figure 3.5:** Top: GC-MS TIC profiles (ZBWax column) of 70 separate virgin *Gonipterus* sp. 2 males and females, sampled for 15 minutes with a SPME-fiber. Peak numbers correspond to tentative identities in Table 3.1. Top right: A zoomed-in trace of the male sample overlaid with the reference standard containing cis-verbenol, trans-verbenol and verbenone. Bottom: An example of a match of the mass spectrum of peak 7 to aromandendrene on the NIST database, with a low match factor of 76.5%.

- **Figure 3.6:** Left: GC-MS TIC profiles (ZBWax column) of six virgin *Gonipterus* sp. 2 males (n = 1) and seven virgin females (n = 1) sampled with SPME. Peak numbers correspond to tentative identities in Table 3.2. The traces are overlaid with the reference standard containing cis-verbenol, trans-verbenol and verbenone. Top right: The zoomed-in elution profile of the different samples with the reference standard. No sample peak elutes where cis-verbenol elutes (11.798 min, I_K = 1659), a sample peak (9) co-elutes with trans-verbenol (12.031 min, I_K = 1681) and sample peaks eluting at 12.414 min (I_K = 1714, 10) and 12.447 min (I_K = 1717, 11) both have offset co-elutions to the S-(-)-verbenone reference standard (12.407 min, I_K = 1713). The peak eluting at 12.831 min (I_K = 1751) was tentatively identified as a naphthalene derivative, and was present in all samples including the reference standard and blank. Bottom right: Deconvolution of peaks 10 and 11 with ions including m/z = 57, 85, 97, 69, 105 and 134.
- **Figure 3.7A:** GC-EAD responses from female *Gonipterus* sp. 2 antennae (purple, n = 4) and male antennae (red, n = 6) to multiple injections of the same DHS extract from 75 untreated female *Gonipterus* sp. 2 weevils (n = 1, FID trace in black). Some repeated responses from female antennae include those with retention indexes of I_K = 1202, 1396, 2112, 1569, 1614, 1723, 1803 and 1947; and from male antennae, I_K = 1274, 1402, 1580, 1614, 1772, 1955 and 2112 (shown with arrows).
- **Figure 3.7B:** GC-EAD responses of female *Gonipterus* sp. 2 antennae (purple, n = 7, 5 shown) and male antennae (red, n = 6) to multiple injections of the stimulus of a DHS extract from 75 untreated *Gonipterus sp. 2* male weevils (n = 1, FID trace in black). Some repeated responses from female antennae include those with retention indexes of I_K = 1521, 1684 and 1971; and from male antennae, I_K = 1283, 1521, 1617, 1842 and 1947 (shown with arrows). Deflections were mostly below detection limits of the EAD software.
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- **Figure 3.7D:** GC-EAD results to stimulus of multiple injections of a DHS extract of 75 JHIII-treated *Gonipterus* sp. 2 males (n = 1, FID trace in black) by female *Gonipterus* sp. 2 antennae (purple, n = 5) and male antennae (red, n = 5). Some repeated responses from female antennae include those with retention indexes of I_K = 1319, 1606 and 1853; and from male antennae, I_K = 1250, 1409, 1684, 1702, 2084 and 2180 (shown with arrows on the figure).
- **Figure 3.8:** Top: Overlaid GC-MS TIC traces of SPME samples of 75 untreated (♀ or ♂) or JHIII-treated ((♂) or (♀)) *Gonipterus* sp. 2 weevils. Top right: An overlaid trace of the reference standard of S-cis-verbenol (1), trans-verbenol

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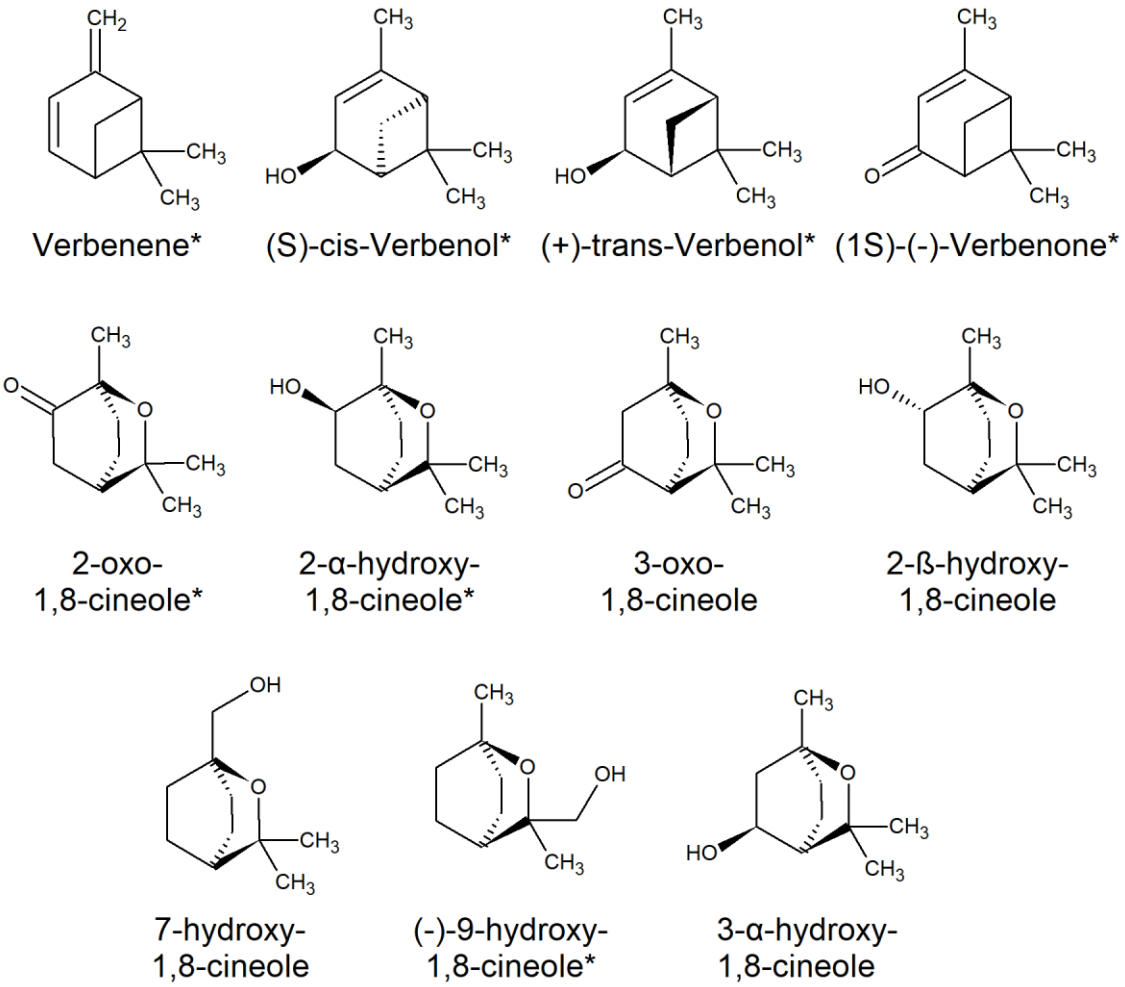
- **Figure 3.10:** Deflections of female *Gonipterus* sp. 2 antennae to 1 µl of a 100 ppm solution of (S)-cis-verbenol (1), with co-elutions of the reference standards of trans-verbenol (2) and S-(-)-verbenone (3). Response sizes could not be determined as they were mostly below the EAD software detection limits (20 µV).

Figures



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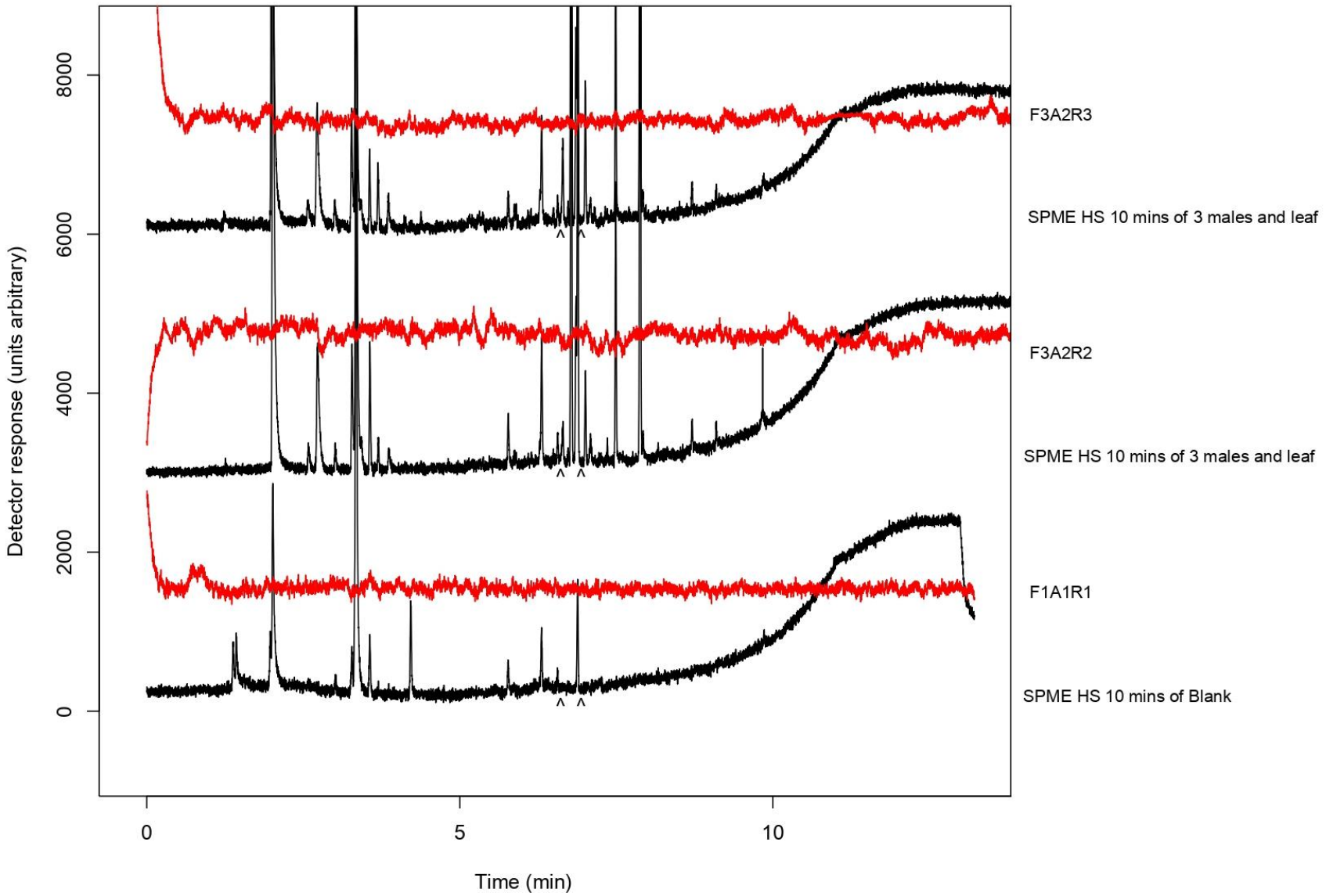

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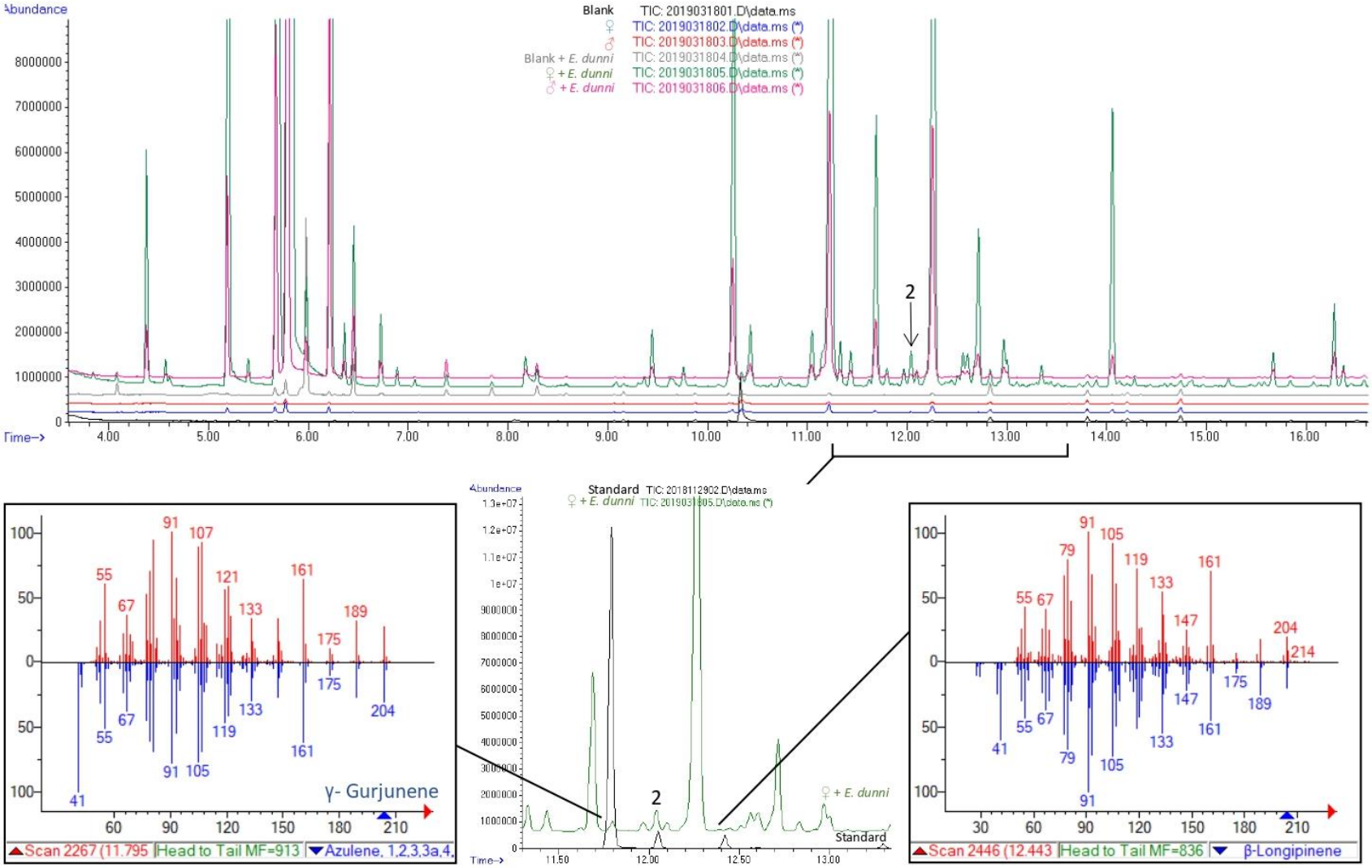

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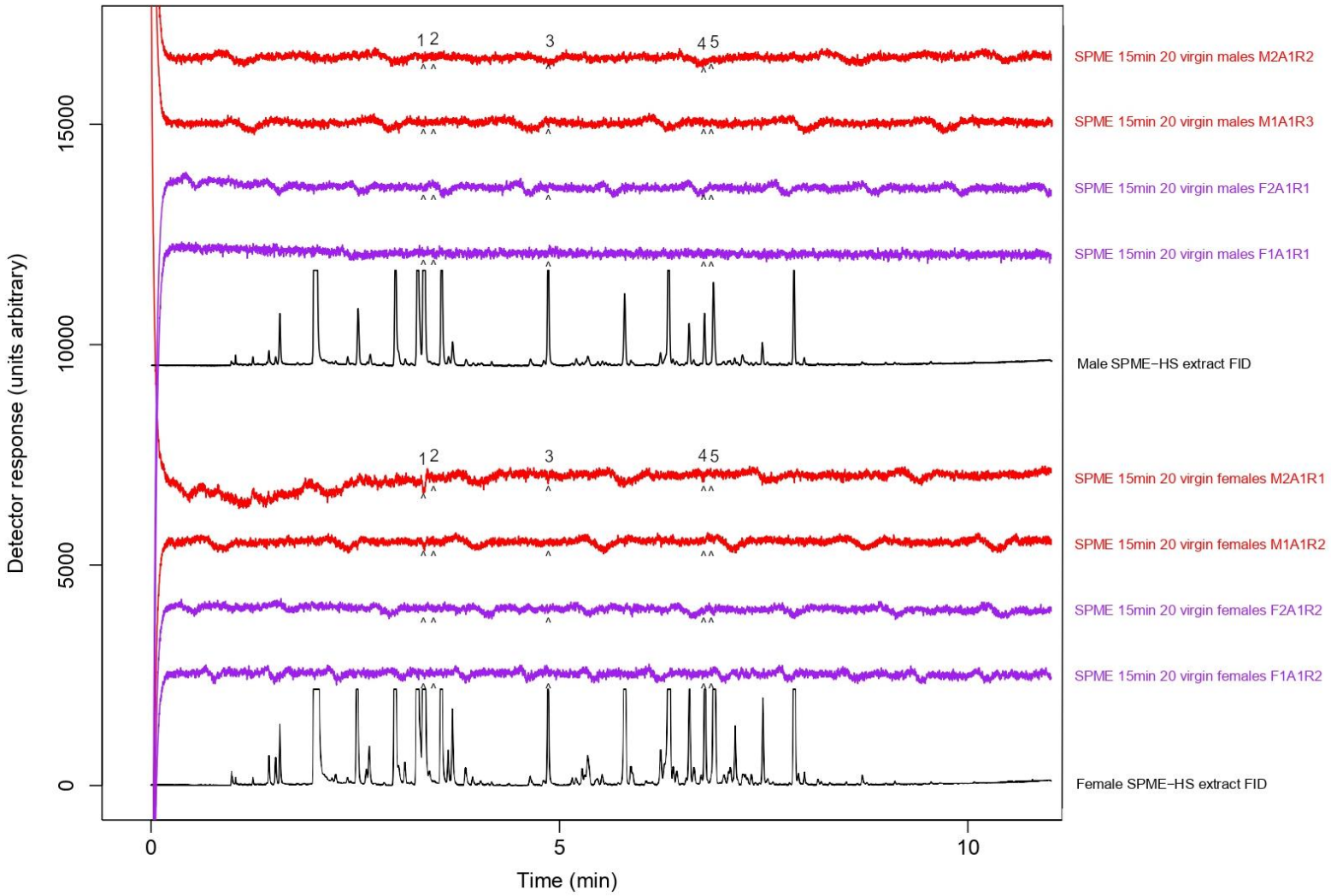

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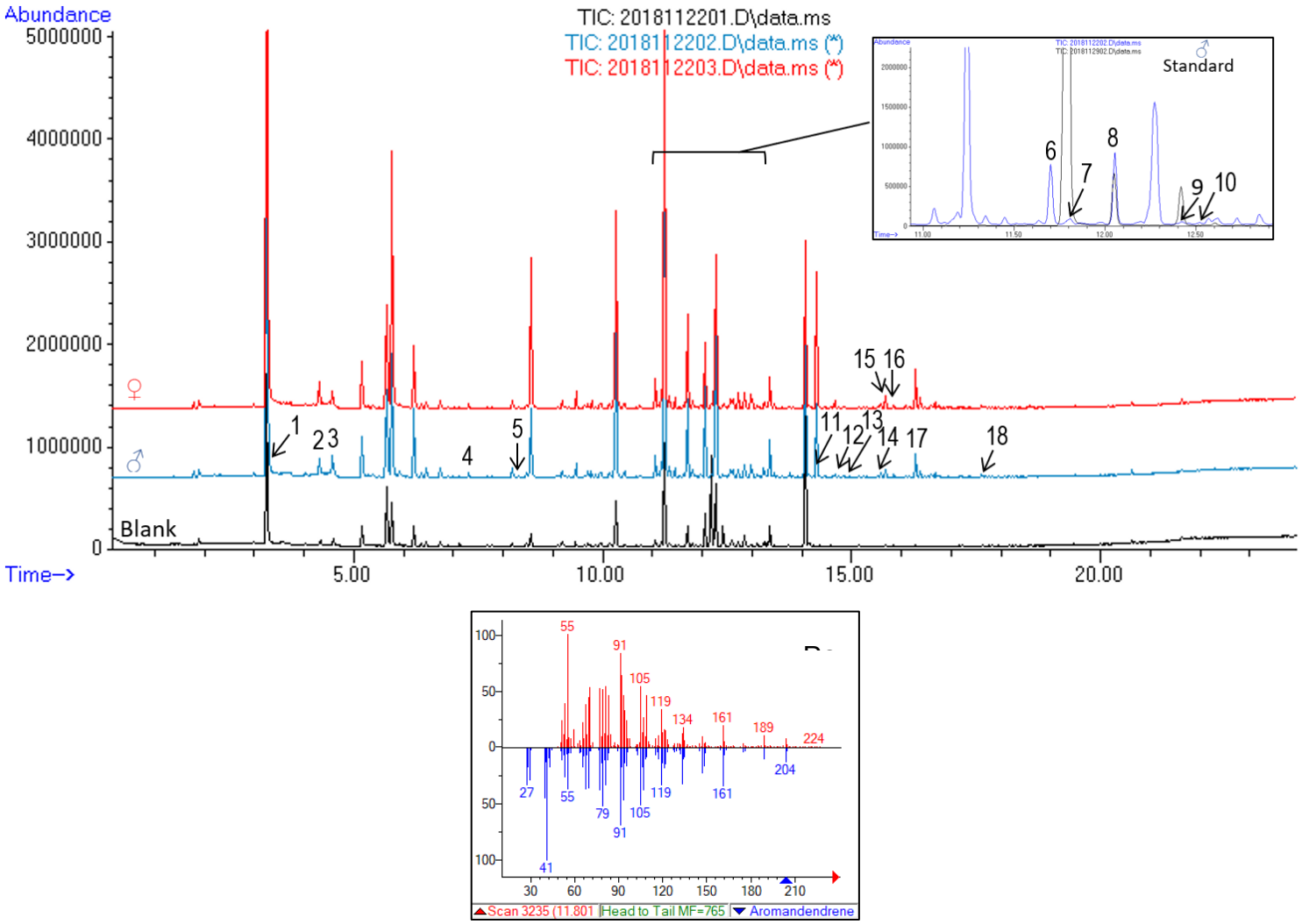

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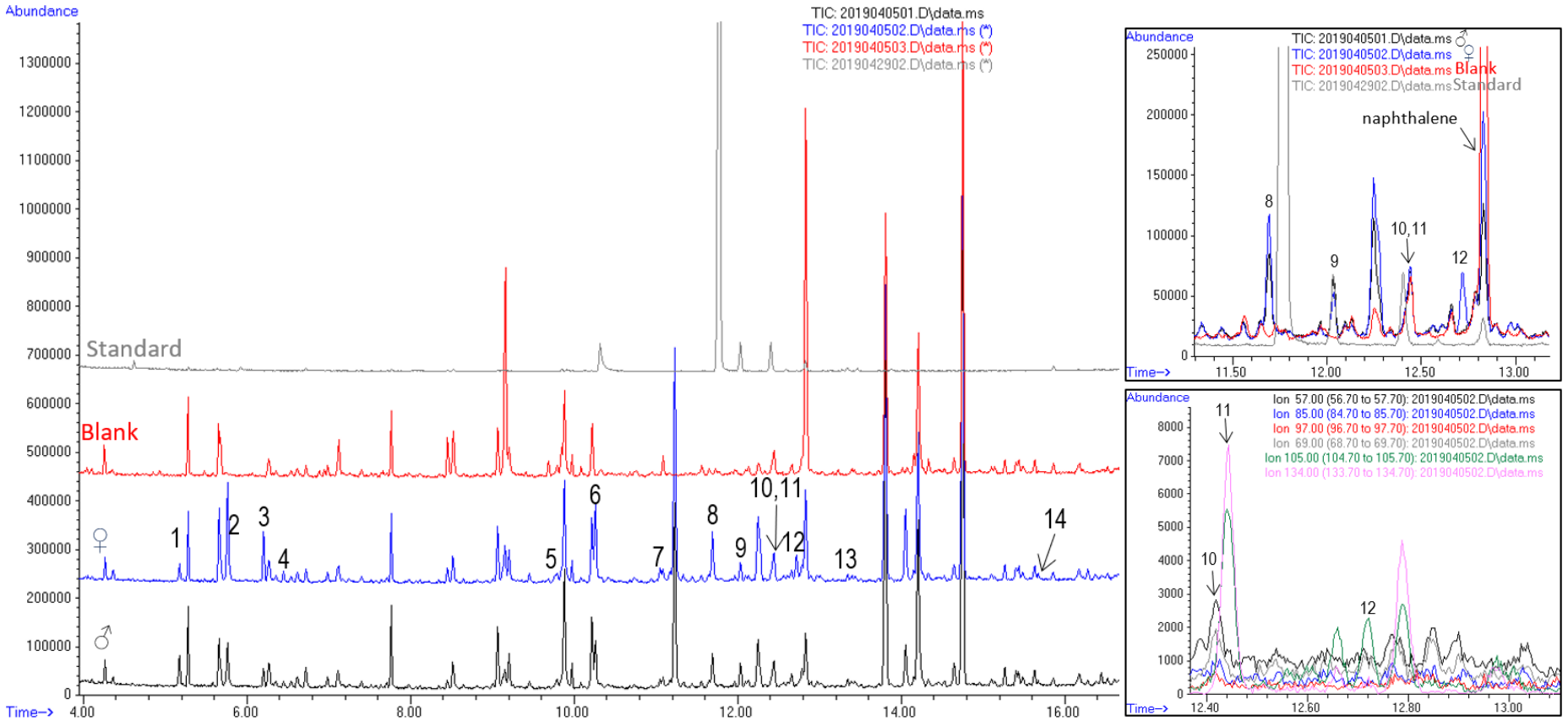

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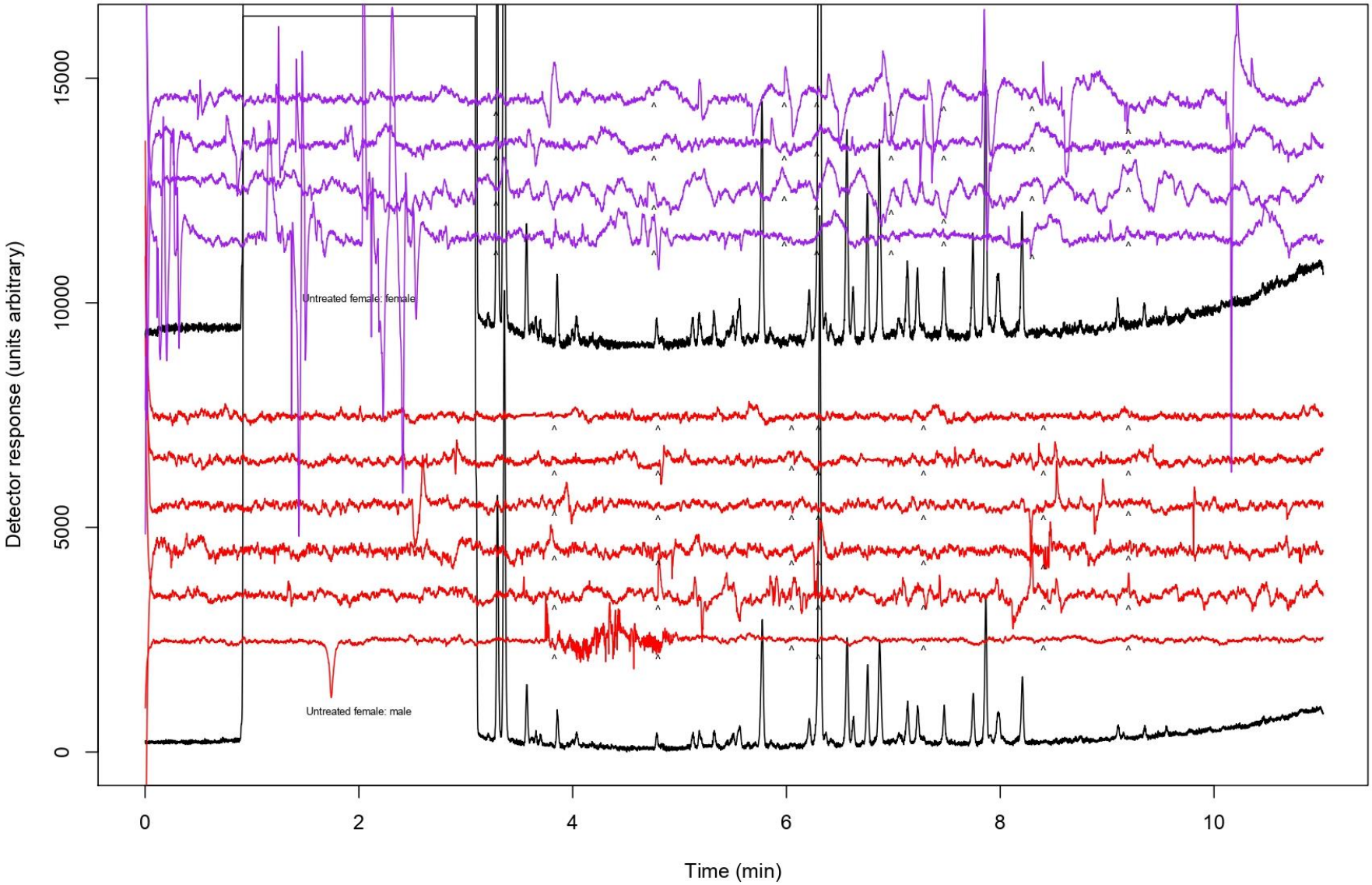

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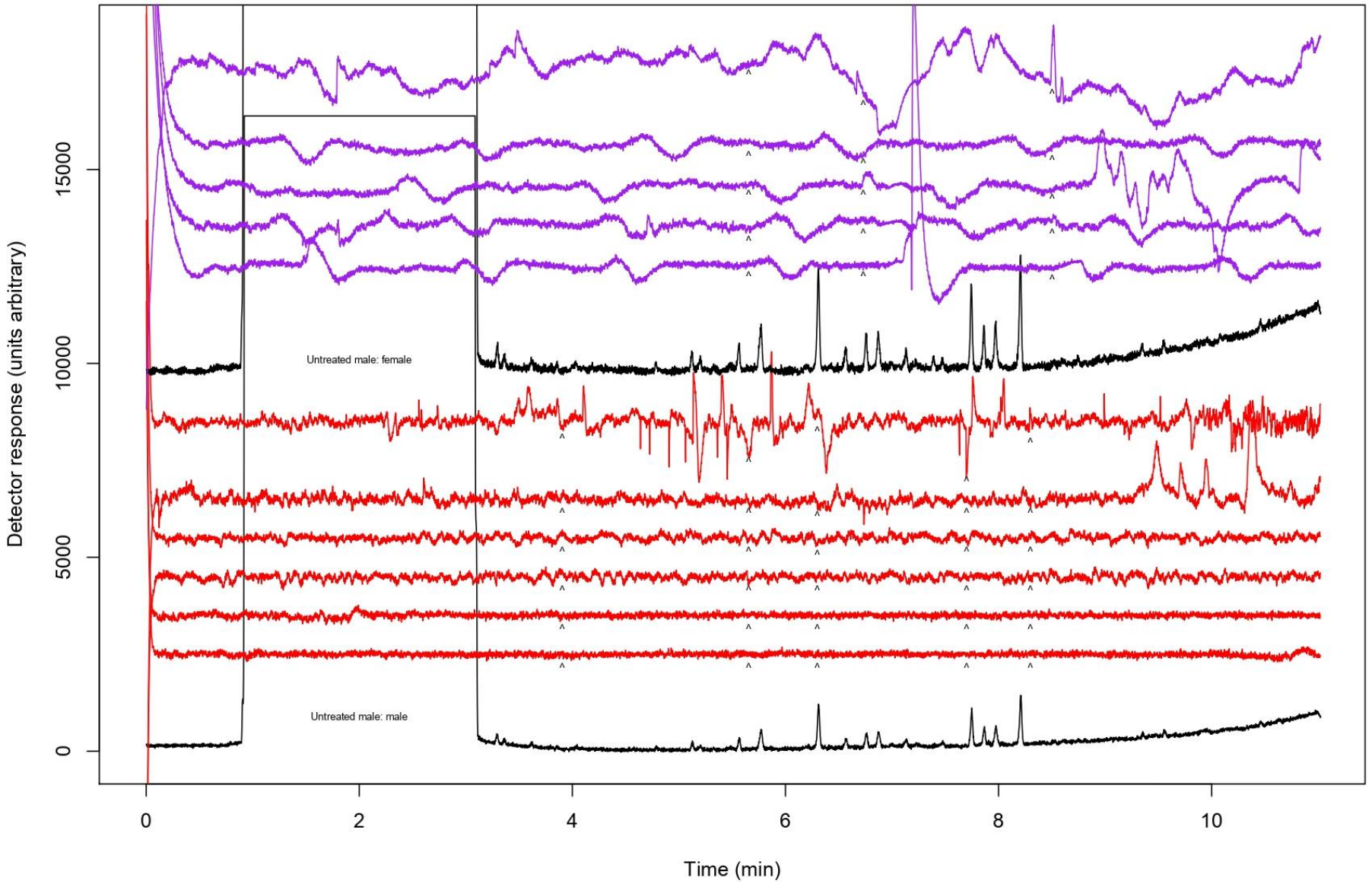

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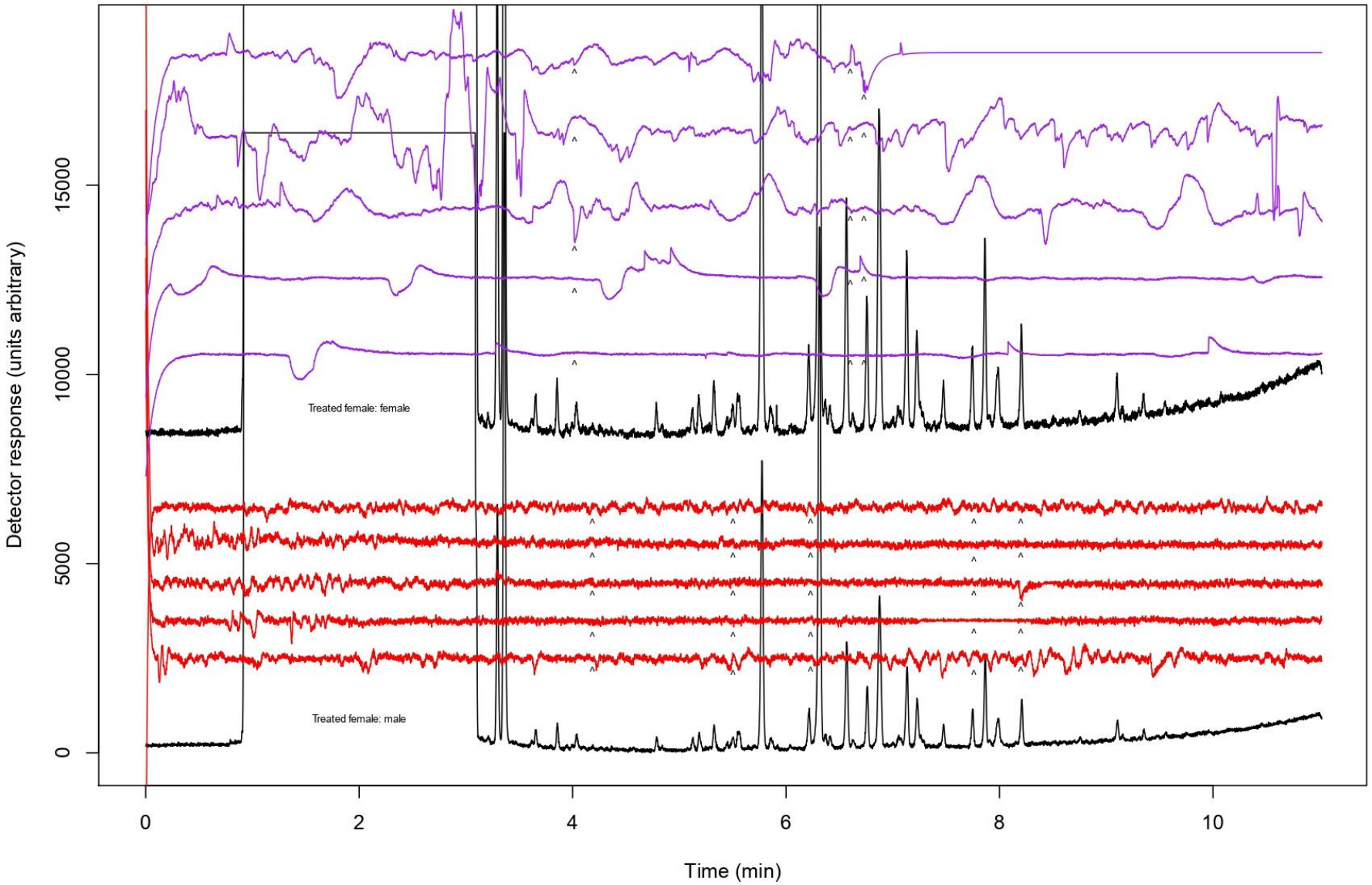

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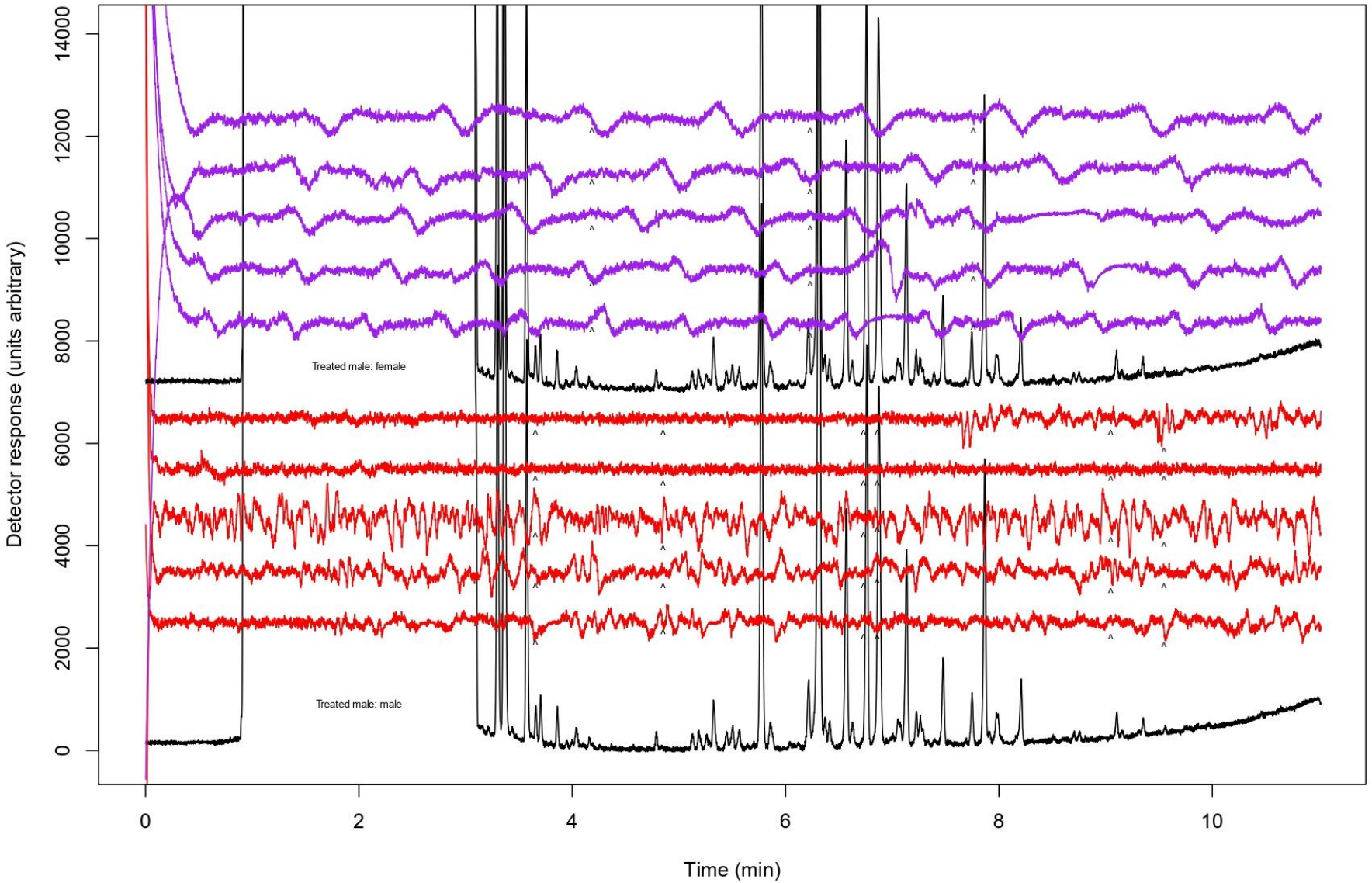

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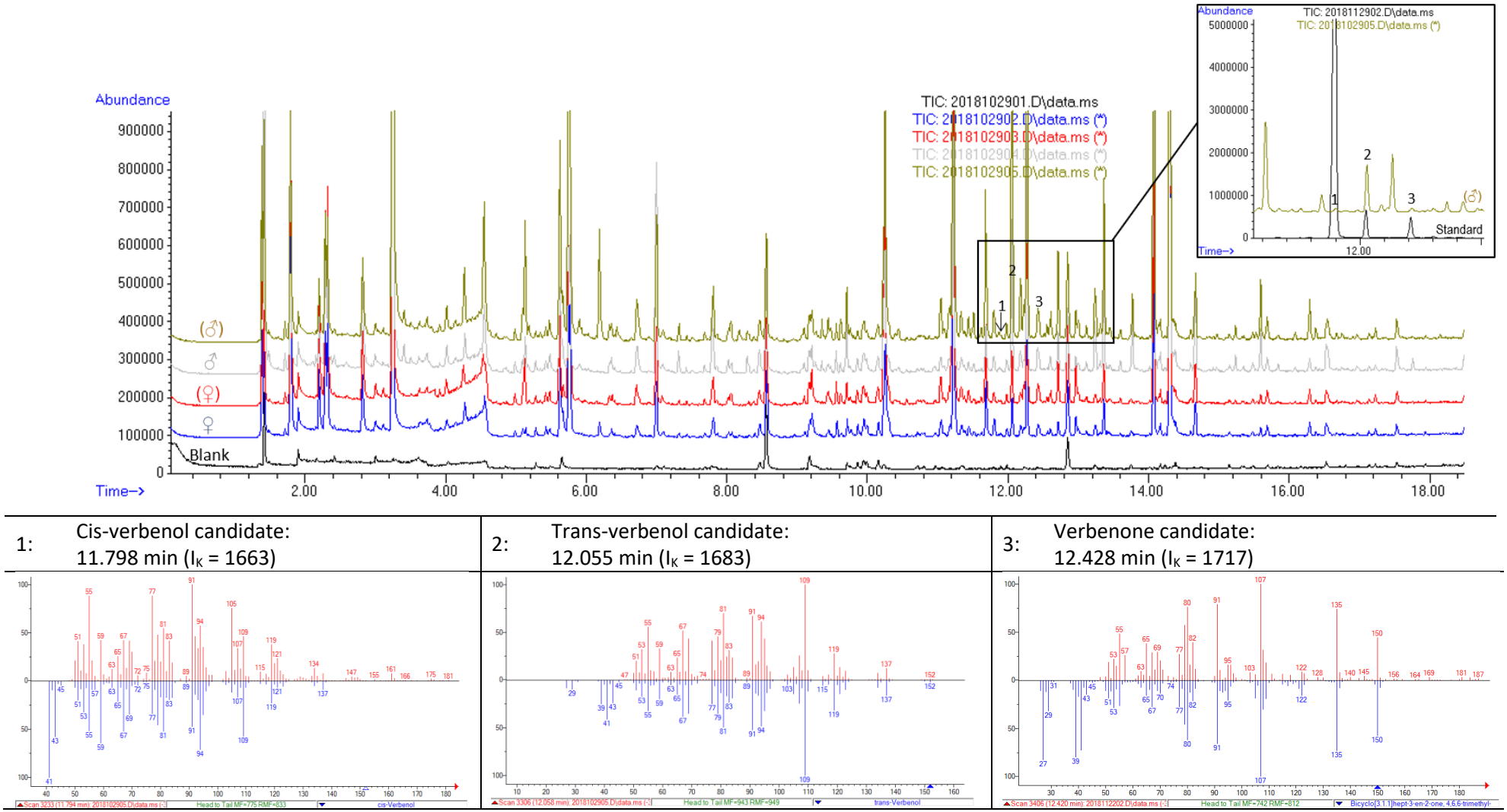


Figure 3.8: Top: Overlaid GC-MS TIC traces of SPME samples of 75 untreated (♀ or ♂) or JHIII-treated ((♂) or (♀)) *Gonipterus* sp. 2 weevils. Top right: An overlaid trace of the reference standard of S-cis-verbenol (1), trans-verbenol (2) and S-(-)-verbenone (3), showing identical retention indexes of three peaks in the treated male sample ((♂)). Bottom: Mass spectral matches of sample TIC peaks to cis-verbenol, trans-verbenol and verbenone on the NIST library. These literature mass spectra match to our reference standards 1, 2 and 3 (I_K = 1663, 1683 and 1717).

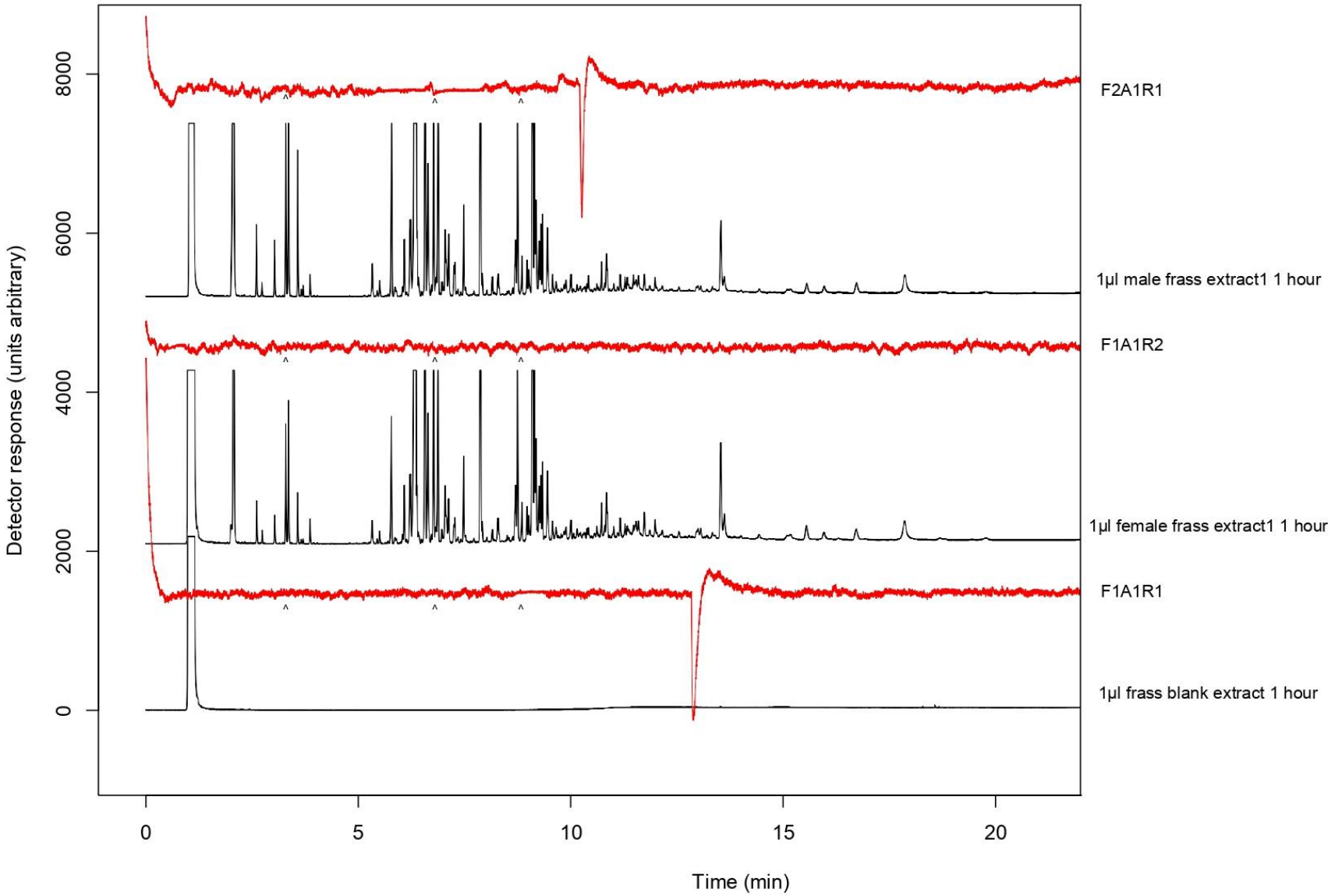


Figure 3.9A: GC-EAD results of male *Gonipterus* sp. 2 frass (top black FID trace) and female frass hexane extracts (middle black FID trace) screened for responses from female *Gonipterus* sp. 2 antennae (red, n = 1), compared to the female response to a solvent blank. Responses with retention indexes of I_K = 1202, 1710 and 2060 are shown with arrows.

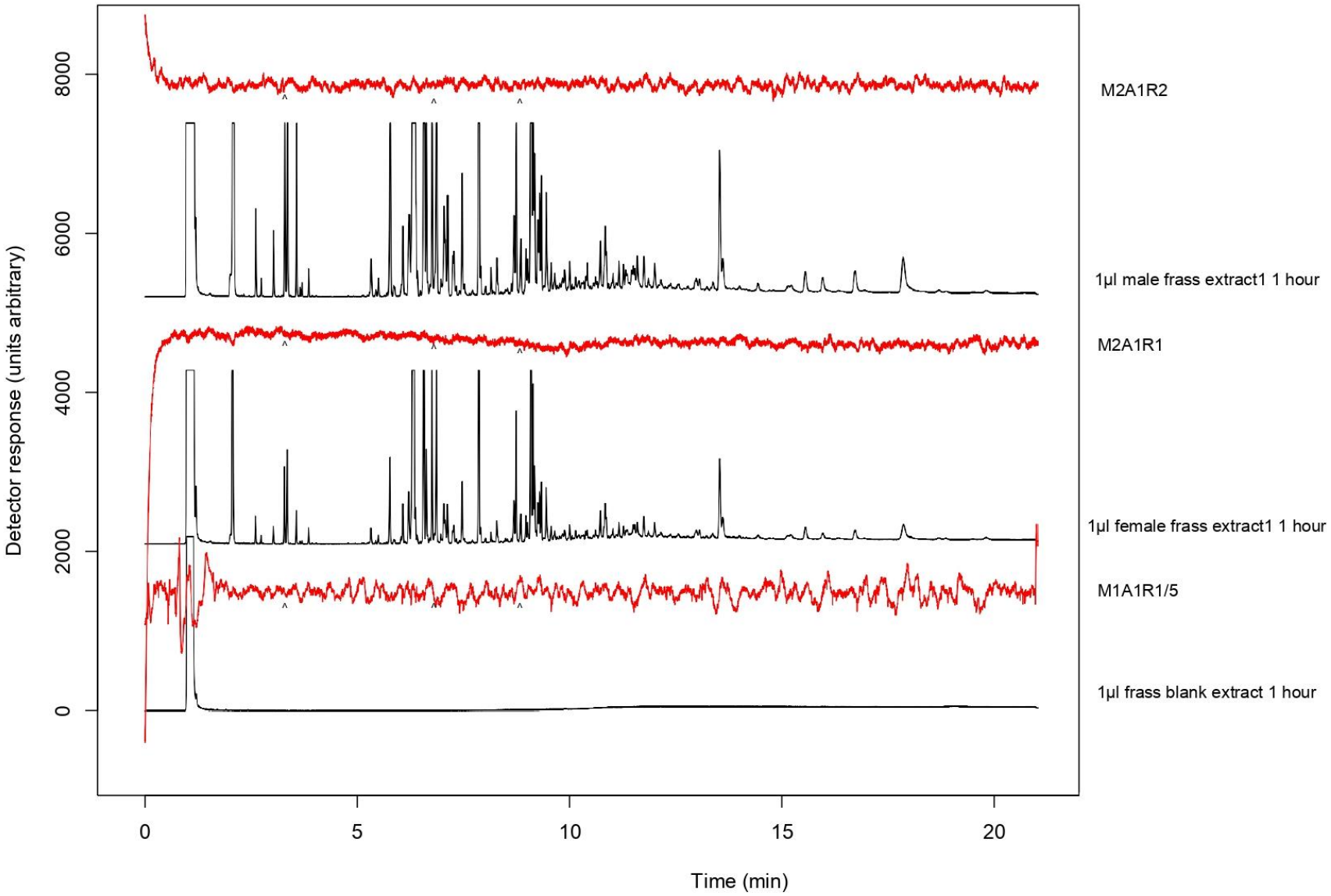


Figure 3.9B: GC-EAD results of male *Gonipterus* sp. 2 frass (top black FID trace) and female frass hexane extracts (middle black FID trace) screened for responses from male *Gonipterus* sp. 2 antennae (red, n = 1), compared to the male response to a solvent blank. Responses with retention indexes of I_K = 1202, 1710 and 2060 are shown with arrows. The antenna response signal to the blank sample was noisy, and required a five-fold reduction the EAD trace output.

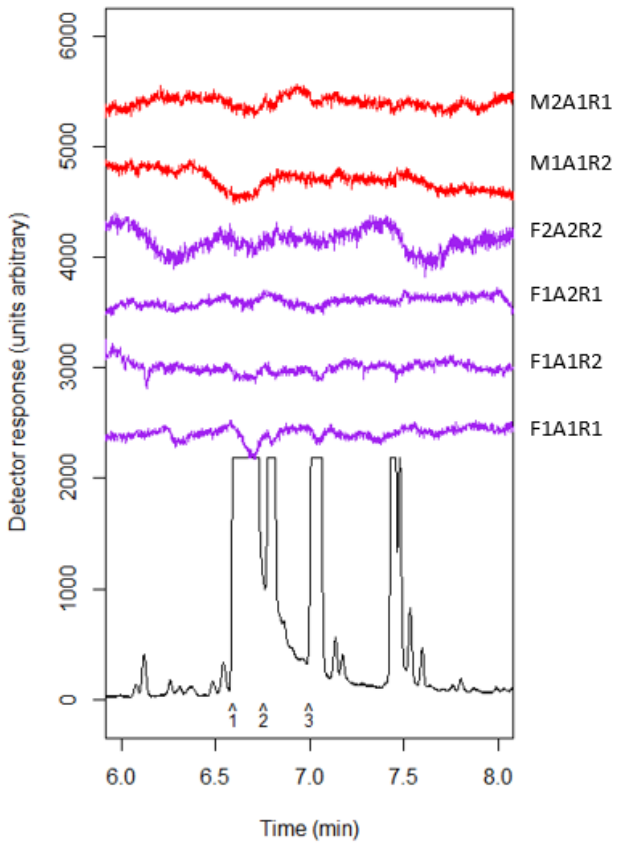
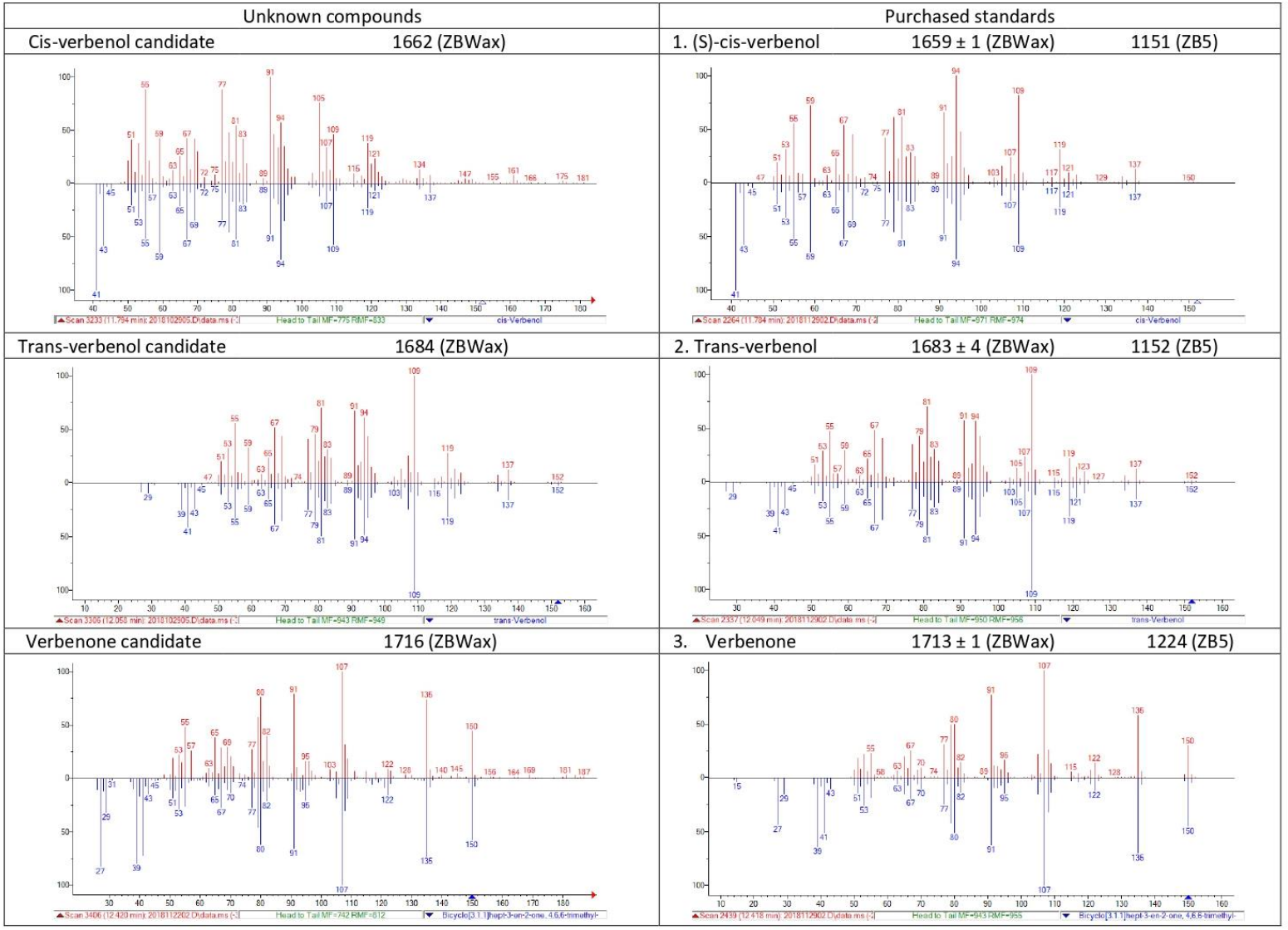


Figure 3.10: Defelctions of female *Gonipterus* sp. 2 antennae to 1 µl of a 100 ppm solution of (S)-cis-verbenol (1), with co-elutions of the reference standards of trans-verbenol (2) and S-(-)-verbenone (3). Response sizes could not be determined as they were mostly below the EAD software detection limits (20 µV).

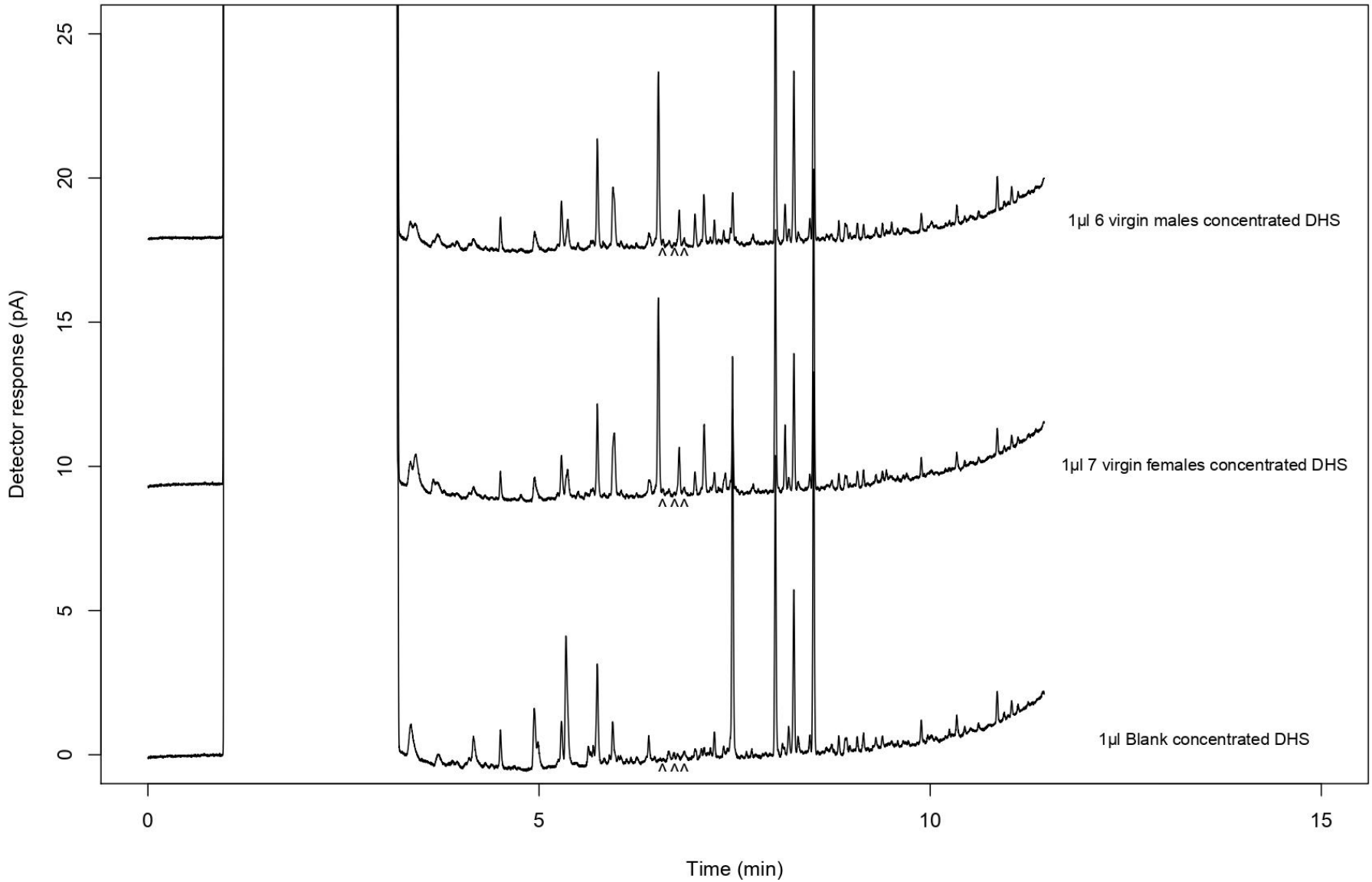
Supplementary figure captions

- **Supplementary Figure 3.1:** Mass fragmentation patterns of three headspace sample components from *Gonipterus* sp. 2 (red spectra) compared to NIST library matches (blue spectra) of S-cis-verbenol, trans-verbenol and S-(-)-verbenone, respectively. The reference standards are confirmed to be the same components that the library matched to the sample components. A reverse match factor (RMF) of 100 represents a perfect match of a library fragmentation pattern to a sample peak. The retention indexes of the sample peaks also match to those of the respective reference standards. This data confirms the identity of these three sample peaks.
- **Supplementary Figure 3.2:** FID traces of the DHS samples of six virgin Gonipterus sp. 2 males (top, n = 1), and seven virgin females (middle, n = 1), compared to the dirty sample blank (bottom). Some peaks in the weevil samples that are not present in the blank, represent retention indexes of cis-verbenol (IK = 1659), trans-verbenol (IK = 1683), and S-(-)-verbenone (IK = 1713) (shown with arrows).
- **Supplementary Figure 3.3:** Top: A comparison between the FID traces of the DHS extract from 75 untreated Gonipterus sp. 2 males (n = 1) and a DHS extract of 75 JHIII-treated males (n = 1). Many peaks are present in high abundance after treatment with JHIII, of which some were present in untreated males in lower abundance. Bottom: A comparison between the FID traces of the DHS extract from 75 untreated Gonipterus sp. 2 females (n = 1) and the DHS extract of 75 JHIII-treated females (n = 1). The difference between FID traces from female samples are not as apparent as between male samples (top traces).
- **Supplementary Figure 3.4:** A comparison of the GC-MS TIC traces (ZBWax column) of the same four sampling chambers with the same content, sampled with different sampling methods. The sampling chambers contained 75 untreated Gonipterus sp. 2 females (♀), 75 untreated males (♂), 75 JHIII-treated females ((♀)) or 75 JHIII-treated males ((♂)), respectively. Top: The TIC trace of two replicate SPME samples of the same chambers, sampled for 15 minutes. Middle: TIC traces of DHS samples of sampling chambers after two days of volatile accumulation onto Poropak. Bottom: Overlaid TIC traces of the same sample, containing 75 JHIII-treated Gonipterus sp. 2 males ((♂)), sampled with different methods. The direct solvent wash sampling method revealed semi-volatiles in its TIC elution, with a different elution profile than the extracts obtained from DHS and SPME headspace sampling methods.
- **Supplementary Figure 3.5:** Selected GC-EAD results of three different sampling methods of Gonipterus sp. 2, and the respective EAD responses of either male or female antennae (red) to each FID chromatographic elution profile (black). Top: An EAD signal of a male antenna to a solvent extract of female frass, that was extracted for 1 hour. Middle: An EAD signal of a female antenna to a 30-minute SPME sample of the same female frass. Bottom: An EAD signal of a female antenna to a 10-minute SPME sample of a chamber containing three Gonipterus sp. 2 males and a E. dunni leaf.

- **Supplementary Figure 3.6:** A comparison between typical FID elution profiles between male (black) and female Gonipterus sp. 2 elutions (red) from different sampling methodologies. Overall, samples of female weevils showed higher concentration of chromatographic peaks than male weevils in all these sampling methods. A: FID traces of male (black) and female (red) frass, that were extracted in solvent for one hour. The FID elution was minimized with a 15-fold division of the abundance of all peaks in both traces for ease of visualizing the elution profile. B: FID traces of half-hour SPME samples of male (black) and female frass (red). The FID traces were minimized with a 10-fold division. C: FID traces of 15 minute-SPME samples of 20 virgin males (black) and females (red) are overlaid. FID traces were minimized with a 15-fold division. D: A representative FID elution profile from a sample with three males and a single E. dunni leaf. This experiment was not conducted with females.



Supplementary Figure 3.1: Mass fragmentation patterns of three headspace sample components from *Gonipterus* sp. 2 (red spectra) compared to NIST library matches (blue spectra) of S-cis-verbenol, trans-verbenol and S-(-)-verbenone, respectively. The reference standards are confirmed to be the same components that the library matched to the sample components. A reverse match factor (RMF) of 100 represents a perfect match of a library fragmentation pattern to a sample peak. The retention indexes of the sample peaks also match to those of the respective reference standards. This data confirms the identity of these three sample peaks.



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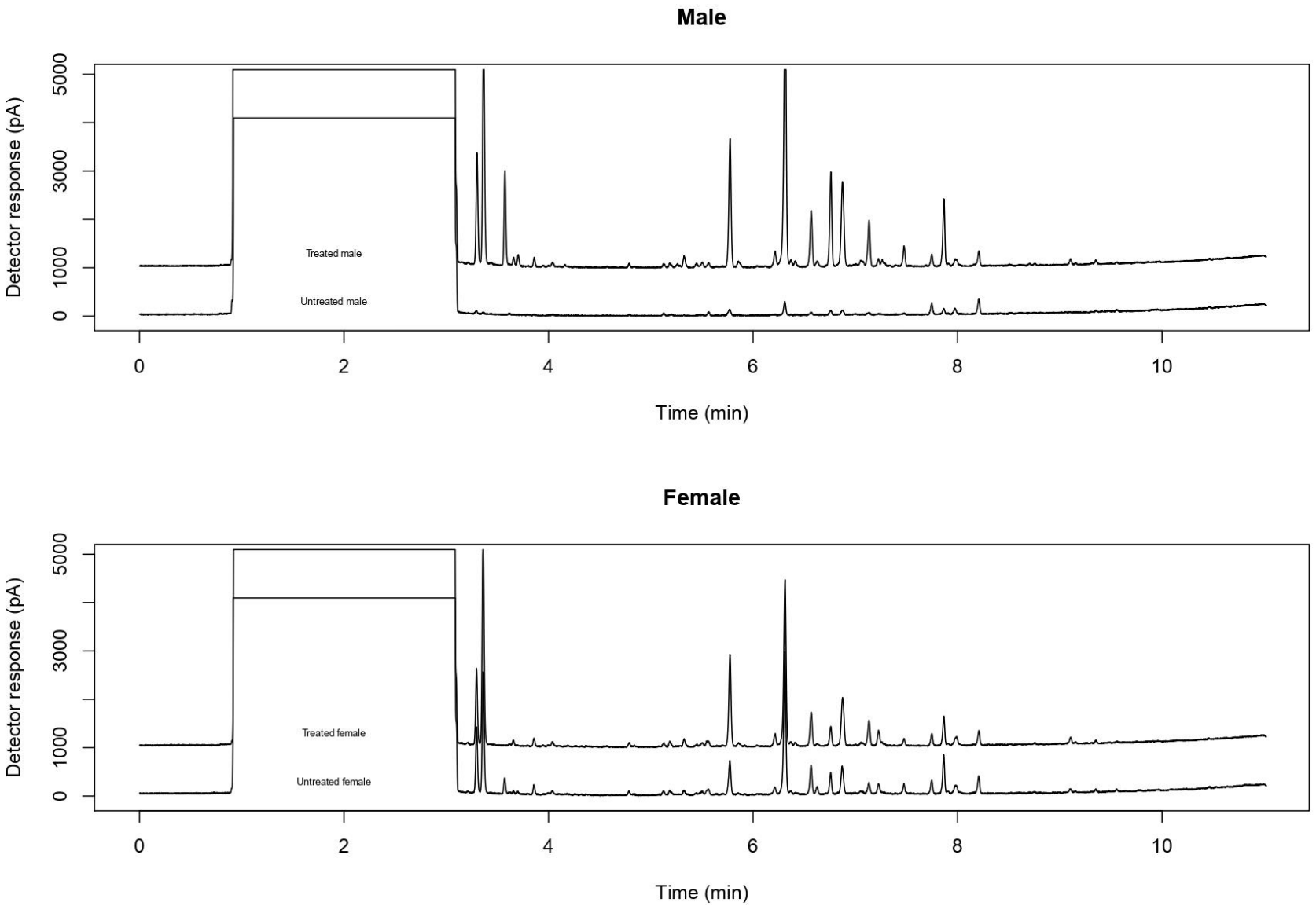
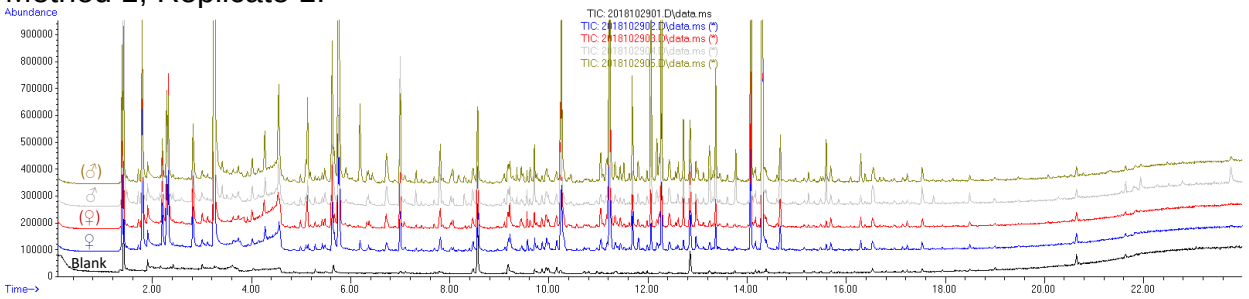
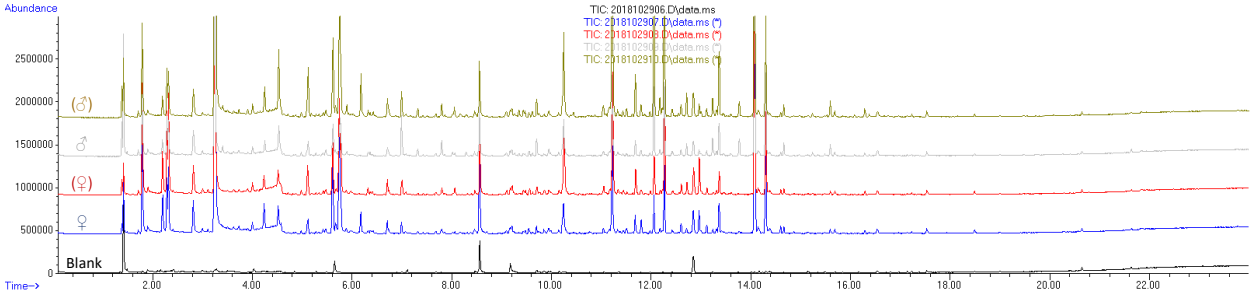


Supplementary Figure 3.3: Top: A comparison between the FID traces of the DHS extract from 75 untreated *Gonipterus* sp. 2 males (n = 1) and a DHS extract of 75 JHIII-treated males (n = 1). Many peaks are present in high abundance after treatment with JHIII, of which some were present in untreated males in lower abundance. Bottom: A comparison between the FID traces of the DHS extract from 75 untreated *Gonipterus* sp. 2 females (n = 1) and the DHS extract of 75 JHIII-treated females (n = 1). The difference between these FID traces are not as apparent as between male samples (top traces).

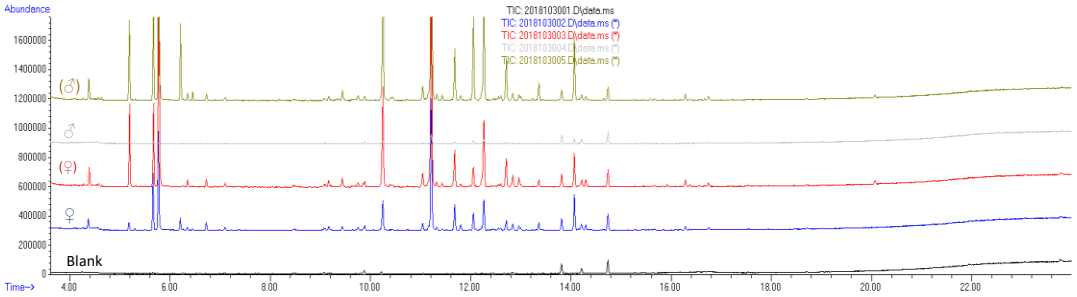
Method 1, Replicate 1:



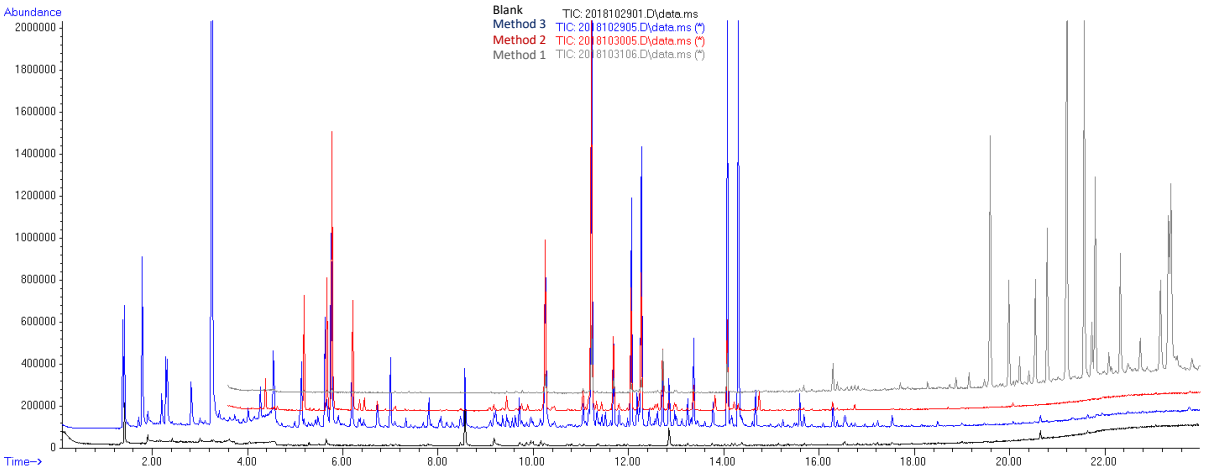
Method 1, Replicate 2:



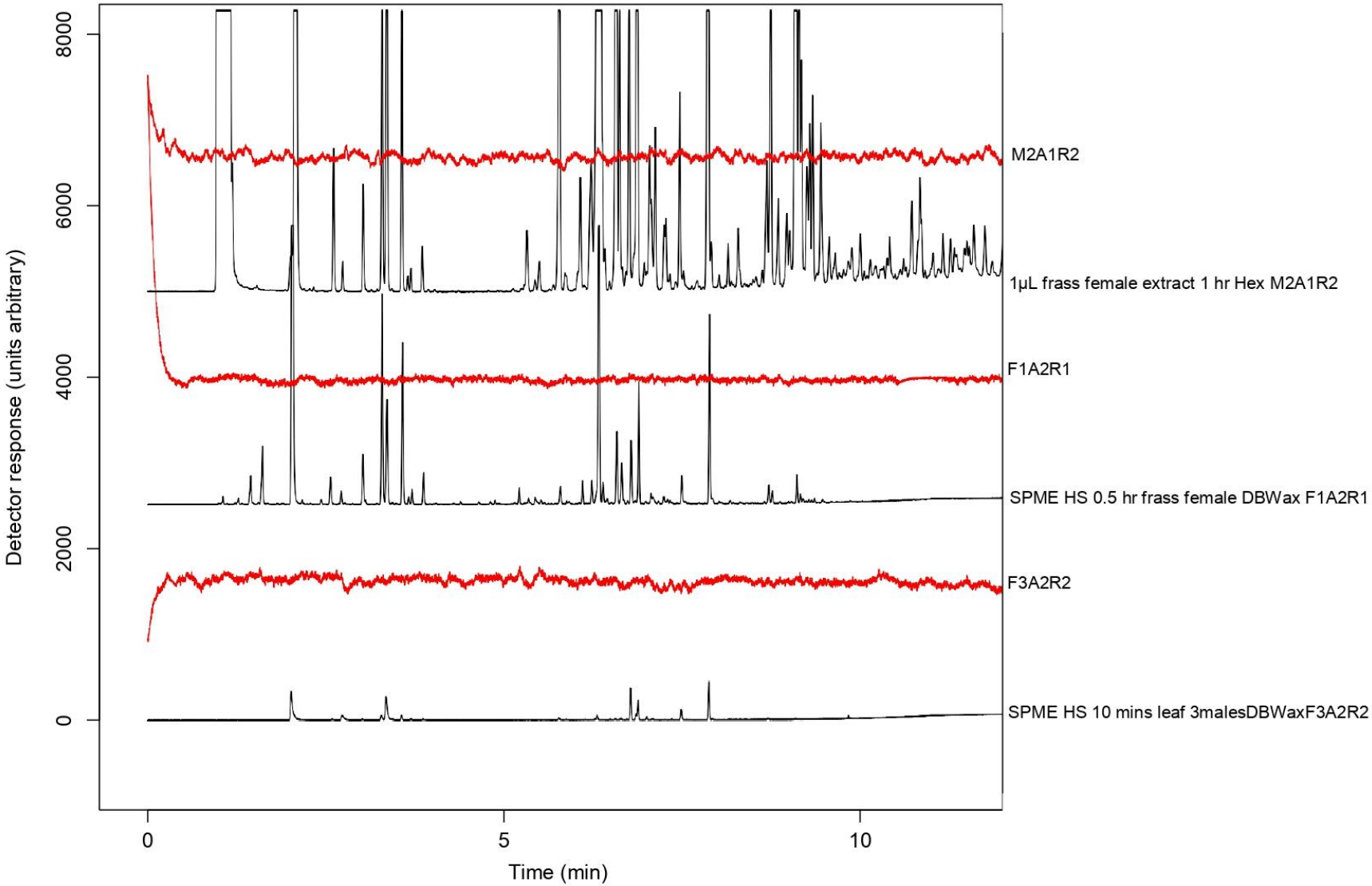
Method 2. DHS sample on GC-MS:



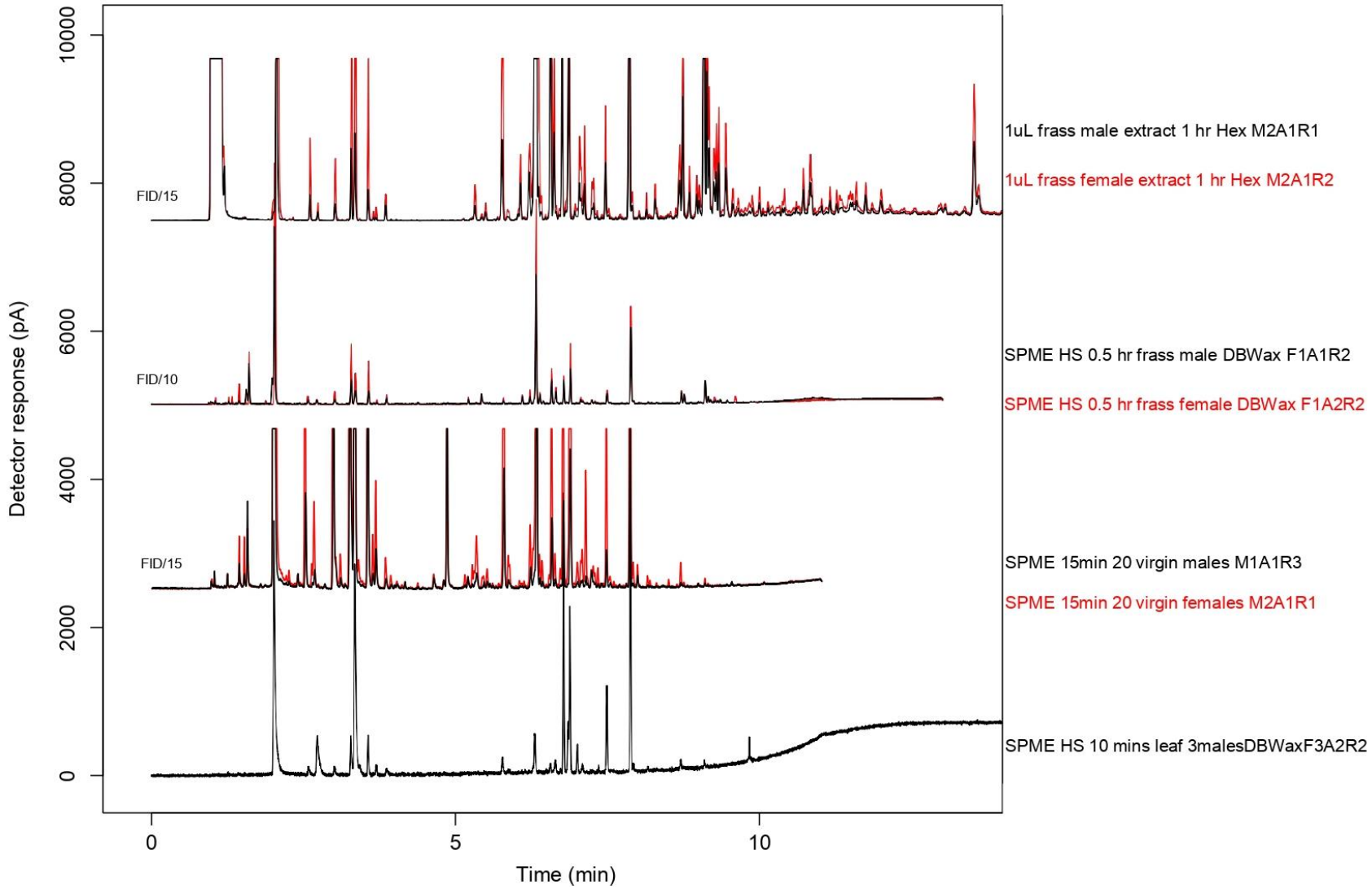
Method 3. Solvent wash on GC-MS, in comparison to headspace samples of treated males (♂):



Supplementary Figure 3.4: A comparison of the GC-MS TIC traces (ZBWax column) of the same four sampling chambers with the same content, sampled with different sampling methods. The sampling chambers contained 75 untreated *Gonipterus* sp. 2 females (♀), 75 untreated males (♂), 75 JHIII-treated females ((♀)) or 75 JHIII-treated males ((♂)), respectively. Top: The TIC trace of two replicate SPME samples of the same chambers, sampled for 15 minutes. Middle: TIC traces of DHS samples of sampling chambers after two days of volatile accumulation onto Poropak. Bottom: Overlaid TIC traces of the same sample, containing 75 JHIII-treated *Gonipterus* sp. 2 males ((♂)), sampled with different methods. The direct solvent wash sampling method revealed semi-volatiles in its TIC elution, with a different elution profile than the extracts obtained from DHS and SPME headspace sampling methods.



Supplemental Figure 3.5: Selected GC-EAD results of three different sampling methods of *Gonipterus* sp. 2, and the respective EAD responses of either male or female antennae (red) to each FID chromatographic elution profile (black). Top: An EAD signal of a male antenna to a solvent extract of female frass, that was extracted for 1 hour. Middle: An EAD signal of a female antenna to a 30-minute SPME sample of the same female frass. Bottom: An EAD signal of a female antenna to a 10-minute SPME sample of a chamber containing three *Gonipterus* sp. 2 males and a *E. dunni* leaf.



Supplemental Figure 3.6: A comparison between typical FID elution profiles between male (black) and female *Gonipterus sp. 2* elutions (red) from different sampling methodologies. Overall, samples of female weevils showed higher concentration of chromatographic peaks than male weevils in all these sampling methods. A: FID traces of male (black) and female (red) frass, that were extracted in solvent for one hour. The FID elution was minimized with a 15-fold division of the abundance of all peaks in both traces for ease of visualizing the elution profile. B: FID traces of half-hour SPME samples of male(black) and female frass (red). The FID traces were minimized with a 10-fold division. C: FID traces of 15 minute-SPME samples of 20 virgin males (black) and females (red) are overlaid. FID traces were minimized with a 15-fold division. D: A representative FID elution profile from a sample with three males and a single *E. dunni* leaf. This experiment was not conducted with females.

Table captions

- **Table 3.1:** Tentative identifications of selected peaks in the samples represented in Figure 3.5. Tentative identities were based on mass spectral hit-similarities and retention indexes as reported in the NIST database. The literature retention indexes from the NIST library on three different column polarities are included for reference. Because the retention index from literature values (polar column) differ to the calculated retention index of peak 7, the tentative identity aromandendrene is not correct and must be verified. We confirmed that trans-verbenol and S-(-)-verbenone were isolated from the SPME samples of 70 virgin *Gonipterus* sp. 2 males and females.
- **Table 3.2:** Putative identifications of selected peaks in the samples of *Gonipterus* sp. 2 represented in Figure 3.6. Tentative identities were based on mass spectral hit-similarities and retention indexes as reported in the NIST database. The literature retention indexes on three different column polarities are included for reference.
- **Table 3.3:** Tentative identifications of TIC peaks from SPME headspace samples of single male or female *Gonipterus* sp. 2 weevils with a leaf of *E. dunni*, shown in Figure 3.3. Tentative identifications were based on mass spectral hit-similarities and retention indexes as reported in the NIST library.
- **Table 3.4:** Retention data from the reference standards of pheromone candidates S-cis-verbenol, trans-verbenol and S-(-)-verbenone and *Eucalyptus* host volatiles that were found in samples of *Gonipterus* sp. 2. The elution times and calculated retention indexes on our instruments are compared to literature values on both polar and semi-non-polar GC column polarities. The complete list is tabulated in Addendum A.
- **Table 3.5:** EAD deflection times and corresponding calculated retention indexes. DHS samples of 75 untreated *Gonipterus* sp. 2 females (n = 1, left) were screened on male (n = 6) and female *Gonipterus* sp. 2 antennae (n = 4), and DHS samples of 75 untreated males (n = 1, right) were screened for antennal responses on male (n = 6) and female antennae (n = 7).
- **Table 3.6:** Chromatographic peak comparisons and tentative identifications from samples of 75 untreated male and female *Gonipterus* sp. 2 weevils and 75 JHIII-treated male and female weevils. These four treatments (n = 1) were sampled with DHS and analyzed on GC-FID (left), or with SPME and analyzed on the GC-MS instrument (right).
- **Table 3.7:** Retention index characteristics of EAD deflections to DHS samples of 75 JHIII-treated *Gonipterus* sp. 2 females (n = 1, left) and 75 JHIII-treated males (n = 1, right). Both of these treatments were screened on male (n = 5) and female *Gonipterus* sp. 2 antennae (n = 5).

Tables

Table 3.1: Tentative identifications of selected peaks in the samples represented in Figure 3.5. Tentative identities were based on mass spectral hit-similarities and retention indexes as reported in the NIST database. The literature retention indexes from the NIST library on three different column polarities are included for reference. Because the retention index from literature values (polar column) differ to the calculated retention index of peak 7, the tentative identity aromandendrene is not correct and must be verified. We confirmed that trans-verbenol and S-(-)-verbenone were isolated from the SPME samples of 70 virgin *Gonipterus* sp. 2 males and females.

2018112201-3: blank, ♂, ♀.							
#	**Peak Rt**	**RI (ZBWax)**	**NIST putative identity**	**Rmatch**	**RI (lit)-polar****	**RI (lit)-semi-standard nonpolar**	**RI (lit)-nonpolar**
1	3.253	<1100.0	α-Pinene*	948	1028±8 (573)	937±3 (995)	933±4 (676)
2	4.375	1106.4	ß-Pinene*	948	1112±7 (518)	979±2 (849)	973±5 (587)
3	4.578	1120.9	Dehydrosabinene*	958	1124±10 (6)	943±4 (14)	956±2 (59)
4	7.319	1314.1	2-Methyl-5-pentanol	930	1315±13 (46)	846±8 (12)	833±7 (20)
5	8.311	1385.2	Cis-3-hexen-1-ol	892	1382±9 (367)	857±3 (169)	839±5 (165)
6	11.714	1653.6	9-Epi-(E)-caryophyllene*	952	1572±9 (3)	1466±3 (24)	1464±3 (4)
7	11.801	1661.2	Aromandendrene	765	1635±2 (3)	1440±1 (5)	1447±8 (2)
8	12.058	1682.8	Trans-verbenol*	945	1683±4 (59)	1144±2 (103)	1131±9 (76)
9	12.417	1713.9	Verbenone*	836	1725±5 (81)	1205±2 (185)	1184±7 (75)
10	12.569	1727.5	β-Eudesmene*	927	1717±13 (166)	1486±3 (349)	1482±5 (163)
11	14.289	1884.6	Benzyl alcohol	962	1870±14 (323)	1036±4 (174)	1012±8 (125)
12	14.658	1919.9	Phenylethyl alcohol	868	1906±15 (423)	1116±5 (262)	1088±8 (159)
13	14.727	1926.6	α-Calacorene	876	1919±21 (107)	1542±3 (219)	1531±5 (59)
14	15.237	1976.6	1,5-Menthadien-7-ol	890	1814±0 (9)	1194±N/A (1)	1191±0 (2)
15	15.589	2011.5	Isocarveol	889	2015±14 (40)	1296±3 (40)	1281±5 (31)
16	16.465	2100.9	Viridiflorol	962	2095±10 (108)	1591±2 (198)	1582±8 (98)
17	16.628	2118.2	Octanoic acid	862	2060±15 (265)	1180±7 (100)	1173±15 (38)
18	17.616	2224.6	Nonanoic acid	931	2171±17 (145)	1273±7 (97)	1272±11 (41)

Rmatch = reverse match factor: the percentage similarity between the mass fragmentation pattern reported in the NIST library compared to the mass fragmentation pattern of the sample peak.
RI = Kovat's retention index (mean ± SD)
RI (lit) = mean Kovat's retention index reported from the NIST library match to corresponding peak's mass spectrum
*: Compounds also found in the sample blank
**: Column polarity comparable to elution profile from the ZBWax column, as shown in Figure 3.4

Table 3.2: Putative identifications of selected peaks in the samples of *Gonipterus* sp. 2 represented in Figure 3.6. Tentative identities were based on mass spectral hit-similarities and retention indexes as reported in the NIST database. The literature retention indexes on three different column polarities are included for reference.

2019040501-3: ♂, ♀, blank.							
Peaks absent, or larger in concentration than in the blank							
#	**Peak Rt**	**RI (ZBWax)**	**NIST putative identity**	**Rmatch**	**RI (lit)-polar****	**RI (lit)-semi-standard nonpolar**	**RI (lit)-nonpolar**
1	5.173	1163.7	ß-Myrcene	866	1161±7 (569)	991±2 (840)	983±3 (580)
2	5.763	1205.5	Eucalyptol	940	1213±9 (356)	1032±2 (580)	1022±5 (394)
3	6.201	1236.0	Cis-ocimene	910	1235±8 (281)	1038±2 (446)	1028±3 (329)
4	6.451	1253.4	Ocimene	853	1250±4 (17)	1037±7 (20)	1037±4 (15)
5	9.453	1471.5	β-Copaene	806	1586±11 (36)	1432±3 (71)	1426±8 (49)
6	10.26	1534.9	α-Gurjunene	890	1528±7 (93)	1409±2 (196)	1406±5 (90)
7	11.049	1597.6	β-Gurjunene	897	1605±12 (46)	1432±3 (234)	1430±5 (84)
8	11.694	1652.1	Alloaromadendrene	923	1650±11 (158)	1461±2 (343)	1460±6 (178)
9	12.031	1680.7	Trans-verbenol	894	1683±4 (59)	1144±2 (103)	1131±9 (76)
10	12.414	1713.8	n-Dodecanal	877	1711±11 (81)	1409±4 (142)	1389±2 (86)
11	12.447	1716.8	4-Ethyl-benzaldehyde	897	1721±2 (3)	1180±16 (5)	NA
12	12.719	1741.0	Bicyclogermacrene	836	1735±14 (199)	1495±4 (386)	1492±4 (177)
13	13.341	1796.4	Myrtenol	829	1796±8 (139)	1195±2 (203)	1181±5 (109)
14	15.673	2020.3	Epiglobulol	695	2025±14 (11)	1585±5 (29)	1554±14 (9)

Rmatch= reverse match factor: the percentage similarity between the mass fragmentation pattern reported in the NIST library compared to the mass fragmentation pattern of the sample peak.
RI = Kovat's retention index (mean ± SD)
RI (lit) = mean Kovat's retention index reported from the NIST library match to corresponding peak's mass spectrum
*: Compounds also found in the sample blank
**: Column polarity comparable to elution profile from the ZBWax column, as shown in Figure 3.5

Table 3.3: Tentative identifications of TIC peaks from SPME headspace samples of single male or female *Gonipterus* sp. 2 weevils with a leaf of *E. dunni*, shown in Figure 3.3. Tentative identifications were based on mass spectral hit-similarities and retention indexes as reported in the NIST library.

2019031804-6: blank, ♀, ♂.							
Peaks absent in the blank							
#	**Peak Rt (min)**	**RI (ZBWax)**	**NIST putative identity**	**Rmatch**	**RI (lit)-polar****	**RI (lit)-semi-standard nonpolar**	**RI (lit)-nonpolar**
1	4.38	1107	ß-Pinene	966	1112±7 (518)	979±2 (849)	973±5 (587)
2	4.57	1121	Sabinene	950	1124±8 (387)	974±2 (619)	967±4 (451)
3	4.61	1124	3-Methylbutyl acetate	948	1122±7 (168)	876±2 (100)	859±4 (82)
4	5.67	1199	D-Limonene	973	1200±7 (759)	1030±2 (1004)	1023±4 (703)
5	5.77	1206	Eucalyptol	965	1213±9 (356)	1032±2 (580)	1022±5 (394)
6	6.73	1273	p-Cymene	975	1272±8 (543)	1025±2 (820)	1014±4 (616)
7	8.18	1376	(4E,6E)-Allocimene	928	1379±13 (4)	1144±1 (4)	1122±1 (5)
8	11.80	1661	γ-Gurjunene	922	1674±13 (20)	1473±2 (85)	1470±6 (28)
9	12.04	1681	Trans-verbenol*	953	1683±4 (59)	1144±2 (103)	1131±9 (76)
10	12.09	1686	(Z)-3,7-Dimethyl-2,6-octadienal	957	1680±13 (161)	1240±3 (168)	1218±5 (125)
11	12.26	1700	α-Terpineol	926	1697±10 (594)	1189±2 (811)	1175±5 (540)
12	12.44	1716	β-Longipinene	881	NA	1403±1 (5)	NA
13	12.56	1727	β-Eudesmene	951	1717±13 (166)	1486±3 (349)	1482±5 (163)
14	12.60	1731	α-Selinene	917	1725±12 (128)	1494±3 (196)	1491±6 (120)
15	13.25	1789	Cubenene	911	1786±13 (65)	1532±1 (119)	1525±5 (41)
16	13.51	1812	(-)-Cis-sabinol	904	1795±9 (6)	1143±0 (7)	1178±1 (5)

Rmatch= reverse match factor: the percentage similarity between the mass fragmentation pattern reported in the NIST library compared to the mass fragmentation pattern of the sample peak
RI = Kovat's retention index (mean ± SD)
RI (lit) = mean Kovat's retention index reported from the NIST library match to corresponding peak's mass spectrum
* present only in the male (♂) sample
**: Column polarity comparable to elution profile from the ZBWax column, as shown in Figure 3.3

Table 3.4: Retention data from the reference standards of pheromone candidates S-cis-verbenol, trans-verbenol and S-(-)-verbenone and *Eucalyptus* host volatiles that were found in samples of *Gonipterus* sp. 2. The elution times and calculated retention indexes on our instruments are compared to literature values on both polar and semi-non-polar GC column polarities. The complete list is tabulated in Addendum A.

Pheromone Standard*	**ZBWax column, GC-MS (n = 4)**			**ZB5 column, GC-MS (n = 1)**			**NIST**
	Rt (min)	**RI**	**lit RI****	**Rt (min)**	**RI**	**lit RI****	**R.match**
S-cis-verbenol	11.771 ± 0.01	1659 ± 1	1663 ± 0 (33)	6.89	1151	1142 ± 2 (87)	975
Trans-verbenol	12.052 ± 0.02	1683 ± 2	1683 ± 4 (59)	6.90	1152	1144 ± 2 (103)	956
S-(-)-Verbenone	12.407 ± 0.01	1713 ± 1	1725 ± 5 (81)	7.47	1224	1205 ± 2 (185)	975
Host volatile Standard***	**ZBWax column, GC-FID (n = 1)**			**ZB5 column, GC-MS (n = 1)**			**NIST**
(-)-ß-pinene	4.27	1108	1112 ± 7 (518)	4.62	980	979 ± 2 (849)	962
Dehydrosabinene	4.50	1124	1124 ± 10 (6)	4.33	955	956 ± 2 (59)	954
Limonene	5.54	1198	1199 ± 6 (6)	5.19	1032	1031 ± 4 (5)	917
Eucalyptol	5.66	1206	1213 ± 9 (356)	5.22	1035	1032 ± 2 (580)	969
p-Cymene	6.58	1270	1272 ± 8 (543)	5.14	1027	1025 ± 2 (820)	955
Cis-3-hexenol	8.12	1380	1382 ± 9 (367)	3.16	857	857 ± 3 (169)	967
Myrtenol	13.16	1789	1796 ± 8 (139)	6.72	1190	1195 ± 2 (203)	917
Phenylethyl alcohol	14.43	1908	1906 ± 15 (423)	6.04	1116	1116 ± 5 (262)	965

All values are mean ± SD, when n > 1
*: Purchased standards of candidate pheromone components reported in Branco *et al.,* 2019
**: Reported retention index from the respective column polarities from NIST library, represented as mean ± SD (number of replicates)
***: Selected *Eucalyptus* volatile standards (see Addendum A) that were found in samples of *Gonipterus* sp. 2

Table 3.5: EAD deflection times and corresponding calculated retention indexes. DHS samples of 75 untreated *Gonipterus* sp. 2 females (n = 1, left) were screened on male (n = 6) and female *Gonipterus* sp. 2 antennae (n = 4), and DHS samples of 75 untreated males (n = 1, right) were screened for antennal responses on male (n = 6) and female antennae (n = 7).

Untreated female sample (n = 1)				Untreated male sample (n = 1)			
Male antennae (n = 6)		Female antennae (n = 4)		Male antennae (n = 6)		Female antennae (n = 7)	
Rt (min)	RI	Rt (min)	RI	Rt (min)	RI	Rt (min)	RI
3.28	1202.3	3.28	1202.3	3.90	1282.6	3.25	<1200
3.74	1261.9	3.35	1211.4	5.06	1437.6	3.78	1267.1
4.80	1401.5	3.40	1217.9	5.18	1454.3	5.30	1471.0
5.20	1454.4	3.60	1243.8	5.52	1501.6	5.52	1501.6
6.28	1614.1	3.83	1273.6	5.65	1520.7	6.10	1586.9
7.28	1772.2	4.70	1388.2	5.85	1550.1	6.66	1672.8
7.50	1808.3	4.76	1396.2	6.30	1617.2	6.73	1683.6
7.55	1816.8	5.42	1487.6	6.50	1648.1	6.85	1702.3
7.60	1825.3	5.56	1507.5	6.55	1655.8	7.20	1759.2
7.97	1888.3	5.68	1525.1	6.73	1683.6	7.90	1876.4
8.12	1914.4	5.98	1569.3	6.85	1702.2	8.50	1970.6
8.30	1946.5	6.05	1579.6	6.98	1723.4	9.85	2239.8
8.35	1955.4	6.30	1617.2	7.70	1842.3	10.05	2280.2
8.52	1985.7	6.40	1632.6	8.05	1902.0		
8.88	2052.0	6.48	1645.0	8.30	1946.5		
9.20	2112.0	6.90	1710.4	8.68	2014.9		
9.65	2199.4	6.98	1723.4	9.06	2085.5		
9.80	2229.7	7.26	1768.9	9.40	2150.9		
10.70	2416.6	7.35	1783.6	9.93	2256.0		
		7.47	1803.2				
		7.85	1867.9				
		7.90	1876.4				
		8.40	1964.3				
		8.58	1996.4				
		9.03	2079.9				
		9.20	2112.0				
		10.20	2310.9				
		10.70	2416.6				

Table 3.6: Chromatographic peak comparisons and tentative identifications from samples of 75 untreated male and female *Gonipterus* sp. 2 weevils and 75 JHIII-treated male and female weevils. These four treatments (n = 1) were sampled with DHS and analyzed on GC-FID (left), or with SPME and analyzed on the GC-MS instrument (right).

FID of DHS sample		TIC of SPME sample		NIST match	
Rt (min)	**RI**	**Rt (min)**	**RI**	**Name (literature RI -polar)**	**Rmatch**
o		4.55	1120.8	Dehydrosabinene (1124)*	959
3.28	1202.3	5.63	1197.5	Limonene (1200)*	937
3.55	1237.3	6.19	1236.7	Trans-ß-ocimene (1250)	938
3.60	1243.8	6.33	1246.5	Cymene (1275)	952
4.15	1315.4	7.32	1315.5	4-Methyl-1-pentanol (1315)	946
4.78	1398.8	8.29	1385.6	/	/
5.25	1464.0	9.35	1465.3	/	/
5.31	1472.4	9.44	1472.2	/	/
5.48	1496.0	9.76	1496.1	/	/
5.55	1506.0	9.81	1500.2	/	/
5.73	1532.5	10.25	1535.1	/	/
5.83	1547.2	10.39	1546.7	/	/
6.20	1601.7	11.10	1603.5	/	/
6.28	1614.1	11.23	1614.2	/	/
6.40	1632.6	11.51	1638.1	/	/
6.60	1663.5	11.80	1662.6	Cis-verbenol (1663)*	853
6.73	1683.6	12.06	1684.3	Trans-verbenol (1683)*	943
o		12.18	1694.7	2-Oxo-1,8-cineole (?)	907
o		12.23	1698.9	β-Longipinene (?)	861
6.85	1702.3	12.27	1702.1	α-Terpineol (1697)	931
o		12.43	1716.7	Verbenone (1725)*	843
7.10	1742.9	12.71	1742.2	Bicyclogermacren (1735)	906
7.20	1759.2	12.85	1754.4	/	/
7.25	1767.3	13.00	1767.9	/	/
7.45	1799.8	13.37	1800.2	Myrtenol (1796)*	940
7.72	1845.7	13.76	1837.5	Cis-verbenol-acetate (?)	769
7.82	1862.8	14.07	1866.3	2-OH-1,8-cineole (1845)	942
8.70	2018.6	15.59	2013.9	/	/
8.74	2026.0	15.69	2023.5	/	/
9.08	2089.2	16.29	2085.0	Globulol (2086)	918
9.15	2102.3	16.38	2094.3	Viridiflorol (2095)	875
9.54	2178.1	17.14	2174.8	/	/

All samples analyzed on a polar ZBWax column
o : Undetected FID peak in DHS sample elution, but detected in SPME samples analyzed on GC-MS
/ : NIST match does not corroborate with sample compound retention index or mass spectrum.
* : Confirmed with reference standard

Table 3.7: Retention index characteristics of EAD deflections to DHS samples of 75 JHIII-treated *Gonipterus* sp. 2 females (n = 1, left) and 75 JHIII-treated males (n = 1, right). Both of these treatments were screened on male (n = 5) and female *Gonipterus* sp. 2 antennae (n = 5).

Treated female sample (n = 1)				**Treated male sample (n = 1)**			
Male antennae (n = 5)		**Female antennae (n = 5)**		**Male antennae (n = 5)**		**Female antennae (n = 5)**	
Rt (min)	**RI**	**Rt (min)**	**RI**	**Rt (min)**	**RI**	**Rt (min)**	**RI**
3.27	1201.0	4.02	1298.2	3.30	1204.9	3.60	1243.8
3.65	1250.3	5.73	1532.5	3.35	1211.4	4.18	1319.3
4.20	1322.0	6.30	1617.2	3.55	1237.3	4.80	1401.5
5.20	1457.1	6.60	1663.5	3.65	1250.3	5.70	1528.1
5.50	1498.8	6.73	1683.6	4.85	1408.5	6.23	1606.3
5.73	1532.5	6.85	1702.3	5.50	1498.8	6.30	1617.2
6.20	1601.7	7.05	1734.8	5.90	1557.5	6.35	1624.9
6.25	1609.4	10.40	2352.9	6.30	1617.2	6.60	1663.5
6.60	1663.5	10.60	2395.0	6.50	1648.1	6.73	1683.6
6.73	1683.6			6.60	1663.5	6.85	1702.3
7.45	1799.8			6.73	1683.6	7.10	1742.9
8.20	1928.7			6.80	1694.4	7.76	1852.6
				6.92	1713.7	7.98	1890.0
				7.20	1759.2	9.02	2077.1
				7.65	1833.8	9.50	2167.3
				7.70	1842.3		
				7.84	1866.2		
				7.90	1876.4		
				9.05	2083.6		
				9.10	2092.9		
				9.55	2180.0		
				10.15	2300.4		
				15.50	>3000		

Supplementary table captions

- **Supplementary Table 3.1:** EAD deflections of male and female *Gonipterus* sp. 2 antennae in response to FID peaks from SPME samples of 20 virgin *Gonipterus* sp. 2 males (n = 5) or 20 virgin females (n = 4). Response sizes were below detection limits of the EAD software.
- **Supplementary Table 3.2:** The complete list of detectable chromatographic peaks in samples of untreated and JHIII-treated *Gonipterus* sp. 2 weevils, sampled with DHS (left) or SPME (right) and analyzed with GC-FID (left) or GC-MS (right). Qualitative peak height analysis was performed, with 0 to 2 representing relative peak heights from absent to high abundance, respectively. The tentative identifications of some chromatographic peaks were determined based on sample peak fragmentation pattern matches to a compound reported in the NIST library. Excel spreadsheet attached in separate document ("Supplementary Table 3.2").
- **Supplementary Table 3.3**: EAD deflections of male (n = 3) and female *Gonipterus* sp. 2 antennae (n = 3) in response to frass extracts from male and female *Gonipterus* sp. 2 weevils.

Supplementary tables

Supplementary Table 3.1: EAD deflections of male and female *Gonipterus* sp. 2 antennae in response to FID peaks from SPME samples of 20 virgin *Gonipterus* sp. 2 males (n = 5) or 20 virgin females (n = 4). Response sizes were below detection limits of the EAD software.

Responses to male sample		Responses to female sample	
Rt (min)	**RI**	**Rt (min)**	**RI**
2. 52	<1200	2.64	<1200
3.30	1204.9	3.31	1206.2
3.98	1293.0	3.45	1224.0
4.28	1332.6	4.55	1368.3
5.02	1432.1	5.32	1473.8
5.23	1461.3	5.75	1535.4
5.50	1498.8	6.23	1606.3
5.75	1535.4	6.60	1663.5
6.60	1660.4	6.76	1688.3
6.86	1703.9	6.86	1703.9
6.93	1715.3	7.25	1767.3
6.98	1723.4	7.89	1874.7
7.42	1795.0	8.92	2059.5
7.50	1808.3	9.30	2131.5
8.10	1910.9	9.80	2229.7
8.20	1928.7	10.10	2290.3
8.62	2003.7		
8.84	2044.6		
10.08	2286.3		

Supplementary Table 3.2: The complete list of detectable chromatographic peaks in samples of untreated and JHIII-treated *Gonipterus* sp. 2 weevils, sampled with DHS (left) or SPME (right) and analyzed with GC-FID (left) or GC-MS (right). Qualitative peak height analysis was performed, with 0 to 2 representing relative peak heights from absent to high abundance, respectively. The tentative identifications of some chromatographic peaks were determined based on sample peak fragmentation pattern matches to a compound reported in the NIST library. Excel spreadsheet attached in separate document ("Supplementary Table 3.2").

FID chromatographic peak relative sizes to one another								TIC chromatographic peak relative sizes to one another										
		Males		Females						Males		Females						NIST data
Rt (min)	RI	Untreated	Treated	Untreated	Treated	(♂)	(♀)	Rt (min)	RI	♂	(♂)	♀	(♀)	In blank	(♂)	(♀)	Rmatch	Name (literature RI on polar column)
Undetected								4.553	1120.783	1	2	0	0.5		increase	increase	959	Dehydrosabinene (1124)
Undetected								5.349	1177.438	0.5	1	0	0.5		increase	increase		
3.15	<1200	0	1	0	0	increase	-	5.432	1183.345	1	1	1	1		-	-		
3.2	<1200	0	1	1	1	increase	-	5.479	1186.69	1.5	1.5	0.5	1		-	increase		
3.28	1202.332	1	2	1	1	increase	-	5.631	1197.509	1	2	1	1		increase	-	937	Limonene (1200)
3.33	1208.808	1	2	1	2	increase	increase											
3.42	1220.466	0.5	0.5	0	0	-	-	5.903	1216.493	0	0.5	0	0		increase	-		
3.55	1237.306	0	1	1	0	increase	decrease	6.193	1236.674	0	2	0.5	0		increase	decrease	938	trans-ß-ocimene (1250)
3.6	1243.782	0.5	0.5	0.5	0.5	-	-	6.334	1246.486	1.5	2	0.5	1		increase	increase	952	Cymene (1275)
3.65	1250.259	0	1	1	1	increase	-	6.363	1248.504	1.5	1.5	1	1		-	-		
3.68	1254.145	0	1	1	0	increase	decrease	6.435	1253.514	0	1	0	0		increase	-		
3.84	1274.87	1	2	1	1	increase	-	6.725	1273.695	1	2	0.5	1	y	increase	increase		
3.93	1286.528	1	2	1	1	increase	-											
3.98	1293.005	0.5	0.5	0.5	0.5	-	-	6.996	1292.554	2	1	0.5	0.5	y	decrease	-		
4.02	1298.187	0	1	1	1	increase	-	7.087	1298.887	1	1	0.5	1	y	-	increase		
4.15	1315.364	0	1	0.5	0.5	increase	-	7.319	1315.54	1	1	0.5	0.5		-	-	946	4-Methyl-1-pentanol (1315)
Undetected								8.025	1366.331	1.5	1.5	0.5	1		-	increase	938	3-Pentanon-2-ol (1366)
Undetected								8.065	1369.209	1.5	2	0.5	1.5		increase	increase		
Undetected								8.177	1377.266	0	1	0.5	0		increase	decrease		
4.78	1398.808	0	1	1	1	increase	-	8.293	1385.612	1.5	1	0	0		decrease	-		
5.1	1443.194	1	1	1	1	-	-	9.1	1446.055	1	1	0.5	1		-	increase		
5.16	1451.528	0	1	1	1	increase	-	9.212	1454.552	1	1	1	1		-	-		
								9.267	1458.725	1	1	0	0		-	-		
5.25	1464.028	0	1	0	0	increase	-	9.354	1465.326	1	2	0	0		increase	-		
5.31	1472.361	1	2	1	1.5	increase	increase	9.444	1472.155	0.5	2	0.5	1		increase	increase		
Undetected								9.477	1474.659	1	1.5	1	1		increase	-		
Undetected								9.564	1481.259	1	1	1	1		-	-		
5.43	1489.028	0	1	1	1	increase	-	9.625	1485.888	1	2	0.5	1		increase	increase		
5.48	1495.972	0	1	1	1	increase	-	9.759	1496.055	1	1.5	1	1		increase	-		
5.55	1506.029	1	1	1	1	-	-	9.813	1500.161	1	1	0	0		-	-		
5.73	1532.5	1	2	1	2	increase	increase	10.248	1535.072	1	2	1	1.5		increase	increase		
5.83	1547.206	0	1	0	1	increase	increase	10.393	1546.709	0.5	1	0.5	1		increase	increase		
Undetected								10.451	1551.364	1	2	0.5	1		increase	increase		
Undetected								11.048	1599.278	0.5	2	0.5	1		increase	increase		
6.2	1601.7	0	1	1	1.5	increase	increase	11.099	1603.547	1	2	0.5	1		increase	increase		
Undetected								11.168	1609.375	1	2	1	1		increase	-		
6.28	1614.065	0	1	1	1.5	increase	increase	11.225	1614.189	1	2	1	1.5		increase	increase		
Undetected								11.334	1623.395	1	1.5	1	1		increase	-		
6.37	1627.975	0	1	1	1	increase	-	11.388	1627.956	1	1	1	1		-	-		
Undetected								11.439	1632.264	1	2	1	1.5		increase	increase		
6.4	1632.612	0	1	1	1.5	increase	increase	11.508	1638.091	1	2	1	1		increase	-		
Undetected								11.62	1647.551	0.5	1.5	0.5	1		increase	increase		
6.53	1652.705	1	2	1	1	increase	-	11.685	1653.041	1	2	1	1.5		increase	increase		
6.6	1663.524	1	2	1	0.5	increase	decrease	11.798	1662.584	1	1.5	1	0.5		increase	decrease	853	cis-verbenol (1663)
6.73	1683.617	1	2	1	1	increase	-	12.055	1684.291	1.5	2	1	1		increase	-	943	trans-verbenol (1683)
Undetected								12.178	1694.679	0.5	1	0.5	0.5		increase	-	907	2-oxo-1,8-cineole (unknown)
Undetected								12.228	1698.902	0.5	1	0	0		increase	-	861	β-Longipinene (unknown)
6.85	1702.276	1	2	1	1.5	increase	increase	12.265	1702.139	1	2	0.5	1.5		increase	increase	931	α-terpineol (1697)
Undetected								12.428	1716.667	0	1.5	0	0		increase	-	843	verbenone (1725)
Undetected								12.561	1728.52	0.5	1	0.5	0.5		increase	-		

(Cont.)

Supplementary Table 3.2: (Continued)

FID chromatographic peak relative sizes to one another								TIC chromatographic peak relative sizes to one another									
		Males		Females						Males		Females				NIST data	
Rt (min)	RI	Untreated	Treated	Untreated	Treated	(♂)	(♀)	Rt (min)	RI	♂	(♂)	♀	(♀)	In blank	(♂)	(♀)	Rmatch	Name (literature RI on polar column)
7.05	1734.797	0	1	1	1	increase	-	12.609	1732.799	1	2	0.5	1		increase	increase		
7.1	1742.927	1	2	1	2	increase	increase	12.714	1742.157	1	2	0.5	1		increase	increase	906	Bicyclogermacren (1735)
7.2	1759.187	0	1	1	1.5	increase	increase	12.851	1754.367	1	1.5	1	1.5	y	increase	increase		
Undetected								12.963	1764.349	1	1.5	1	2		increase	increase		
7.25	1767.317	0	1	1	2	increase	increase	13.003	1767.914	1	1.5	1	1		increase	-		
Undetected								13.119	1778.253	1	1.5	1	1		increase	-		
7.38	1788.455	1	1	1	1	-	-	13.239	1788.948	2	2	1	1	y	-	-		
Undetected								13.311	1795.365	1.5	1.5	1	1		-	-		
7.45	1799.837	1	2	1	1	increase	-	13.365	1800.187	1.5	2	1	1		increase	-	940	Myrtenol (1796)
Undetected								13.449	1808.052	1	2	0.5	0.5		increase	-		
Undetected								13.601	1822.285	1.5	1.5	1	1		-	-		
7.72	1845.748	1	1	1	1	-	-	13.764	1837.547	1	1	0	0		-	-	769	Cis-verbenol-acetate (unknown)
7.82	1862.755	1	2	1	1	increase	-	14.071	1866.292	1.5	2	0.5	1		increase	increase	942	2-OH-1,8-cineole (1845)
7.89	1874.66	1	1.5	1	1	increase	-	14.144	1873.127	1.5	1.5	1	1		-	-		
7.96	1886.565	1	1	1	1	-	-	14.303	1888.015	1	1	1.5	1		-	increase		
8.18	1925.134	1	1	1	1	-	-	14.665	1922.829	1.5	1.5	1	1		-	-		
Undetected								15.237	1978.634	1.5	1.5	0	1		-	increase		
Undetected								15.527	2007.26	0.5	2	0.5	0.5		increase	-		
8.7	2018.587	0	0.5	0	0	increase	-	15.592	2013.906	1	1.5	0.5	0.5		increase	-		
8.74	2026.022	0	0.5	0.5	0.5	increase	-	15.686	2023.517	1	1.5	1	1	y	increase	-		
Undetected								16.084	2064.213	1	1.5	0.5	0.5		increase	-		
9.08	2089.219	0	1	1	1	increase	-	16.287	2084.969	1	2	1	1		increase	-	918	Globulol (2086)
9.15	2102.33	0	0.5	0	0.5	increase	increase	16.378	2094.274	1	2	1	1		increase	-	875	Viridiflorol (2095)
Undetected								16.682	2126.355	0.5	1	0.5	0.5		increase	-		
9.33	2137.282	1	2	1	1	increase	-	16.754	2134.006	0.5	1	0	0		increase	-		
9.54	2178.058	1	1	1	1	-	-	17.138	2174.814	1	1	1	1	y	-	-		

Supplementary Table 3.3: EAD deflections of male (n = 3) and female *Gonipterus* sp. 2 antennae (n = 3) in response to frass extracts from male and female *Gonipterus* sp. 2 weevils.

Rt (min)	RI	Rt (min) (continued)	RI
3.28	1202.3	9.45	2160.6
3.84	1274.9	9.55	2180.0
4.16	1316.7	9.60	2189.7
4.35	1341.9	9.68	2205.5
4.40	1348.5	9.83	2235.6
4.45	1355.1	10.08	2286.3
4.58	1372.3	10.21	2313.0
4.80	1401.5	10.35	2342.4
4.85	1408.5	10.50	2374.0
5.17	1452.9	10.60	2395.0
5.25	1464.0	10.87	2453.6
5.45	1491.8	10.92	2464.5
5.60	1513.4	11.13	2509.9
5.65	1520.7	11.30	2545.5
5.80	1542.8	11.43	2572.7
6.00	1572.2	11.67	2621.2
6.05	1579.6	11.78	2642.3
6.08	1584.0	11.82	2650.0
6.20	1601.7	11.86	2657.7
6.32	1620.2	11.95	2675.0
6.55	1655.8	12.00	2684.6
6.80	1694.4	12.15	2712.0
6.90	1710.4	12.35	2746.3
7.00	1726.7	12.50	2772.0
7.26	1768.9	12.58	2785.8
7.31	1777.1	12.80	2820.2
7.85	1867.9	12.95	2842.4
8.08	1907.3	13.00	2849.8
8.45	1973.3	13.10	2864.5
8.65	2009.3	13.35	2901.2
8.70	2018.6	13.50	2919.8
8.82	2040.9	13.61	2933.3
8.92	2059.5	13.70	2944.4
9.10	2092.9	13.82	2959.2
9.15	2102.3	13.98	2979.0
9.25	2121.7	14.15	3000.0
9.29	2129.5		

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Chapter 4

Sex pheromones of *Nudaurelia clarki* (Lepidoptera: Saturniidae) and their use in field trapping

Abstract

Nudaurelia sp. are sporadic pests of pine plantations in South Africa. Uncertainty in the species identity across South Africa exists and has limited the development and implementation of pheromone-based management tactics. We analyzed female ovipositor extracts with gas-chromatography electroantennographic detection (GC-EAD) and gas-chromatography-mass spectroscopy (GC-MS). We also compared cytochrome oxidase I gene sequences for moths from the Western Cape, KwaZulu-Natal (KZN) and Mpumalanga regions. Results show that *Nudaurelia clarki* (Saturniidae) male antennae respond to two compounds in female ovipositor extracts. One of these compounds was confirmed to be (Z)-dec-5-en-1-yl-3-methylbutanoate with a synthetic standard. The second compound identity could not be confirmed. Both male and female antennae responded to four structurally related compounds in the synthetic pheromone standard. The fact that *N. clarki* females are able to recognize the same pheromone also isolated from their ovipositors through electrophysiological techniques, presents the first evidence of female autodetection in Saturniidae. Field trials with custom-made traps confirmed attraction of *N. clarki* males to pheromone-baited polydimethylsiloxane and polyisoprene lures. Cytochrome oxidase I gene sequences were identical for moths from the Western Cape, KwaZulu-Natal (KZN) and Mpumalanga regions. On the basis of shared pheromone identity, *Nudaurelia* spp. (*N. clarki* and *N. cytherea*) from the Western Cape, KZN and Mpumalanga may be the same species. In addition to its potential role in monitoring *N. clarki,* the sex pheromone has potential for mass-trapping and mating-disruption of this pest.

Introduction

Saturniids (Saturniidae: Lepidoptera) are among the largest known insects in the world. The large moths have characteristic eye spots that are surrounded by concentric rings on their wings. In fact, the rings were used in their family name in reference to the planet Saturn (reviewed in Powell, 2003). The subfamily Saturniinae are a subfamily of the Saturniidae and are commonly known as emperor moths. The larvae of many emperor moths in Southern Africa are edible, and known as “mopane worms”. Some of these mopane worms include the larvae of *Gonimbrasia belina* (= *Imbrasia belina*), *G. zambesina*, *Gynanisa maja* (Kachapulula et al. 2018) and *Bunaea alcinoe* (Amadi et al. 2005). In fact an estimated 1600 tons of dry caterpillars were sold in 1982 in South Africa (Greyling et al. 2001).

Most South African emperor moth species are polyphagous (Supplementary Material from Staude et al. 2016; Van den Berg, 1973a, 1974, 1990). For example, the poplar emperor moth, *Pseudobunaea irius*, has four indigenous and 13 exotic plant species that it feeds on (Hepburn et al. 1966). This species is known to defoliate *Pinus*, *Eucalyptus* and *Acacia* trees in South Africa (Hurley et al. 2017; Van den Berg, 1973a, 1979b). Another common example in South Africa is *Nudaurelia cytherea* (Fabricius 1775), which occur on native plant hosts such as *Rhus angustifolia*, *Rapanea melanophloeos*, *Euclea schimperi*, *Protea repens* and *Watsonia* spp., and several others (Geertsema, 1970; Pinhey, 1956). *Nudaurelia* species have also undergone host range expansion onto exotic plantation trees where they have become a pest (Hurley et al. 2017; Sims, 1903; Tooke, 1935; Tooke et al. 1941; Van den Berg et al. 1973). *Nudaurelia* larvae can be found predominantly on *Pinus radiata* and *P. patula* (Van den Berg, 1973b).

There is uncertainty in the number of *Nudaurelia* species feeding on pine because there are many apparent synonyms for this genus in the literature. Synonyms of these moths include *Antherea cytherea (Sims, 1903)*, *Imbrasia cytherea (Van den Berg, 1979a, 1979b)*, *Bombyx cytherea*, *Gonimbrasia cytherea* and *Nudaurelia cytherea* (Geertsema, 1970). Two prominent *Nudaurelia* variations were first described as the same species (Pinhey, 1956) and later as subspecies, *N. cytherea cytherea* and *N. cytherea clarki* (Geertsema, 1971). These two variants were also described as *N. cytherea* and *N. clarki*, respectively (Supplementary Material from Staude *et al.*, 2016). These separate species names are accepted as true for the purpose of this study.

Nudaurelia cytherea is known along the coastline of the Western Cape through to the Eastern Cape and in Southern KZN (Supplementary Material from Staude *et al.*, 2016; Van den Berg, 1973a) (Supplementary Figure 1). *Nudaurelia clarki* is known in Zimbabwe, Limpopo, North West, KZN and the northern parts of the Eastern Cape, as well as eSwatini, previously known as Swaziland (Van den Berg, 1973a) (Supplementary Figure 1). The *N. cytherea* moths are brown in color and are smaller than the *N. clarki* moths that are predominantly yellow (Geertsema, 1970, 1971). The color of the third ring around the wing eye-spot is white for *N. cytherea* and pinkish or maroon for *N. clarki* (Geertsema, 1971; Supplementary Material from Staude *et al.*, 2016) (Supplementary Figure 2).

The first report of *N. cytherea* as a pest was an outbreak at the Fort Cunyghame plantation in 1896 on *Pinus insignis* that lasted for seven years (Sims, 1903). These moths were rarely seen in subsequent years and damage to pine plantations now occurs sporadically (Govender, 2011; Tooke, 1935). Defoliation, especially of the crown, of pine trees can cause stunted growth and subsequent economic loss (Van den Berg, 1973a). It has been reported that 126 *N. cytherea* larvae (Figure D.K) can totally defoliate a large *Pinus patula* tree with crown depth of 7 m (Van den Berg, 1979b). Although historically infrequent, when infestation of pines occurs these moths can cause serious economic loss if populations are not managed (Van den Berg, 1979b).

Methods that have previously been used for control of emperor moths in plantations include cultural control, biological control and insecticide application. Cultural control involves ploughing the undergrowth in pine plantations to destroy pupae and manually monitoring for infestation by counting frass droppings or dead moths underneath pine trees (Geertsema, 1980; van den Berg et al. 1974). When a threshold of frass or dead moths is realized, these monitoring methods trigger subsequent application of other pest control methods such as insecticides or biocontrol agents. Natural control agents include pigs, chacma baboons, birds and other larger predators (Geertsema et al. 1973). Larval viruses of have also been studied for development and use in biological control (Hendry et al. 1985; Munshi et al. 1998; Tripconey, 1970). Application of pesticides is the preferred control method in pine plantations because it is fast-acting and can prevent damage (Donald, 1963; Govender, 2011; Van den Berg, 1979b). Pyrethroids and organochlorine pesticides

have been used to manage larvae (Donald, 1963; Govender, 2011; Kirsten et al. 2000). These pesticides are in fact more effective against young larvae (Donald, 1963), but are known to have many non-target effects (Quinn et al. 2011). These control measures can be aided with pheromones in integrated management schemes. Pheromones can be useful for monitoring adults and can be used to directly control populations once optimal attraction and species-specificity is attained.

The pheromone of *N. cytherea* was identified in the early 1970's as (Z)-dec-5-en-1-yl-3-methylbutanoate (Henderson, 1972; Henderson et al. 1972, 1973). The pheromone was used experimentally to trap *Nudaurelia* species found in the Cape region in the 1970's (Henderson et al. 1972, 1973). A water trap was used in that study, and although it was effective, many moths from the family Geometridae, flies and the butterfly, *Dira clytus* (Nymphalidae: Satyrinae) were also trapped. The low specificity of these pheromone-baited traps is hypothesized to be the by-product of the liquid soap-paraffin mixture in the open bucket traps (Henderson et al. 1972). No subsequent pheromone field trials or commercial use have been reported for this moth in South Africa.

It is unknown if (Z)-dec-5-en-1-yl 3-methylbutanoate is a pheromone attractant for *N. clarki*. The objective of this study was to identify the sex pheromone of *N. clarki* collected from pine plantations in northeastern South Africa (Figure 1a). The effectiveness of the luring capability of the previously identified and synthesized (Z)-dec-5-en-1-yl 3-methylbutanoate pheromone compound was tested with various pheromone dispensers in the field. Furthermore, in order to determine whether COI gene sequences are able to differentiate between the moths collected from different South African regions, this mitochondrial gene was sequenced and compared between moths collected from the Cape, KZN and Limpopo areas (Supplementary Figure 1).

Methods

Insects

Adult moths were collected by hand from Jessievale (-26.221804, 30.468118), Bulwer (-29.8049195, 29.7553241) and Pringle Bay (-34.3390330, 18.8309181), South Africa (Figure 4.1). Moth legs were used for DNA extraction and were obtained from individuals collected in April-May in 2018 from Bulwer (n = 5) and Jessievale (n

= 10) and April-May in 2019 from Bulwer (n = 5) and Pringle Bay (n = 5). Dynamic headspace sampling ensued with field collected females (n = 5) and reared virgin female moths (n = 7) from Bulwer and Jessievale. Rearing involved manual collection of pupae from the undergrowth of a pine plantation in Bulwer between April to May in 2018, and keeping pupae undisturbed in the soil from the collection site in an unsealed plastic container until moths emerged (Figure D.O). The soil was kept damp by spraying with distilled water. Rearing conditions were 22 ± 2°C, 12:12 L:D cycle, and 65 ± 3 % relative humidity in a fine-mesh cage in an insectarium at the FABI Biocontrol Center on the University of Pretoria experimental farm.

DNA sequencing

The legs of the collected moths were stored in 95% ethanol and freeze dried for 24 hours prior to DNA extraction. DNA was extracted with the phenol-chloroform method (Pacific Biosciences - Shared Protocol). Gene amplification reactions were performed in a total volume of 25 µL that consisted of 2.5 µL of 10x PCR Buffer, 2.5 µL (0.25 mM) of dNTPs, 3 µL (3 mM) of $MgCl_2$, 1 µL (10 µM) of each Lepidopteran-specific COI mitochondrial gene primer (LepF1 5'-ATTCAACCAATCATAAAGATA TTGG-3' or LepR1 5'-TAAACTTCTGGATGTCCAAAAAATCA-3') (Hebert et al. 2004), 0.2 µL (5 U/µl) FastStart Taq Polymerase, 10.8 µL sterile PCR grade SABAX water and 1 µL (50 ng) DNA. PCR amplifications were done with a ProFlex PCR instrument with an initial denaturation temperature at 95°C for 7 min. This was followed by 94°C for 30 sec; 50 or 52 or 56°C for 30-60 sec; 72°C for 30 sec, and these steps were repeated for 36 cycles. The final extension temperature was 72°C for 7 min. Polyacrylamide gel electrophoresis was used to visualize and compare amplified fragment lengths. Sequencing was done on an ABI Prism 3130 XL capillary sequencer. Sequences were inspected and trimmed in ClustalX 2.1 (Larkin et al. 2007) and aligned with default parameters using Mega 7.0.26. The sequences were compared to sequences in GenBank through a BLAST search, and to each other. The base-pair sequences of samples from each respective region were identical. A neighbor-joining tree was thus constructed with representative samples from each region, via Mega 7.0.26 with the default parameters and COI gene sequences from GenBank.

Electrophysiology

GC-EAD

An Agilent 6890N gas chromatography system (Chemetrix, Midrand, South Africa) was coupled to an electroantennographic detection system (Syntech, Hilversum, The Netherlands) for GC-EAD recordings. The GC oven temperature for the ZBWax column (30 m x 0.32 mm ID, 0.25 µm, 7HG-G007-11, Zebron™) was held at 50°C for 1 minute and ramped at a rate of 20°C/min to 250°C and held for 10 minutes. The method on the mid-polar ZB5 column (30 m x 0.32 mm ID, 0.25 µm, 7HM-G002-11, Zebron™) was held at 50°C for 2 minutes and ramped at a rate of 20°C/min to 300°C and held for 5 minutes. Sample screening was performed on two column polarities to corroborate retention characteristics of respective chromatographic peaks. Solvent-based samples were injected into the GC-EAD system splitlessly (injector temperature at 250°C) to screen for repeatable electrophysiological responses from male antennae. The effluent was split with a Y-quartz splitter (Agilent, PN:5181-3398) at the end of the GC-column to the FID detector (300°C) and antennal preparation in a 50:1 split ratio, respectively. The transfer line was kept at 250°C. Samples that gave responses were combined and concentrated and rescreened to ascertain whether previously undetected peaks were concentrated enough for detection with the FID. Kovat's retention indices (I_K) were calculated for all chromatographic peaks.

Antennae were removed with dissecting scissors at the pedicel. The terminal antennal nerve endings were exposed by cutting off the antennal tip. Fine hairs on the male bipectinate antennae were removed at the terminal ends where the antennae were connected to the glass capillary electrodes (Figure D.H), to minimize mechanic perturbations of the antenna that could introduce noise in antennal recordings. Each antenna was oriented with the tip connected to the recording electrode of the EAD. The antennal preparation was moved to within 5 mm of the stimulus delivery system of the EAD. Micro electrodes were made from pulled glass capillaries (Hirschmann, 120 mm) and Ag/AgCl wire electrodes. The capillaries were filled with Beadle-Ephrussi Ringer electrolyte solution (129 millimolar NaCl, 4.7 millimolar KCl, and 1.9 millimolar CaCl) and connected to a manually adjustable micro-manipulator at each electrode. All direct current recordings were made with a GcEad32 V4.3 system (Syntech, Hilversum, The Netherlands), and amplified ten times with external amplification.

Baseline drift was removed by plotting the derivative of the EAD data as described in Slone and Sullivan (2006).

Extracts and Headspace Samples

Gland extracts of dissected ovipositors were made into 500 µl hexane (n = 26) from field collected female moths from Bulwer and Jessievale upon arrival at the lab. When moths were not used immediately, they were kept in the insectarium under rearing conditions. Dynamic headspace sampling was performed by aerating reared female moths individually in modified 1-liter CONSOL jars with bottled air (Figure D.G). All jars were cleaned with soap and water and dried overnight in a drying oven (110°C) before use. Air was passed through a hydrocarbon trap (Supelco Superpure HC, #2-2445-U) for purification and distilled water for humidification with teflon tubing (1.8" OD, Supelco, #20532). The air then moved through bulkhead union fittings (6.35 mm or ¼ inch), attached to the custom modified lids of each Consol jar. The airflow, at a flow rate of 6.2 ± 2.1 ml/min (mean ± SD), was manually regulated with an adjustable glass bubble flow meter. Volatiles were trapped onto PoropakQ adsorbent cartridges (Supelco ORBO 1103, 50/80, 150/75 mg) from 08h00 to 08h00 the next morning. This procedure was repeated for a the same reared female for three consecutive nights after emergence onto different adsorbent cartridges. Each poropakQ adsorbent was desorbed three times with double distilled n-hexane (500 µl, 30 min). Desorbed fractions were combined and the solvent was transferred to new vials. These extracted samples were stored at 4°C in a fridge before analysis.

EAG Dose Response

A serial dilution of the pheromone, (Z)-dec-5-en-1-yl-3-methylbutanoate, was prepared in dichloromethane. Concentrations ranged from 10^{-3} ppm to 10^{-8} ppm. A volume of 1 µl of each concentration was pipetted onto filter paper strips (Whatman 1, 0.5 x 2.2 cm) and inserted into Pasteur pipettes. The control stimulus was impregnated with double-distilled dichloromethane. Puffs (1 second) were generated with the help of a Syntech stimulus controller (CS-55) at a flow rate of 159.8 ± 0.6 l/hour. All concentrations were puffed over the same antenna with thirty seconds intervals between stimuli. Electroantennographic responses were recorded from male (n = 10) and female (n = 2) antennae. Puffs were presented in increasing order over five male antennae, and in decreasing order over five male antennae. The response size for

each puff was measured from direct current data (GcEad32 V4.3, Syntech, Hilversum, The Netherlands). Kruskal-Wallis non-parametric significance tests were performed with blank-subtracted antennal response data in R version 3.5.2.

GC-EAD Dose Response

A serial dilution of between 0.001 ppm to 10 ppm of the synthetic pheromone, (Z)-dec-5-en-1-yl-3-methylbutanoate, was prepared in n-hexane. A volume of 1 µl of the respective standards was injected into the GC-EAD system in splitless mode, and the same separation method was used as for GC-EAD extract analyses (ZBWax). Response sizes of male antennae (n = 4) were measured for each dilution. The experiment was repeated for both increasing and decreasing concentration increments in time. The antennal response sizes were measured from the direct current data with GcEad32 V4.3 software, and blank-subtracted (Syntech, Hilversum, The Netherlands). The response data was analyzed in R version 3.5.2.

GC-MS screenings

An Agilent 7890B gas chromatography system was coupled to a 5877B MSD mass spectrometer detector. Female ovipositor extracts (1 µl) were injected in splitless mode into the GC-MS (purge vent at 2 minutes, 50 ml/min). The oven was held at 50°C for 1 minute, and ramped (20°C/min) to 250°C where it was held for 6 minutes for the ZBWax column (30 m x 0.25 mm ID, 0.25 µm, 7HG-G007-11, Zebron™). The temperature program for the HP5 column (30 m x 0.25 mm ID, 0.25 µm, 19091S-433UI, Agilent™) was 50°C for 2 minutes and ramped (20°C/min) to 300°C and held for 5 minutes (mass scan range of 30 - 550 m/z). The synthetic pheromone (50 ppm) was injected (1 µl) in splitless mode into the instrument. The 110 m/z-ion was extracted from the total ion chromatogram (TIC) and used as a characteristic ion for (Z)-dec-5-en-1-yl-3-methylbutanoate after DMDS derivatization confirmed the position of the double bond (Addendum B) and mass spectrum of the synthesized pheromone was confirmed to be similar to the fragmentation pattern previously reported (Henderson 1972).

Lure release rate quantitation

Polydimethylsiloxane (PDMS) rings, 0.4 ml polyethylene microcentrifuge tubes (PE) (Thermo-Scientific, Catalog #3485) and polyisoprene red rubber (RR) septa

(Thomas-Scientific, Catalog #1199J62, 10 mm ID) were used as pheromone dispensers (Figure D.F). PDMS rings were made with 1 cm cut glass capillaries (Hirschmann, 120 mm) and 3 cm PDMS tubing (Carlin Medical Extrusions (Pty) Ltd, #195, 1.02 mm ID x 2.06 OD), connected at both ends of the capillaries. Red rubber septa were extracted in DCM for 24 hours prior to loading with the synthetic pheromone. All lures were loaded with 1 µl undiluted (Z)-dec-5-en-1-yl-3-methylbutanoate (>99% pure). Each loaded lure was aged in a fume hood at 25 ± 2°C for 27 days or 32 days for the SPME and DHS analyses, respectively. Lure dispensers were placed in individual metal test tube caps, to ease transferring of the dispensers from the fume hood to sampling chambers without interfering with the dispenser polymer-pheromone equilibrium.

SPME sampling

Lures were placed in custom made glass chambers (100 ml) before sampling. These chambers consisted of a 40 mm bottom female ground joint with a corresponding male 40/38 ground joint adapter. The male adapter had an open screw top that was sealed with a stopper containing a N12, 1.3 mm PTFE septum (Machery-Nagel, #702292). The glass chamber was submerged into a water bath (35˚C) during sampling events. A conditioned SPME fiber (DVB/CAR/PDMS, #57328-U) was exposed to the inside of the glass chamber for 30 minutes. The fiber was desorbed in the GC-MS inlet immediately after sampling.

The oven of the GC-MS was set at 50°C for 2 minutes, and ramped (20°C/min) to 300°C and held for 2.5 minutes. The inlet was operated in splitless mode (vent flow = 100 ml/min at 2 min). A mass scan range of 45 - 550 m/z was used in the mass spectrometer. Separation was done on an HP5 column (30 m x 0.25 mm ID, 0.25 µm, 19091S-433UI, Agilent™). External calibration was achieved by injecting 1 µl of a serial dilution of 0.01 ppm to 100 ppm of (Z)-dec-5-en-1-yl-3-methylbutanoate in n-hexane. Four replicate calibration curves were determined on four separate days during the release rate experiment. The peak area of the 110 m/z ion was integrated and used for calibration.

Dynamic headspace sampling

CONSOL glass jars (1-liter) that were fitted with two Swagelok ports and Teflon O-ring seals were used as chambers for dynamic headspace sampling

(Supplementary Figure 4.1). The Swagelok inlet was used to seal Tygon tubing between a needle valve regulated gas manifold and the CONSOL jar lid. The manifold was connected to a tank of dry air, from where the main airflow was maintained at 60 ml/min for up to five sampling chambers at a time, subject to moth availability. The Swagelok outlet sealed the attachment of a glass tube connected via Teflon tubing to a custom blown glass outlet. Mini multi-channel-traps (mini-MCT) were used to sample headspace volatiles. A mini-MCT consisted of parallel packed conditioned silicone tubing (Sil-Tec®, #602-105) in a glass split liner (Supelco, # SU2879405-U) (Ortner et al. 1996; Van Vuuren et al. 2019). Flat zinc plated washers (Eureka, # 7AD41U, 6 x 22 x 1.5 mm) secured the central position of a mini-MCT in the glass inlet when the mini-MCT was inserted into the washer opening. An unmodified washer was placed vertically in the innermost part of the glass outlet, and a washer with three machined 3 mm openings was placed vertically close to the opening of the glass outlet. A vacuum pump (SKC AirChek 52/Sidekick sample pump, # 224-52MTX) was connected to the mini-MCT's to maintain a sampling flow rate of 30 ml/min. Sampling was performed consecutively for each lure (60 minutes). The mini-MCT's were desorbed similar to the procedure described in Van Vuuren et al. (2019).

A gas chromatography system (GCxGC, Agilent 7890) was coupled to a time-of-flight mass spectrometer (4D Pegasus TOF-MS, LECO) that was fitted with a Rxi-1MS column (30 m, 250 µm ID x 0.25 µm, 19091S-933, Agilent™). The primary oven was set at 40°C for 2 minutes, and ramped (8°C/min) to 190°C, followed by a temperature ramp (25°C/min) to 280°C and held for 3 minutes. The secondary oven was kept at a 20°C offset higher than the primary oven. The modulator temperature offset was maintained at 15°C relative to the secondary oven temperature. The transfer line was kept at 280°C. The MS had a column flow rate of 1 ml/min (He). The ion source was operated at 230°C with electron energy of 70 eV. Mass spectra were acquired between a mass scan range of 40-500 m/z after a time delay of 5 minutes at a rate of 10 spectra/second.

External calibration was achieved by injecting 1 µl of dilutions of the synthetic pheromone in n-hexane, including 0.1 ppm, 0.5 ppm, 1.0 ppm, 5.0 ppm, 10 ppm, 50 ppm and 100 ppm. Three replicate standard curves were determined with different mini-MCT's on three separate days during the release rate experiment. The peak area of the 82 m/z ion was used for quantification.

Weighted calibration curves were constructed (n = 4 for SPME calibrations and n = 3 for DHS calibrations) in R version 3.5.2, using the calplot function in the chemcal package and concentrations in release rate samples were predicted with the inverse.predict function.

Field testing

Traps

Collapsible traps were designed and made to be large enough for pine emperor moths to enter (Supplementary Figure 4.2, Figure D.C and D). Fiberglass mesh (5 mm) was wrapped around a cylindrical shape made from two (37 cm, OD) wire (4 mm) rings and four PVC pipes (50 cm by 20 mm ID) as a support for the structure (Supplementary Figure 4.3). Two fiberglass mesh funnels were knitted into the sides of the cylindrical trap with staples. Lure dispensers were hung in the middle of the traps on a 4 mm wire hook that was suspended from the top PVC pipe through a drilled hole (4.5 mm) (Figure D.F).

Trial design:

The field trial was set up near Bulwer, KZN (29°48'17.7"S 29°45'19.2"E) in a *Pinus patula* plantation. Trial 1 ran from 7-12 March 2019 and trial 2 between 12 March - 8 April 2019. Traps were suspended with cable ties and PVC angle brackets from pine trees at a height of between 2 to 2.5 m. Eighteen traps were set out in a linear transect roughly 5 m from the compartment edge (Figure D.A). Lures were loaded with 1 µl of undiluted synthesized pheromone. Trial one compared PDMS ring lures (n = 6) and field collected females (n = 6, Figure D.E) to blank traps (n = 6). Moth counts were taken after 5 days. In trial 2, PDMS ring (n = 6) and red rubber septum lures (n = 6) were compared to blank (unloaded PDMS lures) traps (n = 6). Captured moths (Figure D.B) were counted and discarded weekly until a period of 27 days had passed. Results were analyzed in R version 3.5.2 with the Kruskal-Wallis test to determine significance between treatments for each trial. Thereafter, a post-hoc pairwise Wilcoxon rank sum test with Bonferroni correction was used to show differences between treatments.

Results

COI DNA sequencing

COI-gene sequences (581 base pairs after alignment and trimming) were identical for moths collected from Bulwer, Jessievale and Pringle Bay (Figure 4.2). No sequences of *N. clarki* or *N. cytherea* were available on Genbank. The sequences from our collected samples grouped with those known for other moths in the Saturniidae family. Only the genera that grouped closest to our samples were used in our phylogenetic tree, including species from the *Nudaurelia*, *Imbrasia*, *Gonimbrasia* and *Antherea* genera. The species in the *Antherea* genus formed the outgroup of our dataset. Species that grouped closest to *N. clarki* were *Nudaurelia kohli*, *N. dione*, *N. amathusia*, and *N. anthinoides* (Figure 4.2 and Supplementary Figure 4.4).

Electrophysiology

GC-EAD

One large and one small male antennal response were present in the GC-EAD recordings (n = 26) of female pheromone gland extracts (Figure 4.3 and Supplementary Figure 4.5). The large response occurred at I_K = 1887 ± 1 on the ZBWax column and the male antenna response size was 135.56 ± 42 µV (n = 9). The same large response occurred at I_K = 1627 ± 3 on the ZB5 column and in this case the male response size was 104.62 ± 91 µV (n = 13). The small response occurred at I_K = 1879 ± 1 on the ZBWax column, and the male response size was 23.33 ± 15 µV (n = 6). On the ZB5 column, the small male response, 22.22 ± 16 µV (n = 7), occurred at I_K = 1621 ± 2. The concentration of both active compounds was below our FID detection limits. We observed no male antennal responses to dynamic headspace samples from field collected or reared virgin females (Supplementary Figure 4.6).

We observed four chromatographic peaks in the reference standard at the 50 ppm concentration level (Table 4.1). Both male and female antennae detect all four of these chromatographic peaks. Responses of female antennae were observable, but often not measurable in the EAD software. Male antennae (842.9 ± 446.2 µV, mean ± SD, n = 7) gave significantly larger (t-test p = 0.0179) antennal responses to the largest chromatographic peak (at 50 ppm) when compared to female antennae (50.0 ± 42.4 µV, mean ± SD, n = 3) (Figure 4.4). Kovat's retention index values of the large antennal

response observed from gland extract analysis (ZBWax) matched with peak four in the pheromone standard solution (Figure 4.5 and Table 4.1).

EAG Dose Response

Electrophysiological responses (315.7 ± 45.8 µV, mean ± SD, n = 10) from male antennae were on average larger than for female antennae (104.7 ± 13.3 µV, mean ± SD, n = 5), but not significantly different (Kruskal p = 0.416) (Supplementary Figure 4.7 and Supplementary Table 4.1). A larger variance in response size was observed for male antennae when compared to female antennae. Subsequent recordings on the GC-EAD confirmed that the antennae can detect the dichloromethane (DCM) solvent that was used to dilute the pheromone standard. Electrophysiological responses measured with this technique were confounded by the response to the DCM solvent. The response to the solvent was removed when the same experiment was done on the GC-EAD.

GC-EAD Dose Response

Dose response data confirmed a log linear regression (F (1,2) = 45.59, MSE = 0.365, p = 0.021) from male antennae on the GC-EAD system (R^2 = 0.958, data not shown). Response sizes increased from 20 ± 40 µV to 540 ± 143.29 µV (mean ± SD) over a 0.01 to 10 ppm concentration range (Supplementary Figure 4.8).

GC-MS

Extracts and standards

The concentration of the electrophysiologically active compounds in female *N. clarki* ovipositor extracts were below detection limits of the GC-MS system (n = 24). This was also true when selected extracts were combined, concentrated and re-analyzed. However, the retention indices of two of the four peaks found in the synthesized pheromone standard were almost identical when compared to retention indices calculated for the electrophysiological responses seen to ovipositor extracts on both the polar (ZBWax) and mid-polar (ZB5) columns (Table 4.1).

Analysis of the (Z)-dec-5-en-1-yl-3-methylbutanoate standard revealed four peaks that were separated on the ZBWax column (I_K (peak **1**) = 1852 ± 0, I_K (peak **2**) = 1873 ± 0, I_K (peak **3**) = 1882 ± 0 and I_K (peak **4**) =1887 ± 1) (n = 3)) (Figure 4.6). The mass spectrum of peak one had a 140 m/z ion, in contrast to peaks two, three and four, that

showed mass spectra with the 138 m/z ion (Figure 4.6). The 140 m/z ion was assigned to the same fragment than the 138 m/z ion plus two hydrogen atoms. The identity of peak one was subsequently assigned to the saturated decenyl-3-methylbutanoate. As expected, decenyl-3-methylbutanoate eluted earlier on the ZBWax column than the unsaturated compounds and did not react during DMDS-derivatization.

The characteristic mass fragments of 159 and 175 m/z for one of the DMDS-derivatized compounds in the synthesized pheromone suggested that dec-2-en-1-yl-methylbutanoate was present in the standard (Addendum B). It is possible that peak two represents this compound, as most expected fragments from the underivatized compound (Figure B.8) is present in this peak's mass spectrum (Figure B.5). Peak two was thus tentatively identified as dec-2-en-1-yl-methylbutanoate. Peak three had a similar fragmentation pattern to (Z)-dec-5-en-1-yl-3-methylbutanoate (peak four), however its mass spectral pattern was sufficiently different from (E)-dec-5-en-1-yl-3-methylbutanoate (Figure 4.6) reported by (Henderson, 1972). We conclude that this peak could be a different isomer.

Peak four had a retention index that matched well with the major antennal response found when gland extracts were analyzed on the GC-EAD system (I_K = 1887 ± 1, Figure 4.5 and Table 4.1). Peak four was the synthesized (Z)-dec-5-en-1-yl-3-methylbutanoate and it had fragmentation masses that included m/z = 240 (0.02%, M^+), 138 (33%), 110 (72%), 95 (67%), 81 (100%, base peak), 67 (86%) and 57 (86%) (Figure 4.6, peak 4). The characteristic 117 and 217 m/z ions of the DMDS-derivatized standard confirmed the double bond at the fifth carbon (Addendum B).

Analysis of the (Z)-dec-5-en-1-yl-3-methylbutanoate standard on the HP5 column showed four distinguishable peaks, including I_K (**A**) = 1611 ± 1, I_K (**B**) = 1617 ± 1, I_K (**C**) = 1624 ± 1, and I_K (**D**) = 1636 ± 0 (Supplementary Figure 4.9 and Table 4.1). The mass spectra of peaks B, C and D on the ZBWax column were identical to the mass spectra of peak three, four and one on the ZBWax column (Table 4.1).

Comparison between the retention indexes of the antennal responses to the ovipositor extracts and the (Z)-dec-5-en-1-yl-3-methylbutanoate standard, revealed consistent differences between the retention indexes of the minor antennal response and peak 3 and peak B on both column polarities. The third peak in the synthesized pheromone standard (Figure 4.6) is thus not the same compound to which a minor

response was seen from male and female antennae in the female gland extracts (Figure 4.3). In contrast, the retention index of peak C was similar to the retention index of the GC-EAD major response toward female ovipositor extracts (I_K = 1627 ± 3, ZB5 column, Supplementary Figure 4.5 and Table 4.1). The same was true for peak four (I_K = 1887 ± 1, ZBWax column, Figure 4.3 and Table 4.1). Peak four (Figure 4.4) and peak C (Supplementary Figure 4.9) represent (Z)-dec-5-en-1-yl-3-methylbutanoate, the major pheromone component present in the female ovipositor extracts of the pine emperor moth (Figure 4.3 and Supplementary Figure 4.5). The compound responsible for the small antennal response remains unknown.

Lure release rates

SPME results

A higher release rate of (Z)-dec-5-en-1-yl-3-methylbutanoate (41.85 ± 26.93 ng/hour on day 2, mean ± SD) was observed from the PE dispenser than the RR (6.62 ± 1.43 ng/hour on day 1, mean ± SD) and PDMS (2.59 ± 2.67 ng/hour on day 1, mean ± SD) dispensers. The release rates were different between the lures from day one to day seven (Figure 4.7A). After day seven the release rates were comparatively stable for the dispensers (PE: 6.06 ± 2.39 ng/hour, RR: 2.73 ± 0.61 ng/hour and PDMS: 4.46 ± 1.76 ng/hour, mean ± SD from day 7 to 27) (Figure 4.7A).

The release rate of (Z)-dec-5-en-1-yl-3-methylbutanoate from the RR dispenser decreased marginally over time. A release rate of 7.51 ± 1.25 ng/hour (mean ± SD) was measured between day zero and day seven. The release rate decreased to 2.82 ± 0.36 ng/hour (mean ± SD) between day eight and day 15 and remained stable at 2.65 ± 0.84 ng/hour (mean ± SD) from day 16 to day 27. The PDMS dispenser had a relatively constant release rate (4.43 ± 1.75 ng/hour from day 0 to 27, mean ± SD) of (Z)-dec-5-en-1-yl-3-methylbutanoate throughout the sampling period.

Dynamic headspace results

Results from the dynamic headspace sampling method show that the release rate of (Z)-dec-5-en-1-yl-3-methylbutanoate was roughly double when compared with the SPME sampling method (Figure 4.7A and 4.7B). The average release rate of the PE dispenser was 52.33 ± 17.56 ng/hour (mean ± SD) over 32 days. The PDMS dispenser had the highest average release rate, 74.16 ± 46.62 ng/hour (mean ± SD), over 32

days. The average release rate of the RR dispenser was 8.53 ± 4.70 ng/hour (mean ± SD) over 32 days. Measurements on the first day from the RR and PDMS dispensers showed release rates of 2.24 ng/hour and 2.74 ng/hour. This was much lower than the recording (46.33 ng/hour) for the PE dispenser.

Larger variations in the release rates occurred for the PE and RR dispensers over the first half of the 32-day sampling period (PE: 60.56 ± 24.67 ng/hour (mean ± SD) from day 0 to 17 and 46.85 ± 8.65 ng/hour from day 18 to 32; RR: 8.01 ± 7.07 ng/hour (mean ± SD) from day 0 to 17 and 8.88 ± 2.63 ng/hour (mean ± SD) from day 18 to 32). An increase in the release rate of the PDMS dispenser occurred as time progressed. Breakthrough studies indicated no breakthrough occurred onto secondary mini-MCT's during the sampling period.

Consistent patterns were observed in pheromone release rates between the lures sampled with the SPME method, compared to lures sampled with the dynamic headspace method. The PE dispenser type released pheromone in the highest quantities initially and stabilized after the first two weeks. This was observed from results of both sampling methods. The release rate results of the RR dispenser showed lower release rates than the other dispensers in both sampling methods throughout the trial and the PDMS dispenser released more pheromone than the other dispensers after day 15 in both methods.

Field testing

Trial 1

A total of 364 male moths were captured in this trial (Figure 4.8 and Supplementary Table 4.2). There were significant differences between treatments (Kruskal p = 0.001, n = 6, df = 2, χ^2 = 13.361). Traps baited with the loaded PDMS pheromone dispensers captured significantly (54 ± 40, median ± interquartile range (IQR)) more moths than those baited with live female moths (pairwise Wilcoxon p = 0.030) and unloaded PDMS blank dispensers (pairwise Wilcoxon p = 0.008). A single female captured 28 males. The other females did not call during their time in the traps.

Trial 2

A total of 517 male moths were captured in this trial (Figure 4.8 and Supplementary Table 4.3). There were also significant differences between treatments

(Kruskal p = 0.001, n = 6, df = 2, χ^2 = 13.414). The number of male moths captured in traps baited with the loaded PDMS pheromone dispensers (49 ± 34, median ± IQR) and RR pheromone dispensers (20 ± 35, median ± IQR) was significantly (pairwise Wilcoxon p = 0.008, n = 6) more than for unloaded PDMS dispensers. The number of captured moths did not differ significantly between PDMS pheromone dispensers and RR pheromone dispensers (pairwise Wilcoxon p = 0.233, n = 6).

Discussion

Pine Emperor moths are periodically pests on plantation pines in regions including the Eastern and Western Cape, Gauteng, KZN, Limpopo, North West, eSwatini, and Zimbabwe (Supplementary Material from Staude et al. 2016; Van den Berg, 1973a). There is taxonomic uncertainty as to whether the two variants in separated regions are one species, or two (Geertsema, 1971; Pinhey, 1956; Supplementary Material from Staude et al. 2016). Because the same mechanisms that mediate mate location also mediate reproductive isolation in moths, studies of pheromone biology have the potential to help resolve issues of species identity (Lassance et al. 2019). This study confirmed that populations in the Cape and northeastern South Africa (KZN), previously described as *N. cytherea* and *N. clarki* respectively, both use (Z)-dec-5-en-1-yl-3-methylbutanoate as at least their primary pheromone component and are indistinguishable by comparison of COI gene regions. This study illustrates the utility of pheromone traits as taxonomic traits, particularly when combined with genomic tools.

Electrophysiological responses toward *N. clarki* female ovipositor gland extracts were investigated and compared to a synthetic standard of the previously identified (Z)-dec-5-en-1-yl-3-methylbutanoate pheromone (Henderson et al. 1972). One repeatable electrophysiological response from male antennae was consistently larger than another response when ovipositor gland extracts were screened. This finding is similar to Henderson (1972) and Henderson et al. (1973)because he also found a chromatographic shoulder peak in the combined gland extracts of the *Nudaurelia* species that he studied, even though GC-EAD was not done in that study. The retention characteristics of the larger response matched to one of four responses found toward four components in the synthetic (Z)-dec-5-en-1-yl-3-methylbutanoate standard. The major response was thus confirmed to be toward the synthesized pheromone, but the identity of the compound responsible for the minor response could

not be confirmed. Previous studies suggested that the pheromone from *N. cytherea* is effective for *N. clarki* (Henderson, 1972). Our results prove this as male *Nudaurelia* moths were successfully captured in custom built traps baited with the (Z)-dec-5-en-1-yl-3-methylbutanoate pheromone in KZN. These results suggest that moth populations in KZN have similar pheromone chemistry to those used in the original pheromone characterization in the Cape, despite morphological differences. Geographic differences such as rainfall and plant growth are generally thought to promote divergence of species populations. This was true in other studies, where distinct differences in the pheromone blend composition were noted between geographically separated populations of a single Saturniid species, *Hemileuca eglanterina* (McElfresh et al. 2001). There have also been cases of interspecific pheromone attraction in the Saturniidae subfamily (Collins et al. 1979). Bulwer falls in the region where overlapping populations of *N. cytherea* and *N. clarki* have been reported (Henderson, 1972). However, moths that were captured, had the pink/maroon coloration of the third ring in the wing eye spots and were similar in size and coloration for *N. clarki*, suggesting the presence of only *N. clarki* in that region (Geertsema, 1971; Supplementary Material from Staude et al. 2016). The field trial results suggest that *N. cytherea* and *N. clarki* may be the same species because they can be trapped with the same pheromone.

Comparison of COI DNA sequence data from moths collected from the Cape, Limpopo and KZN in this study, showed identical gene sequences for both *N. cytherea* and *N. clarki*. This finding is in opposition with observed morphological differences between moths collected from the different regions in South Africa for our study (Supplementary Figure 4.2). These differences include that collected moths from the Cape were smaller, with brown coloration on their wings, and that they have a white third ring in the eyespots of their wings (Geertsema, 1971). These morphological observations are consistent with the characteristic colors of *N. cytherea* (Geertsema, 1971; Supplementary Material from Staude et al. 2016). The KZN moth type was larger with mostly yellow coloration and a pink/maroon third eye spot ring on their wings (Geertsema, 1971; Supplementary Material from Staude et al. 2016). Observations from collected moths from Bulwer and Jessievale for this study were similarly consistent with previous descriptions of *N. clarki* (Geertsema, 1971; Supplementary Material from Staude et al. 2016). There are two scenarios that can explain our

findings. One option is that we faced methodological limitations that did not allow us to separate the two *Nudaurelia* species. The COI gene region is usually sufficiently robust for determining variation between species (Lassance et al. 2019), but it has been shown to be insufficient for Saturniidae species before (Janzen et al. 2012). Otherwise, if our collected moth samples do represent a single species, then more sequences and of different genes may be required to prove this. Nevertheless, COI sequences from all collected moths in our study were identical and assigned *N. clarki*. These sequences grouped sister to *N. kohlli*, a related Saturniid from Tanzania. Other species that grouped with *N. clarki* included *N. dione*, from Kenya and Uganda (Pinhey, 1956; Supplementary Material from Staude et al. 2016), *N. amathusia* from Uganda, Cameroon and Gabon (Pinhey, 1956) and *N. anthinoides* from Gabon. It is known that species in *Nudaurelia* is particularly difficult to separate using morphological data (Pinhey, 1956) or molecular data (Janzen et al. 2012) and this is similar to our results.

Lure release rates from different dispenser materials was investigated to determine the kinetic parameters of pheromone release, and whether the pheromone interacts with the dispensers (McDonough et al. 1992). Different dispenser types release volatiles through different mechanisms, namely diffusion (Butler et al. 1981; McDonough et al. 1992) and permeation (Hofmeyr et al. 1995; Torr et al. 1997). Zero-order release rate kinetics were expected for the PDMS and PE dispenser permeation type dispensers at a constant temperature (Bouwer et al. 2015; Hofmeyr et al. 1995; McDonough et al. 1992; Torr et al. 1997). The polyisoprene dispenser (RR) was expected to follow first order release rate kinetics (Butler et al. 1981; McDonough et al. 1992). From our release rate data, we found no evidence of chemical interaction between the pheromone and either dispenser type. Our results also showed that all the dispensers released pheromone at a zero-order release rate, but that PDMS and PE dispensers required a two-week period before release rates stabilized. Zero order release rate kinetics are desirable because it ensures that the ratio between individual components with similar vapor pressures remain constant over time (Brown et al. 1992; Heuskin et al. 2011). A constant pheromone ratio ensures that species-specificity is maintained as the dispensers age in the field. The fact that male pine emperor moths were captured in comparable numbers between the RR and PDMS

dispensers show that the differences in release rates from these lures are not critical for attracting *N. clarki*.

This study showed the first evidence of autodetection in females in the Saturniidae family, based on the GC-EAD response to the pheromone component also identified from *N. clarki* ovipositors. The small responses measured from female antennae confirm that *N. clarki* female moths are less sensitive to pheromone volatiles than the male moths, similar to previous findings (Bouwer et al. 2015; Holdcraft et al. 2016). Previous studies on autodetection in the Saturninae subfamily relied solely on EAG methods (Maida et al. 2001; Schneider, 1962), but our EAG results were skewed due to solvent interference. Analysis by GC-EAD allowed us to determine that female antennae are not only able to detect the pheromone, but that the antenna can detect three other structurally related compounds in the synthetic (Z)-dec-5-en-1-yl-3-methylbutanoate standard.

There are adaptive benefits to females when they are able to detect their own pheromone (Holdcraft et al. 2016). These benefits may include reduction of competition for a mating partner through dispersal, or physiological priming of females for the correct mating behavior once they detect their own pheromone or pheromone from other individuals, among other benefits (Holdcraft et al. 2016). Female *N. clarki* are not attracted to the artificial pheromone in the field. This suggests that they may instead avoid areas where they detect pheromone (Birch, 1977; Mitchell et al. 1974). Dispersive behavior of females due to pheromone presence has implications for pheromone-based control strategies (Holdcraft et al. 2016). Females may move away from areas where pheromone can be detected and cause them to oviposit in previously unaffected areas (Holdcraft et al. 2016).

Some technical difficulties were encountered when pheromone gland extracts were analyzed. The concentration of the electrophysiologically active compounds in pheromone gland extracts were below detection limits. This has been observed for other Saturniidae as well (Millar et al. 2010; Symonds et al. 2012). It is possible that the pheromone may be synthesized by the female during calling behavior or that the pheromones are effective at very low doses and that moths in the Saturniidae family simply produce minute quantities of pheromone (Symonds et al. 2012). The identities of the active pheromone compounds could only be confirmed by matching the retention indexes of electroantennographic responses to extracts and the compounds

present in the synthesized pheromone standard (Brezolin et al. 2018). Mass spectral information from gland extracts was not obtained due to trace amounts in the gland extracts. This introduces the possibility that the unknown compound eliciting electroantennographic responses in ovipositor extracts, may be part of the pheromone, or an antagonist. The function of this compound can only be identified once the compound structure is elucidated.

The use of pheromone control-strategies in plantations could be cheaper and more environmentally friendly than pesticides (Larsson, 2016; Nadel et al. 2012). We proved that the sex pheromone of the pine emperor moth, (Z)-dec-5-en-1-yl-3-methylbutanoate (Henderson, 1972; Henderson et al. 1973), is effective in attracting *N. clarki* males species-specifically in the field in KZN. The unknown compound eliciting smaller electrophysiological responses from male antennae, may be a necessary part of the pheromone blend for *Nudaurelia* and must be investigated in future studies. Nevertheless, the same pheromone previously identified from *N. cytherea*, also attracts *N. clarki*. Together with identical COI gene regions from samples representing these moth variants, our pheromone-based field trapping results suggest that *N. cytherea* and *N. clarki* might be the same species. The same pheromone blend is proposed to be effective over all regions where these moths occur. This simplifies the implementation of pheromone-based control strategies, in contrast to other Saturniids. Future studies will be aimed at optimizing monitoring traps and to increase efficiency for mass-trapping. There is also a possibility that the pheromone can be used for mating-disruption of *N. clarki*.

Figure captions

- **Figure 4.1:** The described distribution of *N. cytherea* (light grey) and *N. clarki* (mid-grey) in South Africa, with the proposed overlapping region where interbreeding has been reported previously (dark grey) (Geertsema, 1971; Supplementary Material from Staude et al., 2016). Black pins: sampling locations in 2018 and 2019 of live moths for pheromone studies and mitochondrial gene comparisons, white pin: sampling location in 2019 of the Cape syntype for mitochondrial gene comparisons.
- **Figure 4.2:** A neighbor-joining tree of the COI gene consensus sequence of representative samples obtained from *N. clarki* from Bulwer (KZN), Jessievale (Limpopo) and Pringle Bay (Western Cape) in South Africa. All consensus sequences were identical between moths. The position of *N. clarki* is shown in relation to other Saturniidae moth species from *Nudaurelia*, *Imbrasia*, *Gonimbrasia* and *Antherea* genera, from available sequences on GenBank (accession numbers follow species names). The tree was constructed with a bootstrap value of 1000.
- **Figure 4.3:** Electroantennography response of a *N. clarki* male antenna to a female ovipositor extract in n-hexane (n = 8) (ZBWax column). Antenna response sizes were **1** = 135.56 ± 42 µV and **2** = 23.33 ± 15 µV (mean ± SD, n = 10) and occurred at Kovat's index values of (**1**) = 1879 ± 1 and (**2**) = 1887 ± 1.
- **Figure 4.4:** Electroantennography responses from male and female *N. clarki* moth antennae to (Z)-dec-5-en-1-yl-3-methylbutanoate (1 µl, 50 ppm) (top). Zoomed-in figures with an indication where the individual responses of male (n = 8) and female (n = 3) antennae were seen ((**1**) = 1853 ± 1, (**2**) = 1874 ± 1, (**3**) = 1883 ± 1 and (**4**) =1888 ± 1) (ZBWax column) (bottom).
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- **Figure 4.7:** A: The release rates of (Z)-dec-5-en-1-yl-3-methylbutanoate from polydimethylsiloxane (PDMS), polyethylene microcentrifuge tubes (PE), and red rubber polyisoprene pheromone lures (RR). These lures were sampled for half an hour with a SPME fiber over a period of 27 days (mean ± SD, n = 3). B: The release rates of (Z)-dec-5-en-1-yl-3-methylbutanoate from the same lures, sampled with dynamic headspace sampling onto Poropak for an hour each time over 32 days (mean ± calibration curve SD, n = 1).

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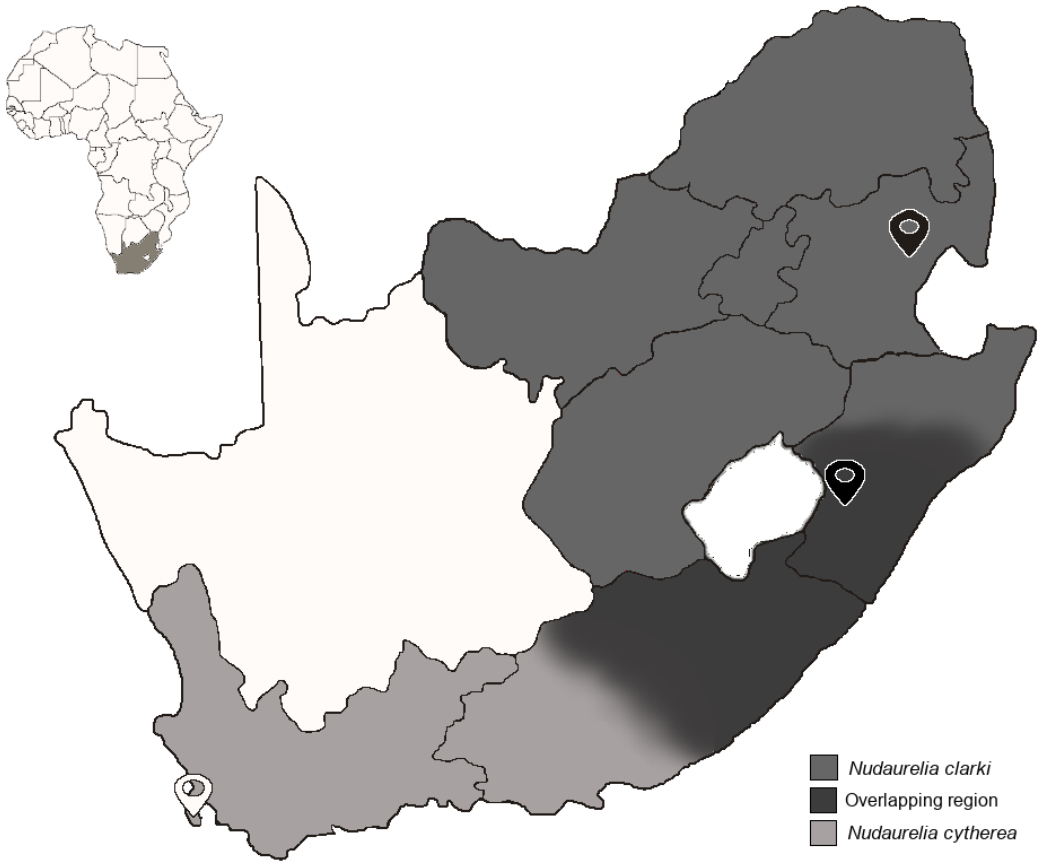
Figures



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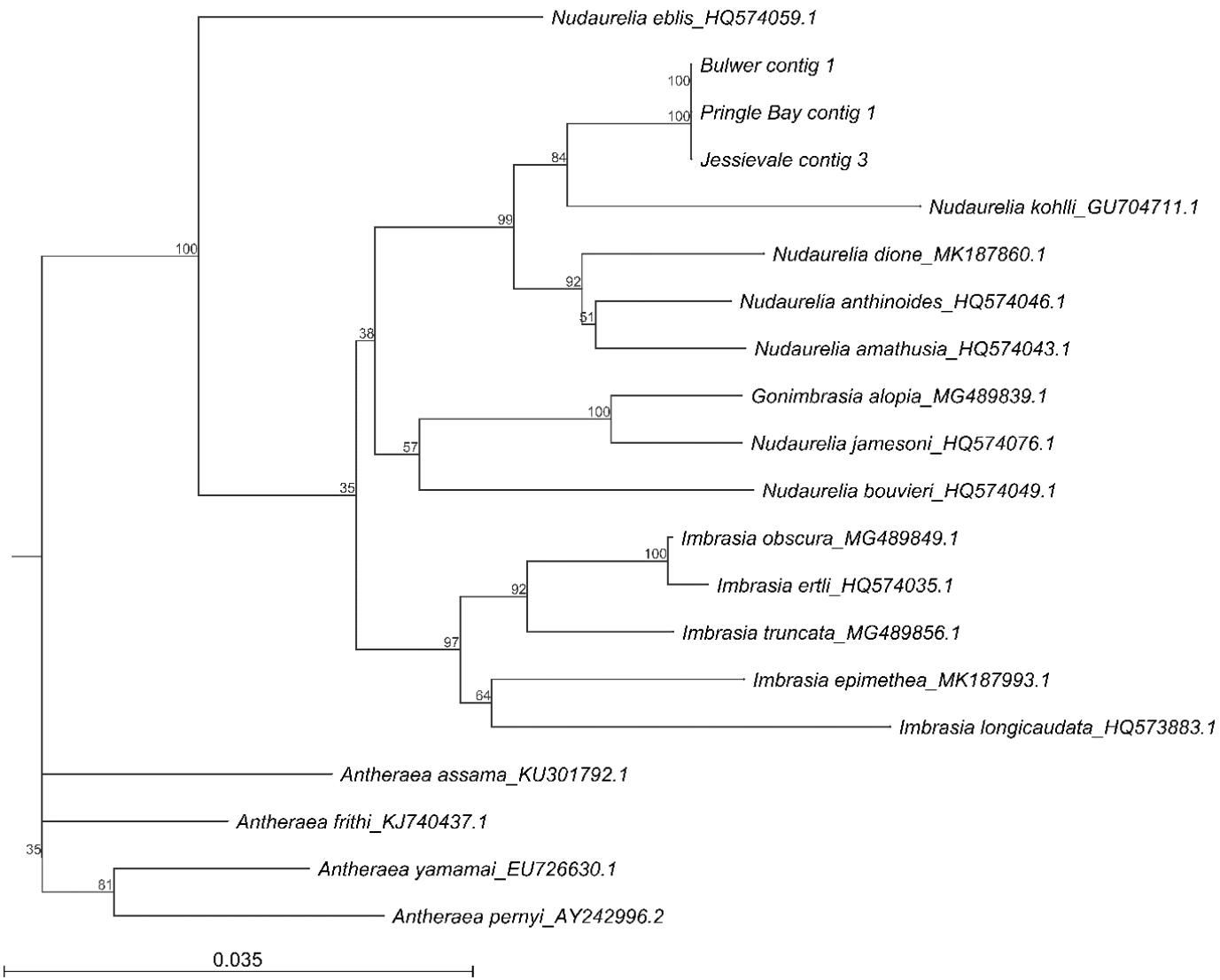

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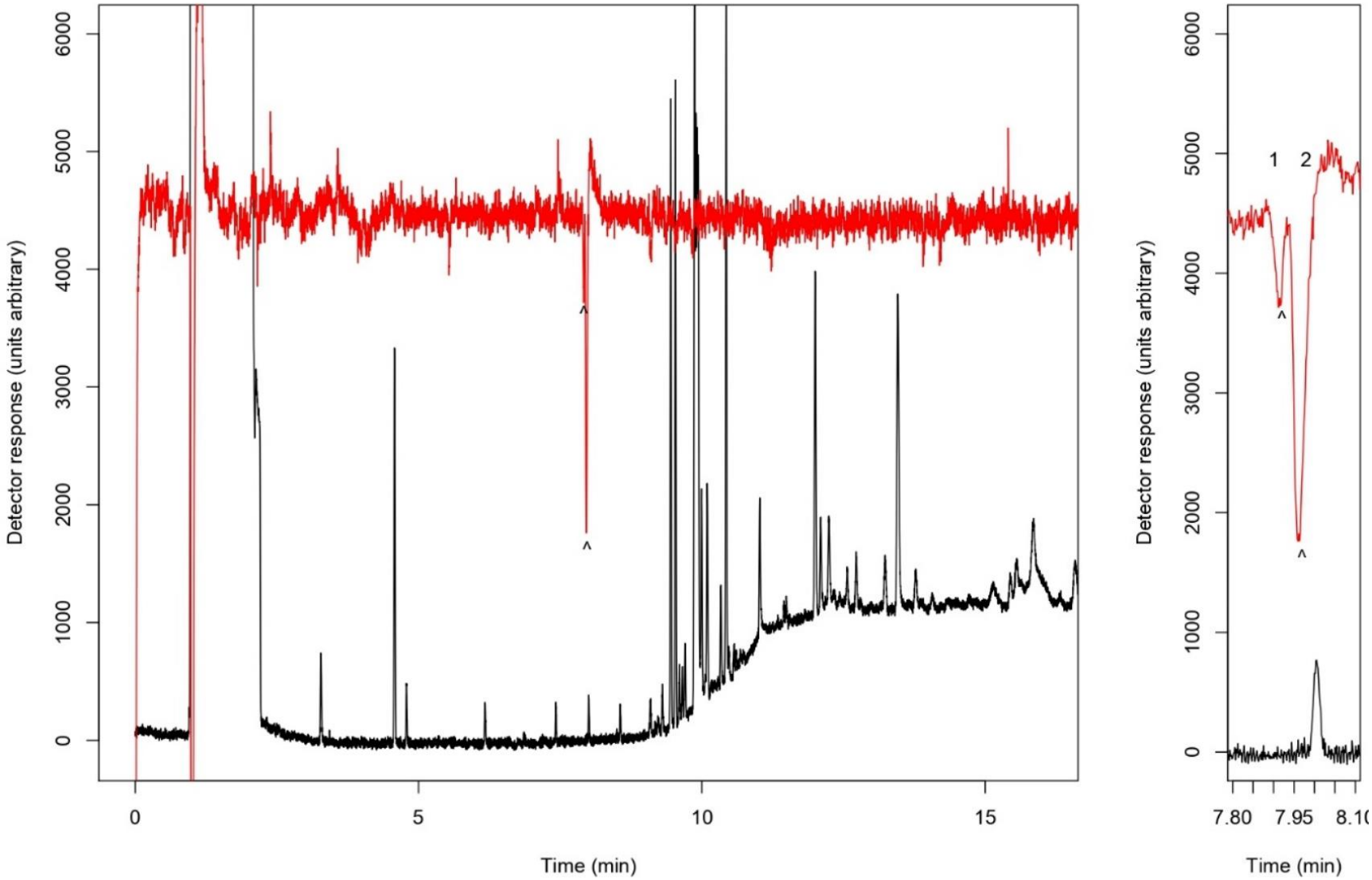

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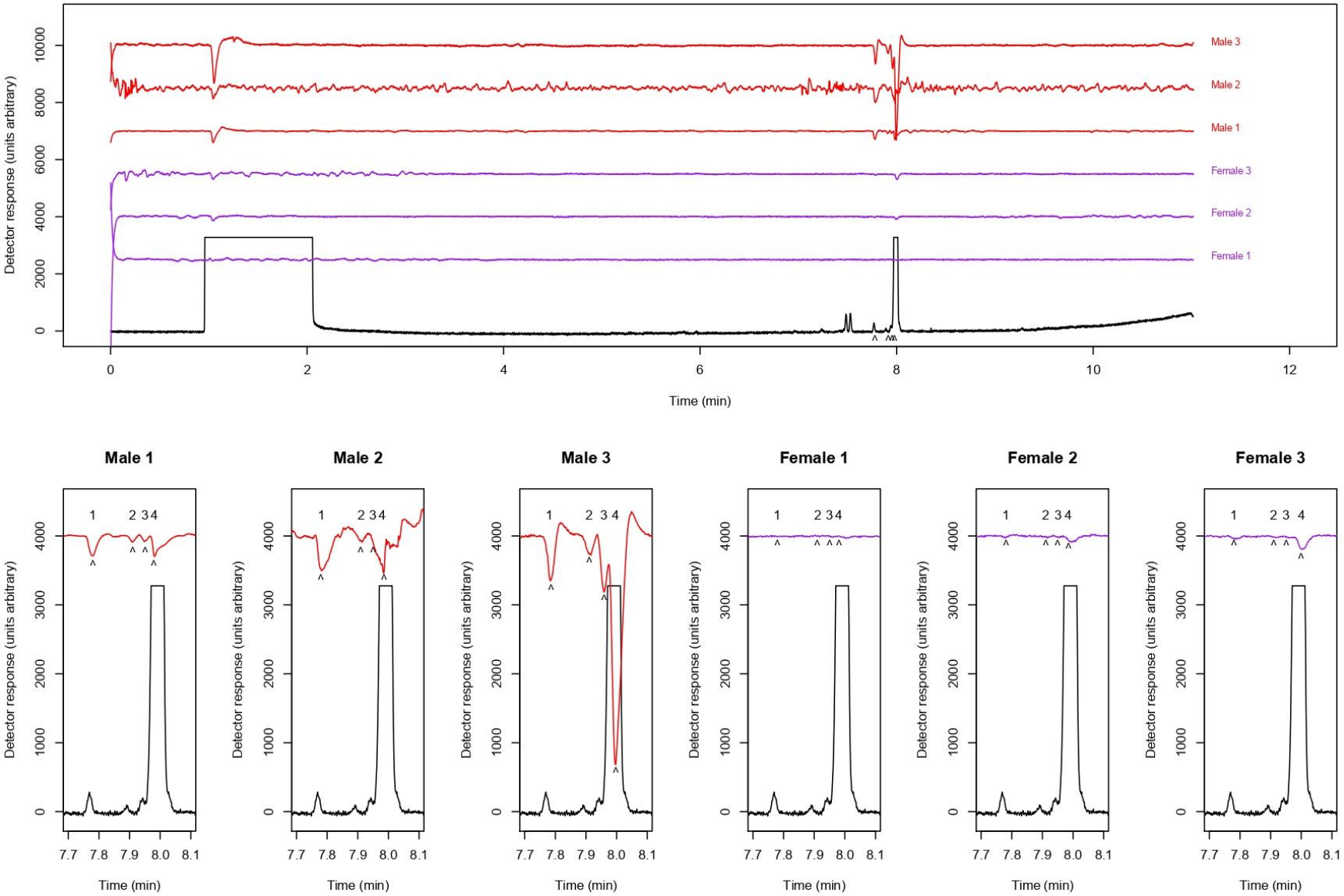

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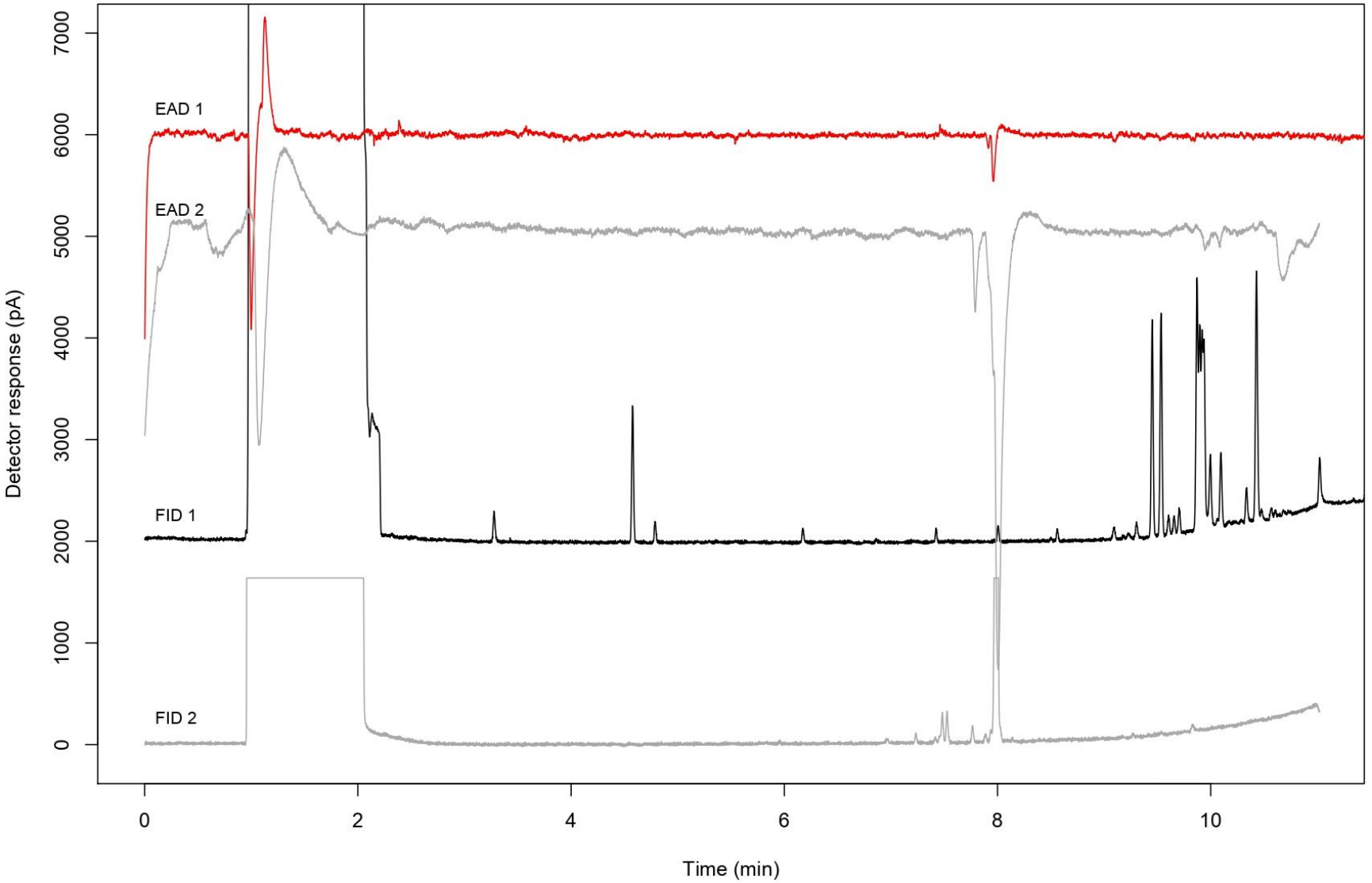

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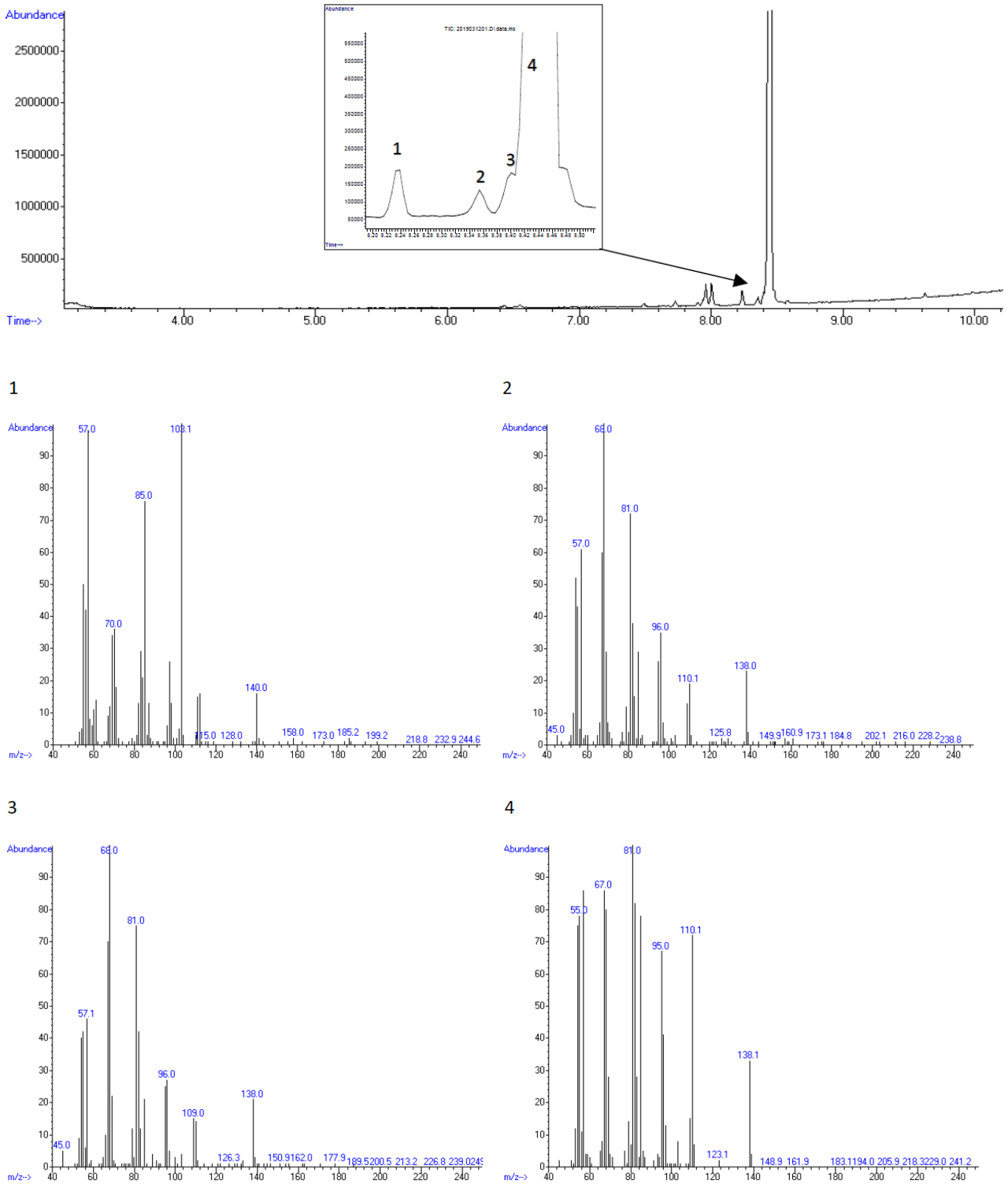

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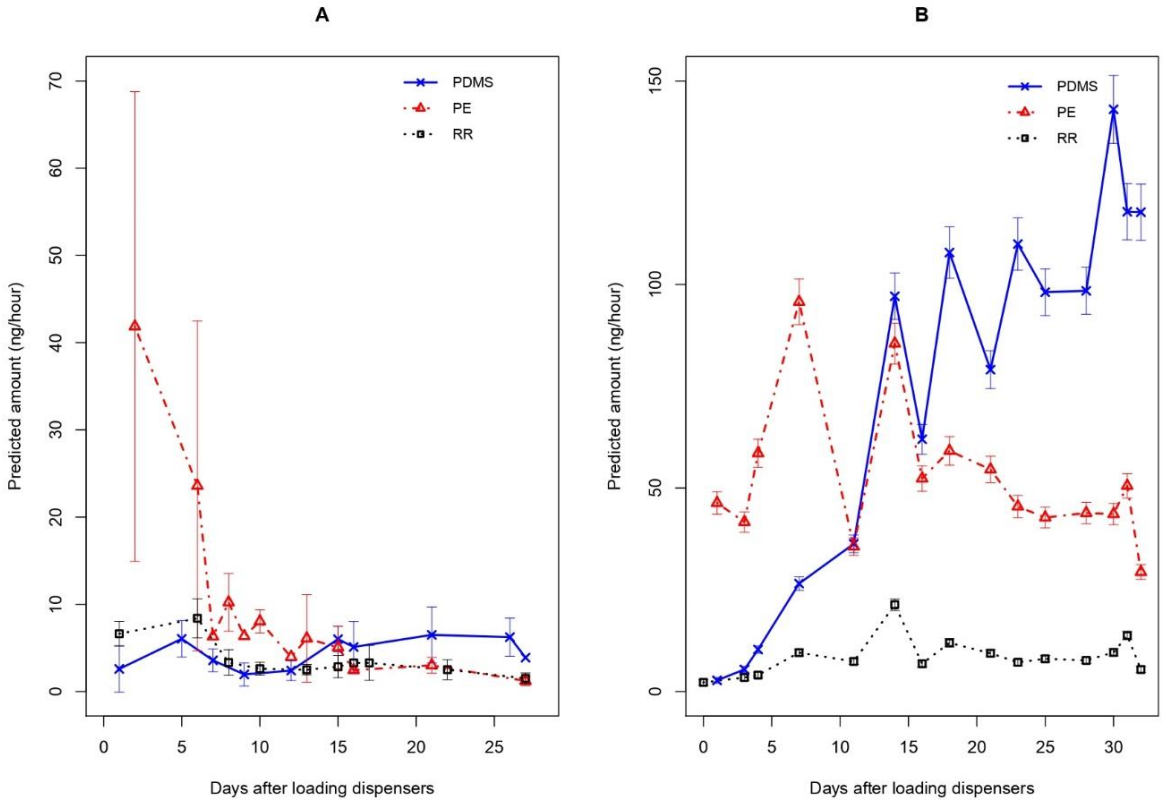

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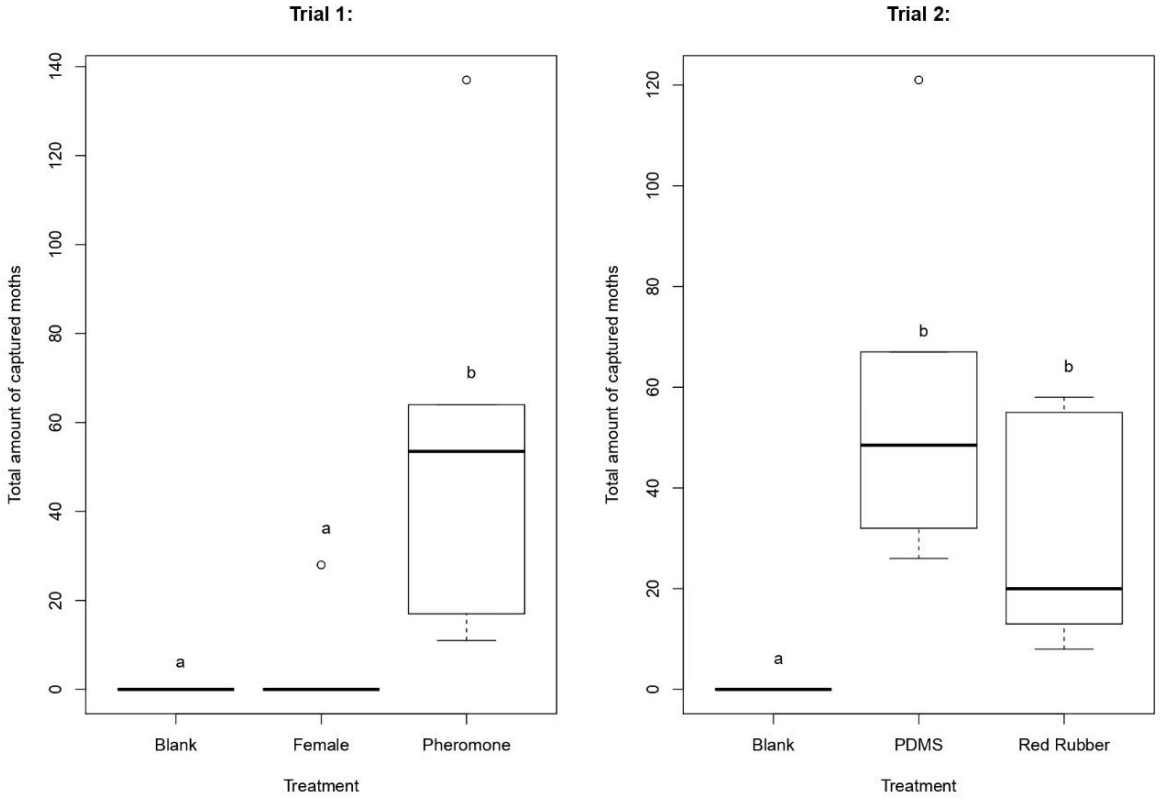

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Supplementary figure captions

- **Supplementary Figure 4.1: a:** Consol glass chambers used for sampling of the release rates of (Z)-dec-5-en-1-yl-3-methylbutanoate from polydimethylsiloxane (PDMS), polyethylene microcentrifuge tubes (PE), and red rubber polyisoprene pheromone lures (RR) in a dynamic headspace sampling facility. **b:** The Swagelok inlet and outlet fittings were sealed with Teflon o-rings (unseen in figure) on the screw-on Consol jar lid. Pheromone dispensers were sampled in metal test tube caps, for ease of manual transferring to and from fume hood, where lures aged. **c:** Machined washers kept the adsorbent cartridge in central position in the exit from the sampling chamber, in a custom blown glass chamber. **d:** Four mini-multi-channel traps (mini-MCT's), each consisting of a glass split liner with packed silicone tubing.
- **Supplementary Figure 4.2:** A visual comparison between two moths respectively collected from Jessievale (Limpopo), *N. clarki* (left), and Pringle Bay (Western Cape), *N. cytherea* (right). The morphological color variations of adults and larvae, when available, were compared to those described in Supplementary Material from Staude et al. (2016) to assign the respective species.
- **Supplementary Figure 4.3:** Photos of the custom-built traps used in field trials in Bulwer, KZN in 2019. An up-close view of the trap construction (left) and the suspension of a trap in a *Pinus patula* tree in a plantation after a day of trapping (right).
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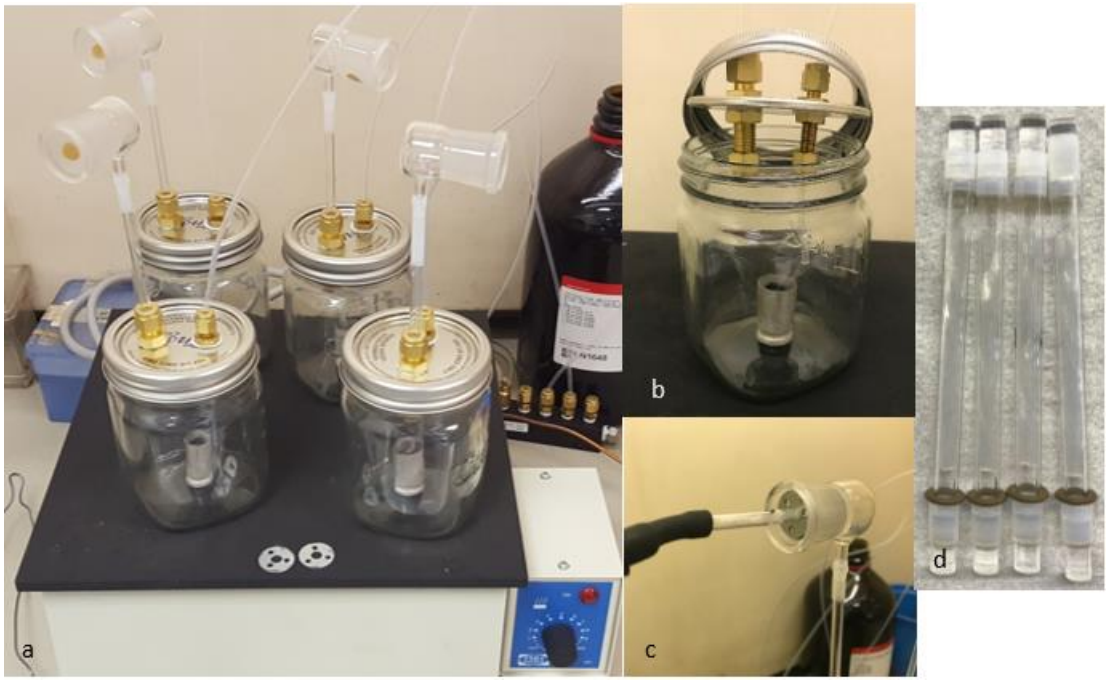
Supplementary figures



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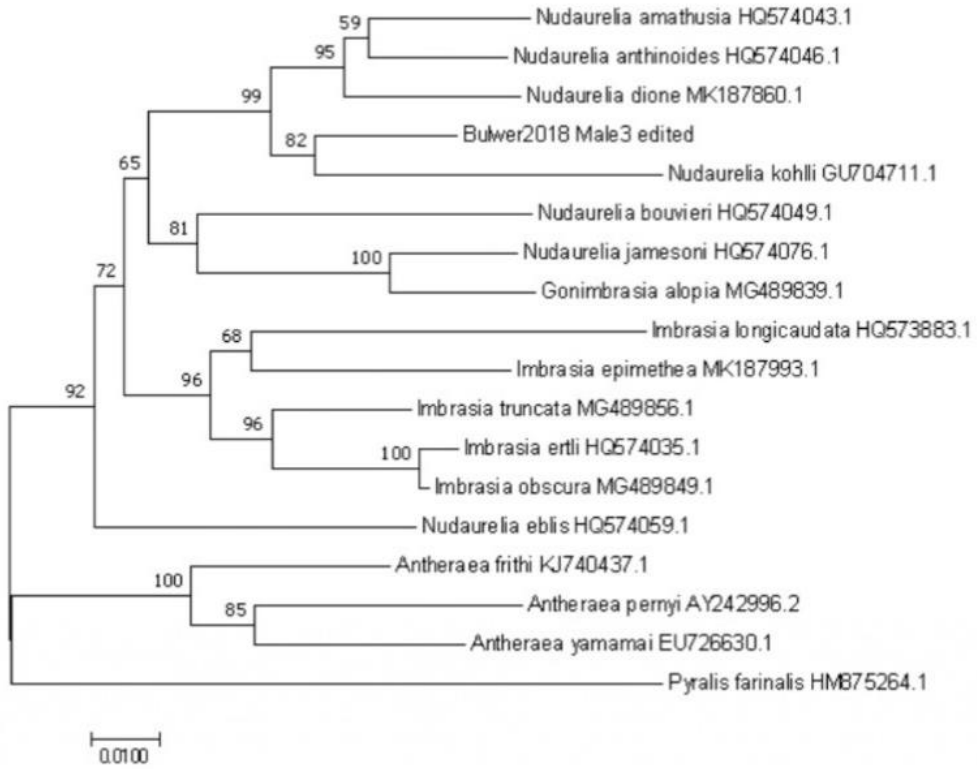

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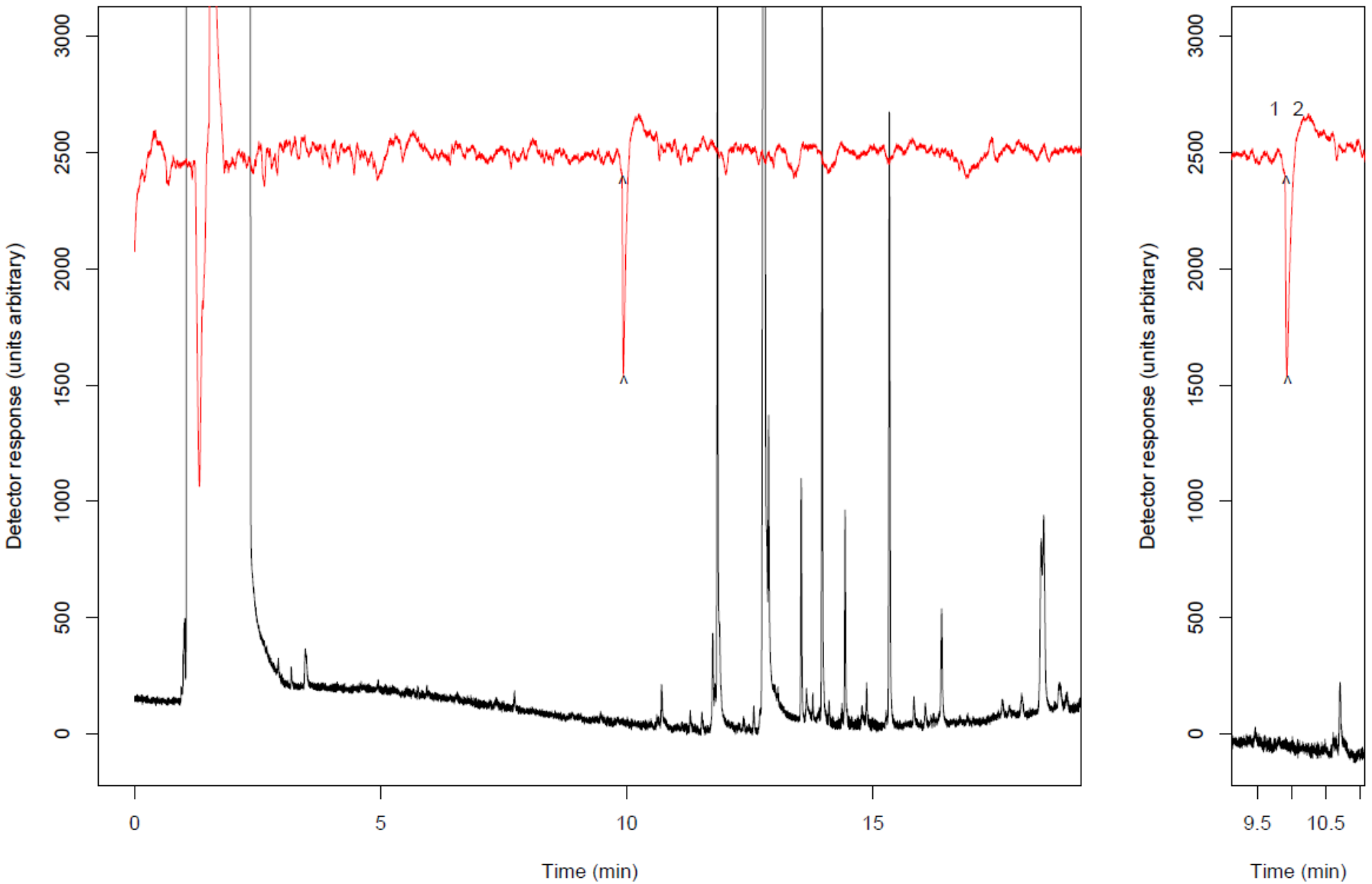

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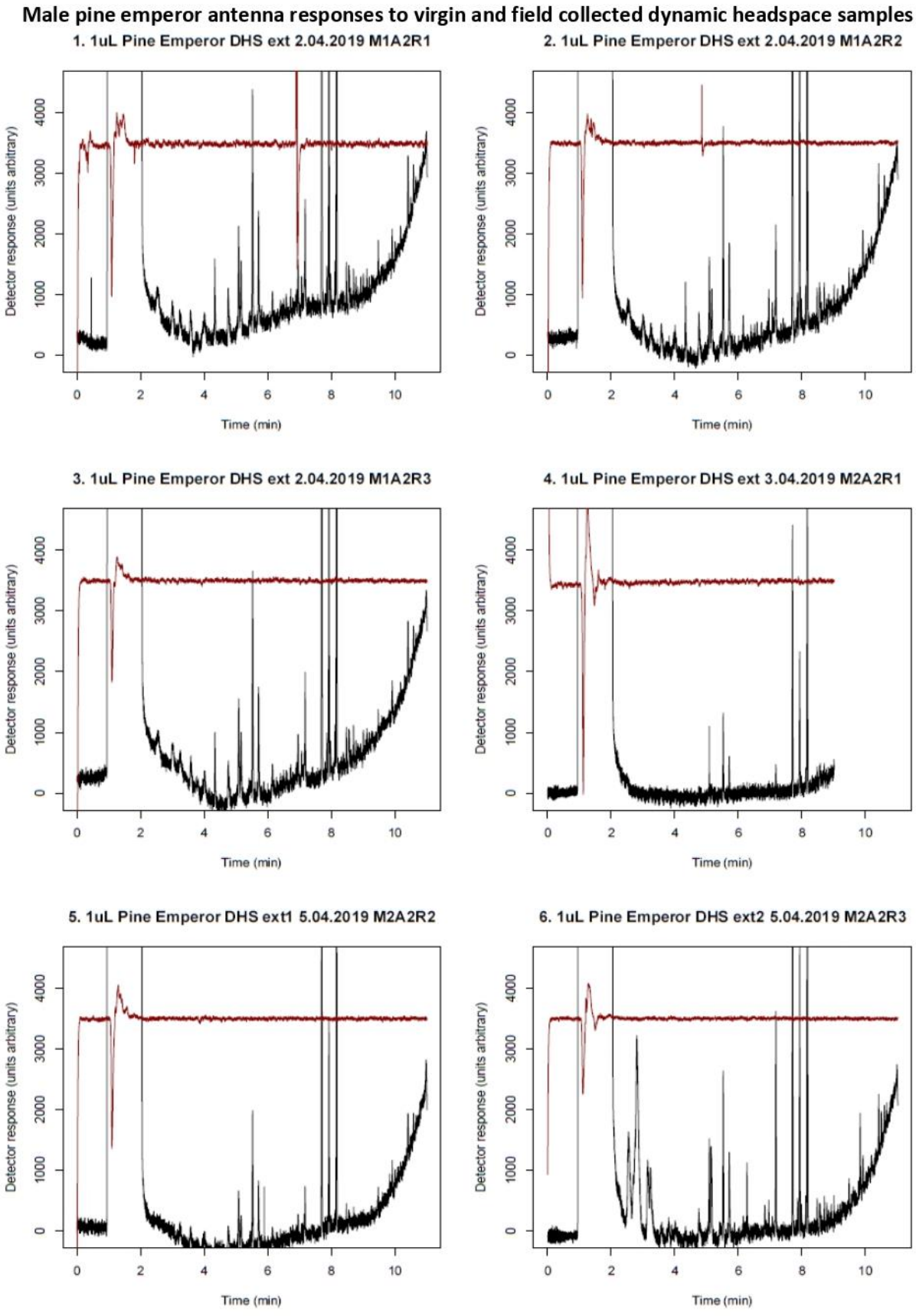

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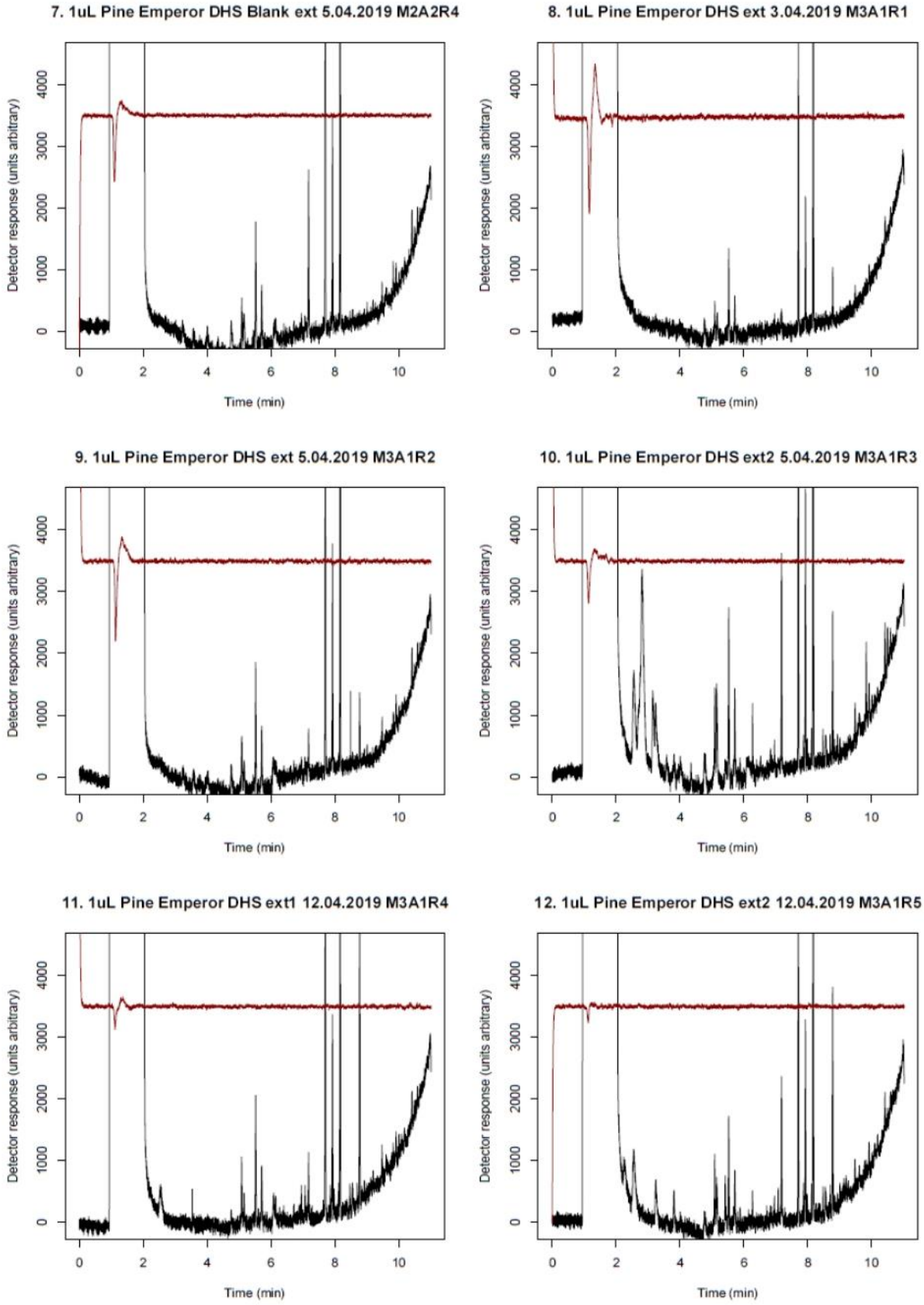
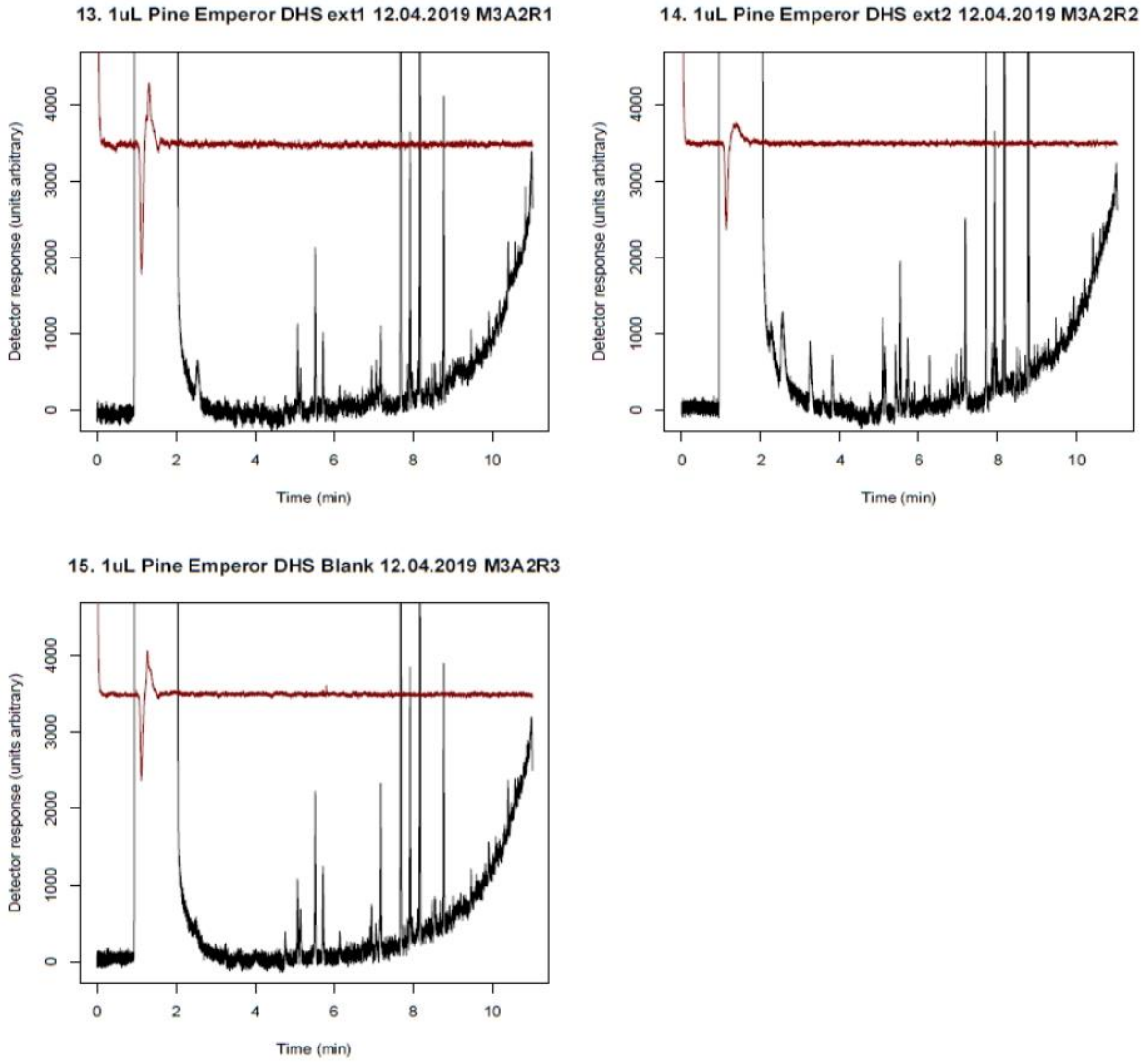
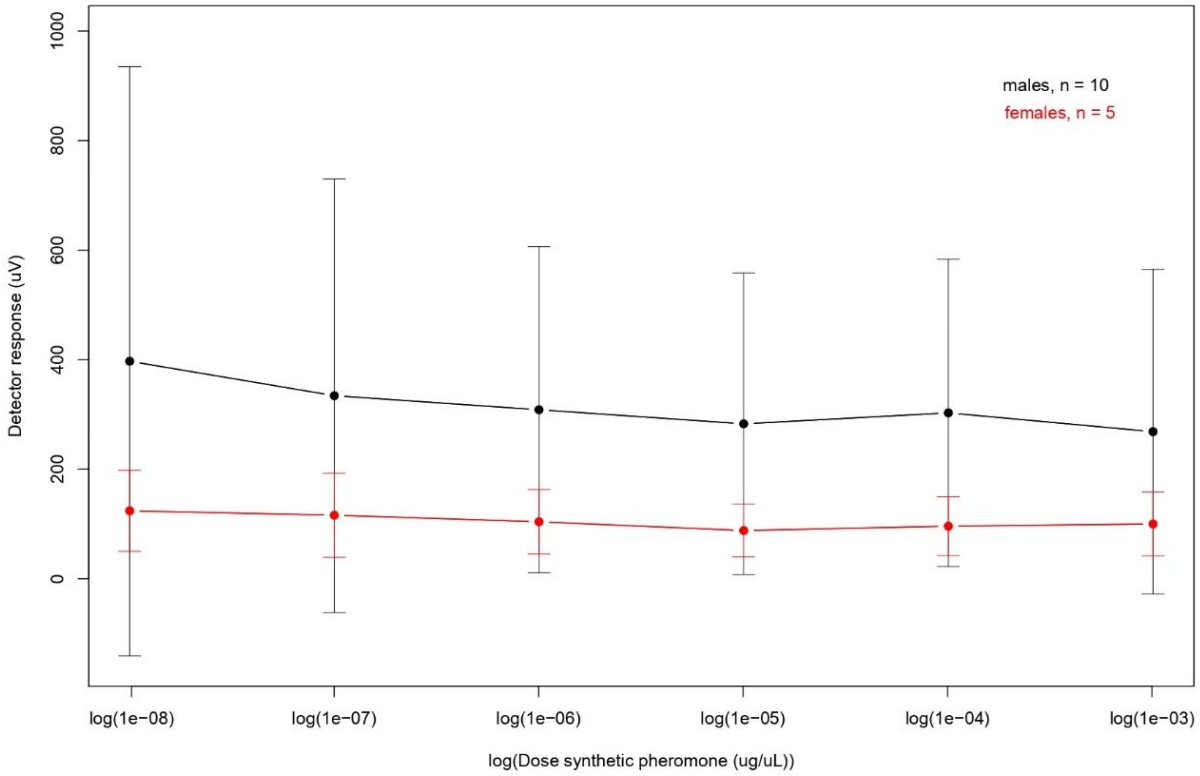


Supplementary Figure 4.6: (Continued)



Supplementary Figure 4.6 (Continued)



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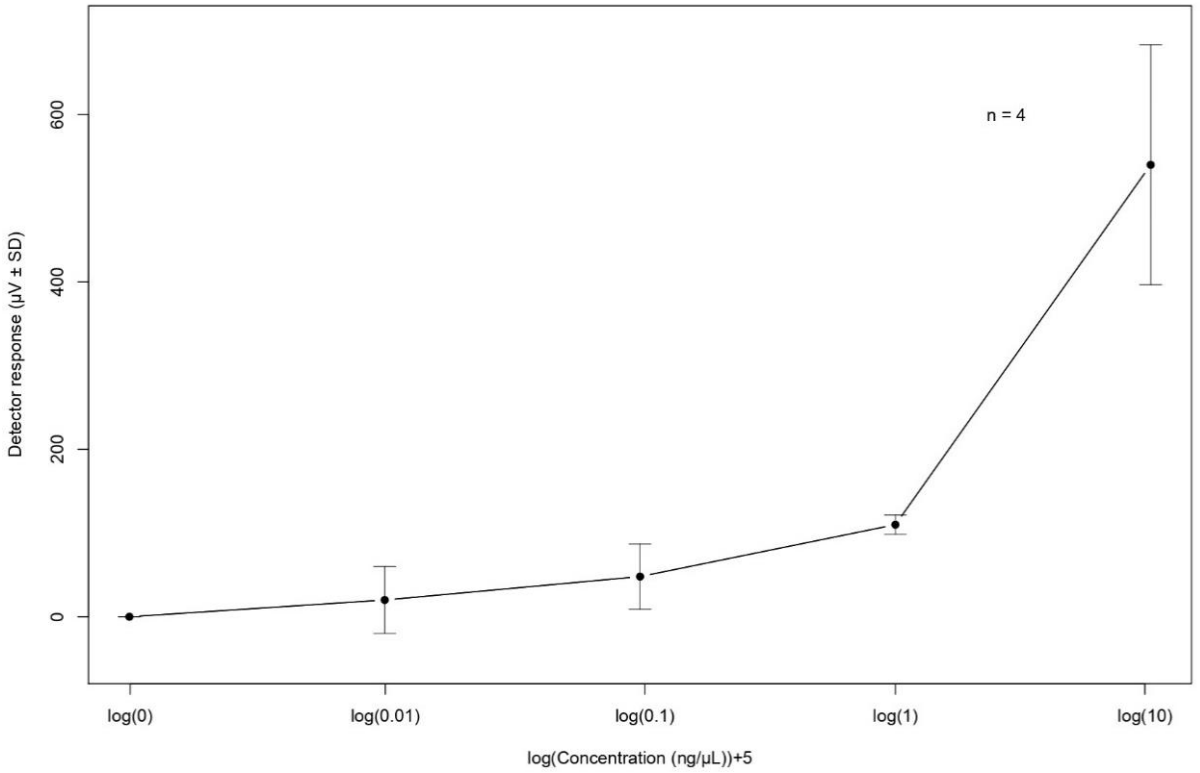

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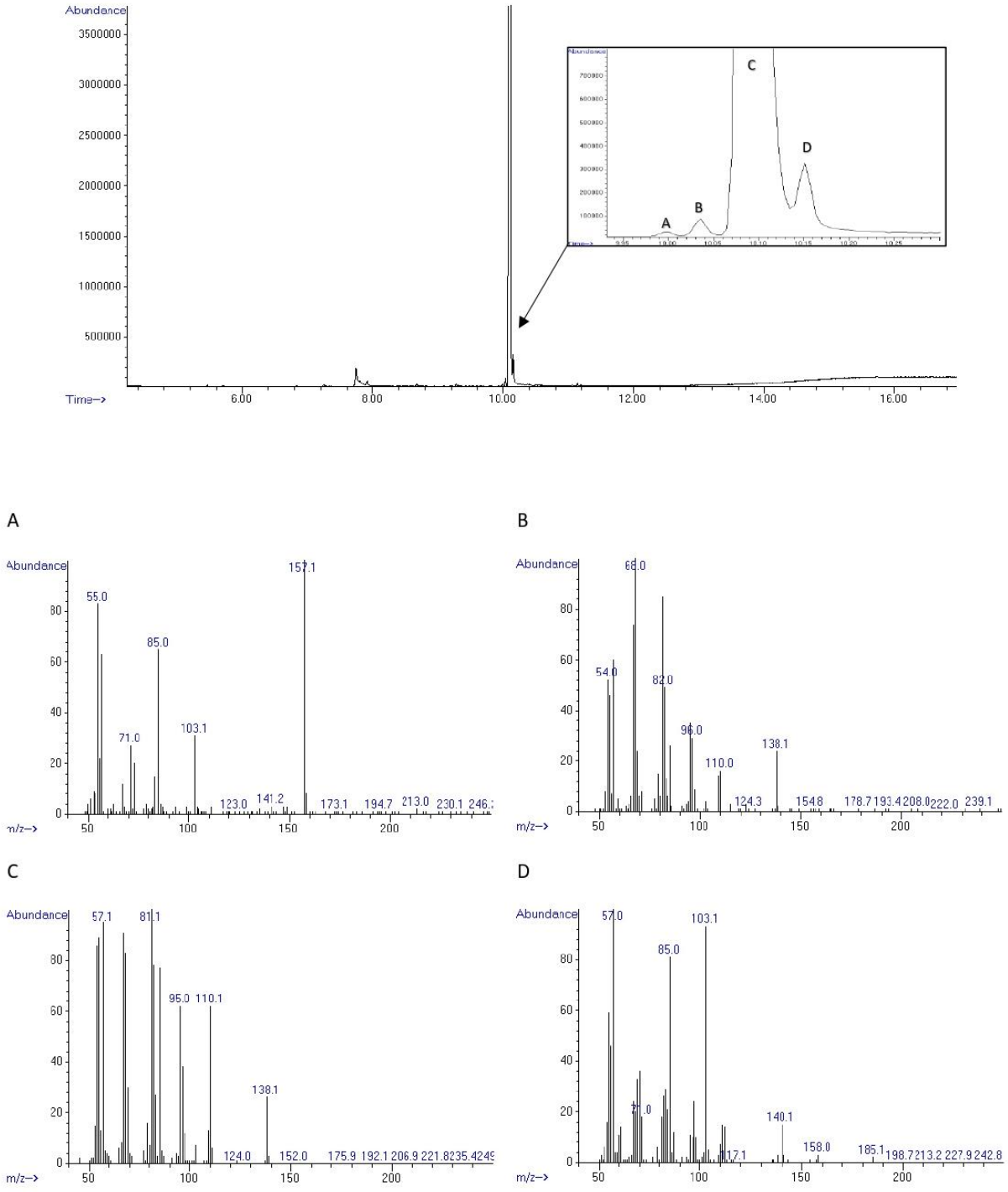

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Table captions

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Supplementary table captions

- **Supplementary Table 4.1:** Statistical results for EAG puffing results. The H_0 critical value was 10.36 in a Kruskal-Wallis test at a 5% confidence level (n = 5, df = 1 per test).
- **Supplementary Table 4.2:** Statistical results for field trial 1. The H_0 critical value was 13.361 in the Kruskal-Wallis test at a 5% confidence level (n = 6, df = 2, p = 0.001). The post-hoc pairwise Wilcoxon Rank sum results, with Bonferroni correction, are shown.
- **Supplementary Table 4.3:** Statistical results of field trial 2. The H_0 critical value was 13.414 in the Kruskal-Wallis test at a 5% confidence level (n = 6, df = 2, p = 0.001). The post-hoc pairwise Wilcoxon Rank sum results, with Bonferroni correction, are shown.
- **Supplementary Table 4.4:** Summary statistics for the dose response curve of male antennae to the synthesized pheromone on the GC-EAD (n = 10).
- **Supplementary Table 4.5**: Data for the calibration curve (R^2 = 0.994) of the 110 m/z-ion of (Z)-dec-5-en-1-yl-3-methylbutanoate (n = 4) on the GC-MS system. All areas were blank corrected. Confidence intervals are at the 98% level. Good prediction accuracy was observed below 5 ng.

Tables

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ZBWax column			**ZB5 column**			**EAD Response size***	
Peak	Elution time (min)	I_K	Peak	Elution time (min)	I_K	Male (µV), n = 7	Female (µV), n = 2
Synthetic pheromone							
1	7.75 ± 0.005	1853 ± 1	D	10.15 ± 0.000	1636 ± 0**	171.43 ± 133.59	x
2	7.88 ± 0.004	1874 ± 1	A	9.99 ± 0.000	1611 ± 1**	57.14 ± 31.47	x
3	7.93 ± 0.003	1883 ± 1	B	10.04 ± 0.000	1617 ± 1**	131.43 ± 107.08	x
4	7.96 ± 0.003^M	1888 ± 1	C	10.09 ± 0.003^M	1624 ± 1**	842.86 ± 460.83	50 ± 42.43
Female ovipositor extracts							
3	7.88 ± 0.02	1879 ± 1	B	9.88 ± 0.01	1621 ± 2	23.33 ± 15	NA
4	7.93 ± 0.02^M	1887 ± 1	C	9.91 ± 0.02^M	1627 ± 3	135.56 ± 42	NA

I_K: Kovat's retention index

All values are mean ± SD

^{M}: Major peak intensity

*: EAD responses determined on ZBWax column

**: Elution time and retention index determined on HP5 by GC-MS

x: Responses below detection threshold (20 µV)

Supplementary tables

Supplementary Table 4.1: Statistical results for EAG puffing results. The H_0 critical value was 10.36 in a Kruskal-Wallis test at a 5% confidence level (n = 5, df = 1 per test).

Concentration (ppm)	Treatment 1	Treatment 2	p-value	Kruskal Wallis chi-squared
1.00E-03	Male	Female	0.255	1.2968
1.00E-04	Male	Female	0.193	1.6938
1.00E-05	Male	Female	0.143	2.1438
1.00E-06	Male	Female	0.166	1.9189
1.00E-07	Male	Female	0.329	0.95278
1.00E-08	Male	Female	0.514	0.42645

Supplementary Table 4.2: Statistical results for field trial 1. The H0 critical value was 13.361 in the Kruskal-Wallis test at a 5% confidence level (n = 6, df = 2, p = 0.001). The post-hoc pairwise Wilcoxon rank sum results, with Bonferroni correction, are shown.

Trial 1	Treatment1.1	Treatment1.2	p-value
1	Blank	Pheromone	0.008
2	Female	Pheromone	0.030
3	Female	Blank	1.000

Supplementary Table 4.3: Statistical results of field trial 2. The H0 critical value was 13.414 in the Kruskal-Wallis test at a 5% confidence level (n = 6, df = 2, p = 0.001). The post-hoc pairwise Wilcoxon Rank sum results, with Bonferroni correction, are shown.

Trial 2	Treatment2.1	Treatment2.2	p-value
1	Blank	RR	0.008
2	PDMS	RR	0.232
3	PDMS	Blank	0.008

Supplementary Table 4.4: Summary statistics for the dose response curve of male antennae (n = 10).

Concentration (ppm)	Mean Response (µV)	Standard deviation (µV)
0.01	20	40
0.10	35	30
1.00	110	11.54701
10.0	540	143.2946

Supplementary Table 4.5: Data for the calibration curve of the 110 m/z-ion of (Z)-dec-5-en-1-yl-3-methylbutanoate (n = 4). All areas were blank corrected. Confidence intervals are at the 98% level.

Concentration (ppm)	Mean Area (counts)	Standard deviation	Negative Confidence limit	Positive Confidence limit
0.00	0	0	0	0
0.01	1185.083	1172.736	12.34699	2357.818
0.05	5207.333	3181.904	2025.429	8389.236
0.10	10913.33	3237.531	7675.802	14150.86
0.50	62555.58	16806.81	45748.77	79362.4
1.00	144361.6	30328.75	114032.8	174690.3
5.00	957370.3	110480.1	846890.2	1067850
10.0	2034514	285907.5	1748606	2320421
50.0	13213982	2058220	11155762	15272202
100	26752336	3243183	23509153	29995520

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Addendum A

Eucalyptus host volatile compound verification from a mixture of reference standards

Introduction

Semiochemical analysis of insect pheromones, such as for *Gonipterus* sp. 2, require reference standards to confirm identities of chromatographic peaks from samples. Reference standards are used to compare peak retention times on the same column polarity, or retention indexes (I_K) for comparison between different column polarity separations. The mass spectrum of an unknown peak in a sample must also be similar to the fragmentation pattern of the reference standard for confirmation of a compound identity. Reference standards of *Eucalyptus* volatiles were analyzed because these kairomones have been shown to elicit responses from male and female *Gonipterus* antennae in the past.

We report here the confirmation of mass spectral and retention data of a blend of *Eucalyptus* volatile reference standards by comparison of similar compounds reported in the NIST library. This blend of standards was used in the study of *Gonipterus* sp. 2 semiochemical explorations, Chapter three.

Methods

Standards

The *Eucalyptus* host volatile blend of reference standards was prepared for previous work in the same laboratory. The concentration of individual standards in the blend was unknown. Due to the possible degrade of some of the volatile components in the blend, GC-MS mass spectral data was examined to confirm the identities of compounds still present in the *Eucalyptus* volatile blend.

GC-MS

A volume of 1 µL of the *Eucalyptus* volatile standard blend (unknown concentrations of reference standards) was injected in spitless mode (vent time = 50 seconds, flow = 100 ml/min) into the GC-MS inlet. A ZBWax column (30 m x 0.25 mm ID, 0.25 µm, 7HG-G007-11, Zebron™) was used for separation of compounds. The GC oven temperature was held at 50°C for 2 minutes and ramped (10°C/min) to 250°C where it was held for 7 minutes. Mass spectral patterns and retention indexes were compared to sample compounds with tentatively assigned identities.

GC-FID

For the ZB5 column (30 m x 0.32 mm ID, 0.25 µm, 19091J-413, Zebron™), the temperature was held at 50°C for 1 minute and ramped to 300°C at a rate of 10°C/min. A volume of 1 µL of the *Eucalyptus* volatile standard blend (unknown concentrations of reference standards) was injected in splitless mode (vent time = 2 minutes, flow = 50 ml/min) under constant column head pressure (16 psi, He).

Results

Analysis of the *Eucalyptus* volatile blend on the GC-MS, revealed twelve distinguishable peaks that were comparable to compounds reported in the NIST database, based on similar mass spectra and retention indexes (I_K) on the ZB5 column (Figure A.1 and A.2).

The same blend was screened on the GC-FID system with the ZBWax column, to determine each component's retention index on a polar column as well. These retention indexes were compared to the expected literature I_K of the different host volatile compounds that were putatively identified (Table A.1). Chromatographic peaks that exhibited similar retention index characteristics to literature values on both columns for a putatively identified volatile compound, were confirmed to be the correct identification.

Table A.1: Retention data of the confirmed identities of chromatographic peaks in the *Eucalyptus* volatile reference blend after analysis with GC-MS and GC-FID instruments.

	Host volatile Standard***	ZBWax column, GC-FID (n = 1)			ZB5 column, GC-MS (n = 1)			NIST
		Rt (min)	RI	lit RI**	Rt (min)	RI	lit RI**	R.match
A	(-)-ß-pinene	4.27	1108	1112 ± 7 (518)	4.62	980	979 ± 2 (849)	962
B	Dehydrosabinene	4.50	1124	1124 ± 10 (6)	4.33	955	956 ± 2 (59)	954
C	Limonene	5.54	1198	1199 ± 6 (6)	5.19	1032	1031 ± 4 (5)	917
D	Eucalyptol	5.66	1206	1213 ± 9 (356)	5.22	1035	1032 ± 2 (580)	969
E	p-Cymene	6.58	1270	1272 ± 8 (543)	5.14	1027	1025 ± 2 (820)	955
F	Cis-3-hexenyl acetate	7.24	1316	1315 ±6 (122)	4.94	1008	1005 ± 2 (74)	946
G	Cis-3-hexenol	8.12	1380	1382 ± 9 (367)	3.16	857	857 ± 3 (169)	967
H	α-Terpinyl acetate	12.08	1694	1692 ± 10 (105)	8.09	1357	1350 ± 3 (156)	955
I	Benzyl acetate	12.47	1728	1720 ± 22 (68)	6.51	1167	1164 ± 2 (64)	976
J	Myrtenol	13.16	1789	1796 ± 8 (139)	6.72	1190	1195 ± 2 (203)	917
K	ß-Phenylethyl acetate	13.44	1815	1813 ± 15 (142)	7.32	1261	1258 ± 3 (76)	972
L	Phenylethyl alcohol	14.43	1908	1906 ± 15 (423)	6.04	1116	1116 ± 5 (262)	965

All values are mean ± SD, when n > 1
*: Purchased standards of candidate pheromone components reported in Branco *et al.,* 2019
**: Reported retention index from the respective column polarities from NIST library, represented as mean ± SD (number of replicates)
***: A blend of *Eucalyptus* volatile standards available from our laboratory

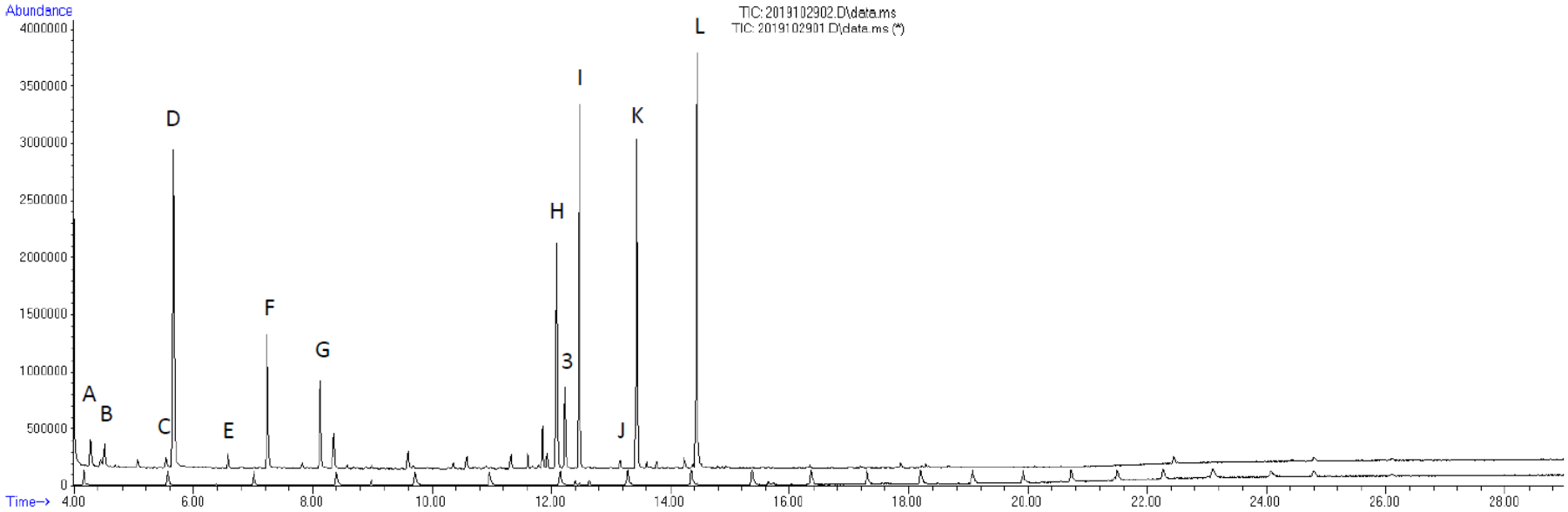


Figure A.1: The TIC trace of the mixture of twelve reference standards of *Eucalyptus* volatiles on the GC-MS (ZBWax column). The letters, A to L indicate the same components in this figure and Figure A.2, and are based on retention index, and mass spectral data from the NIST library. These are (-)-ß-pinene (A), dehydrosabinene (B), limonene (C), eucalyptol (D), p-cymene (E), cis-3-hexenyl acetate (F), cis-3-hexenol (G), α-terpinyl acetate (H), benzyl acetate (I), myrtenol (J), ß-phenethyl alcohol (K) and phenethyl alcohol (L).

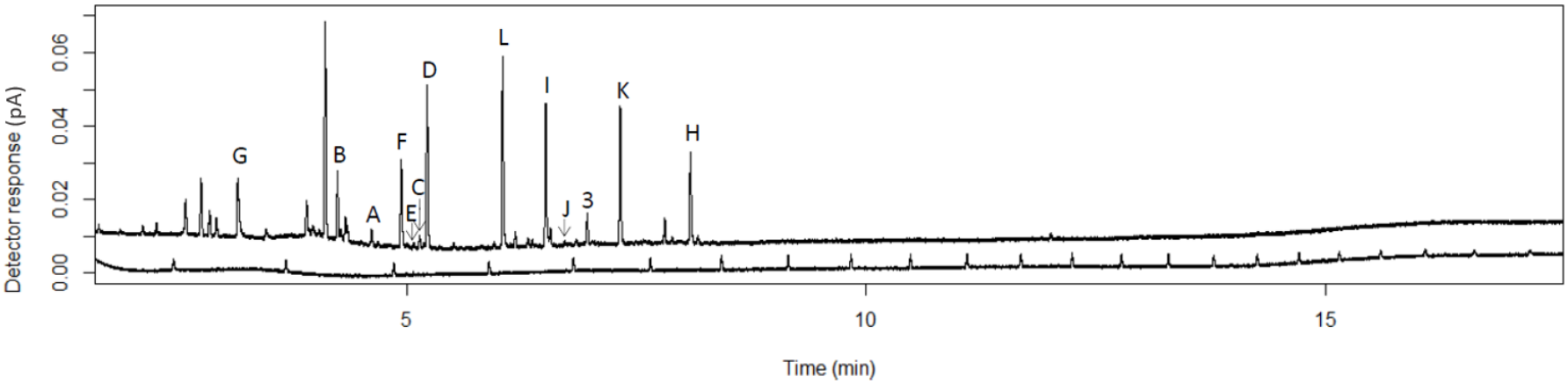


Figure A.2: The FID trace of the same mixture of *Eucalyptus* reference standards analyzed on the GC-FID (ZB5 column).

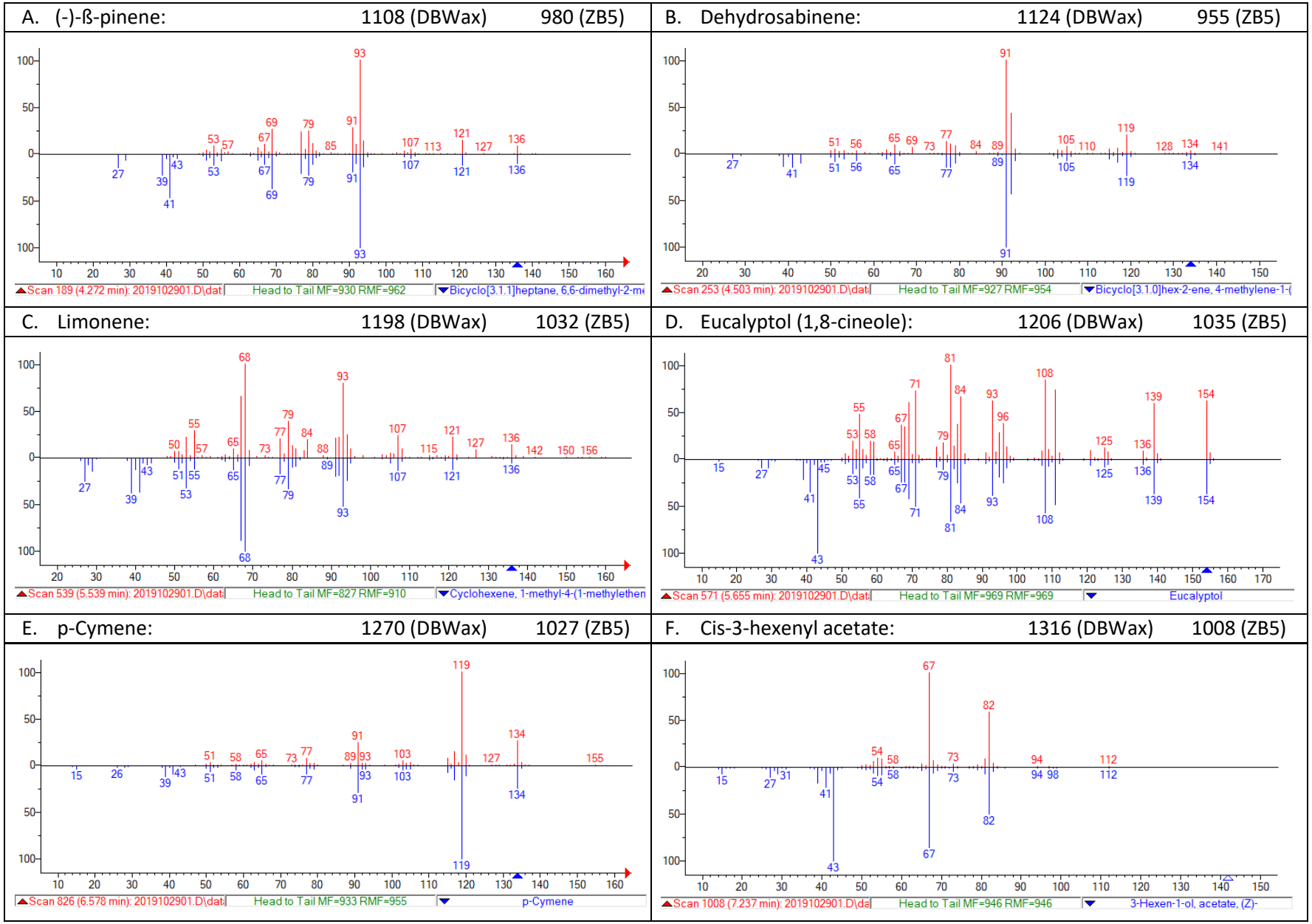
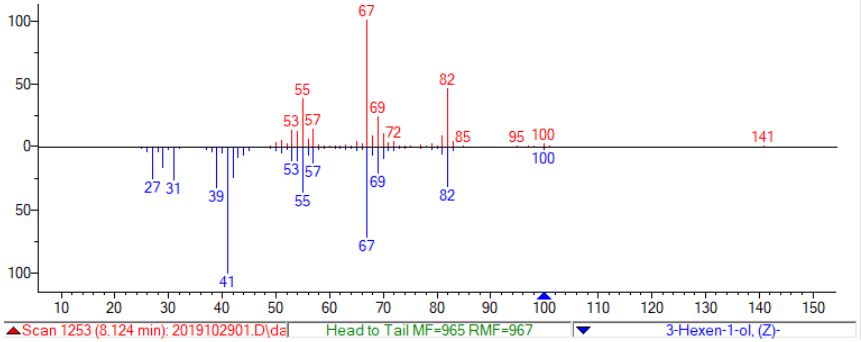
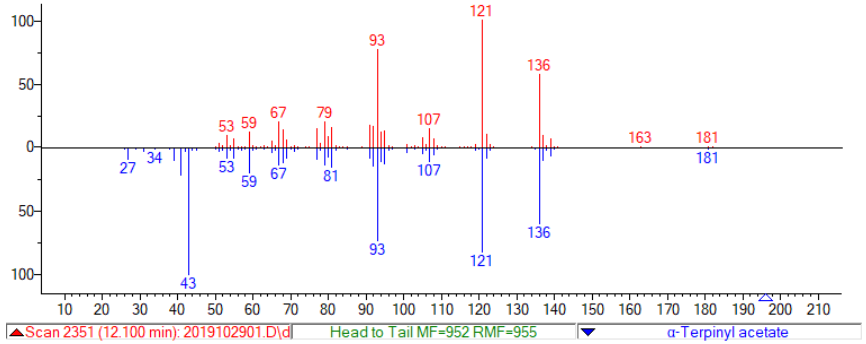


Figure A.3: Mass fragmentation comparison of each of the twelve *Eucalyptus* volatile reference standards to spectra from the NIST database. Calculated retention indexes on ZB5 and ZBWax is shown in the same order as for Figure A.1.

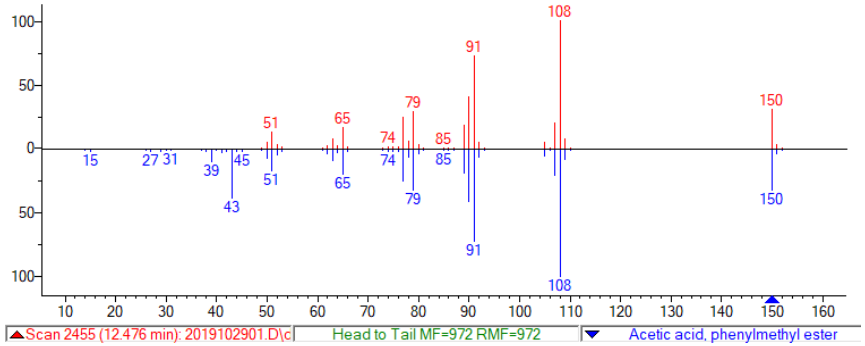
G. Cis-3-hexenol: 1380 (DBWax) 857 (ZB5)



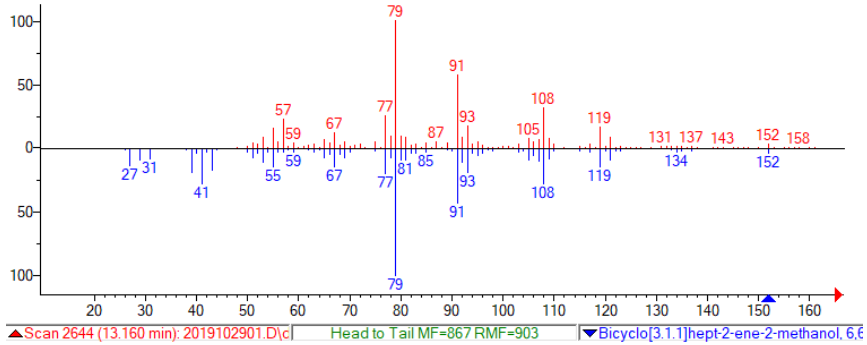
H. α-terpinyl acetate: 1694 (DBWax) 1357 (ZB5)



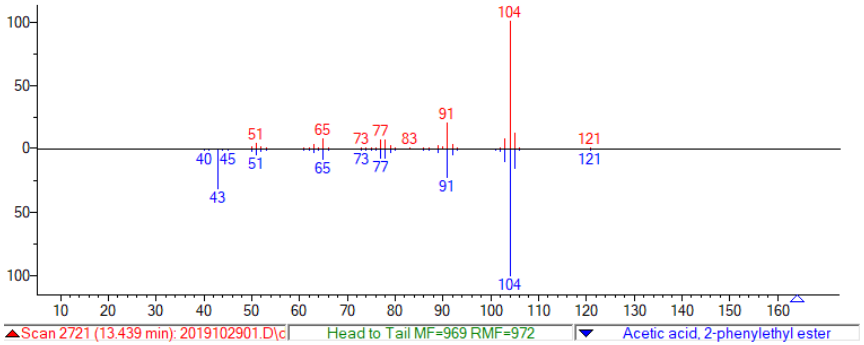
I. Benzyl acetate: 1728 (DBWax) 1167 (ZB5)



J. Myrtenol: 1789 (DBWax) 1190 (ZB5)



K. ß-phenylethyl alcohol: 1815 (DBWax) 1261 (ZB5)



L. Phenylethyl alcohol: 1908 (DBWax) 1116 (ZB5)

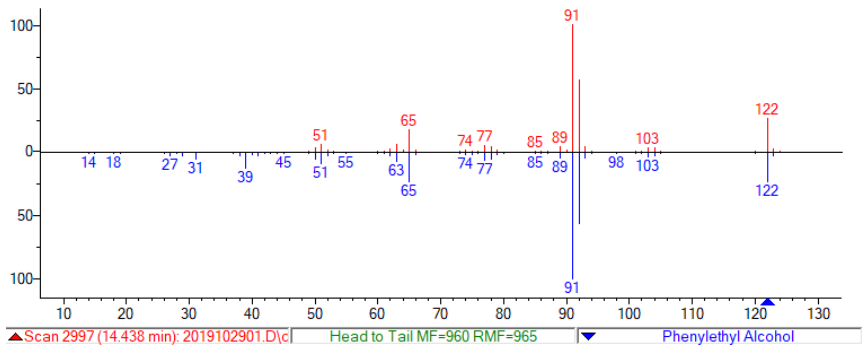


Figure A.3: (Continued)

Discussion

Confirmation of compound identities in a reference standard is not common procedure. However, available reference standards at our disposal were used to verify the identities of multiple *Eucalyptus*-specific volatiles in samples of *Gonipterus* sp. 2, which would otherwise not have been possible in a short period of time. This mixture of reference standards was kept in a fridge for multiple years before use in our sampling procedures, and the composition and concentration of the respective reference standards may have deteriorated in time. This study showed that the integrity of twelve reference standards was retained in the mixture of reference *Eucalyptus* volatiles, and was adequate for use as reference standards, as reported in Chapter three.

Addendum B

DMDS derivatization analysis of Z-dec-5-en-1-yl-3-methylbutanoate

Introduction

The synthesized pheromone of the pine emperor moth, Z-dec-5-en-1-yl-3-methylbutanoate, was treated with DMDS similar to literature (Buser et al. 1983). Due to the induced structural changes of the derivatized pheromone, the elution time of the treated pheromone is altered relative to the untreated pheromone (Buser et al. 1983). Analysis of the mass fragmentation pattern of the treated pheromone with GC-MS, can identify the location of the double bond in this straight-chain ester.

The expected mass-to-charge ratios were determined theoretically (Figure B.1). Because the pheromone is expected to indicate that the correct molecule was indeed synthesized, the expected prominent mass-to-charge fragments are A^+-SCH_3:117 m/z and B^+-SCH_3: 217 m/z, that would indicate the double bond at C5 from the ether functional moiety (X = 5, Figure B.1). The pheromone has previously been shown to have Z-conformation (Henderson et al. 1973), and is expected to show threo-addition of DMDS to the double bond (Buser et al. 1983).

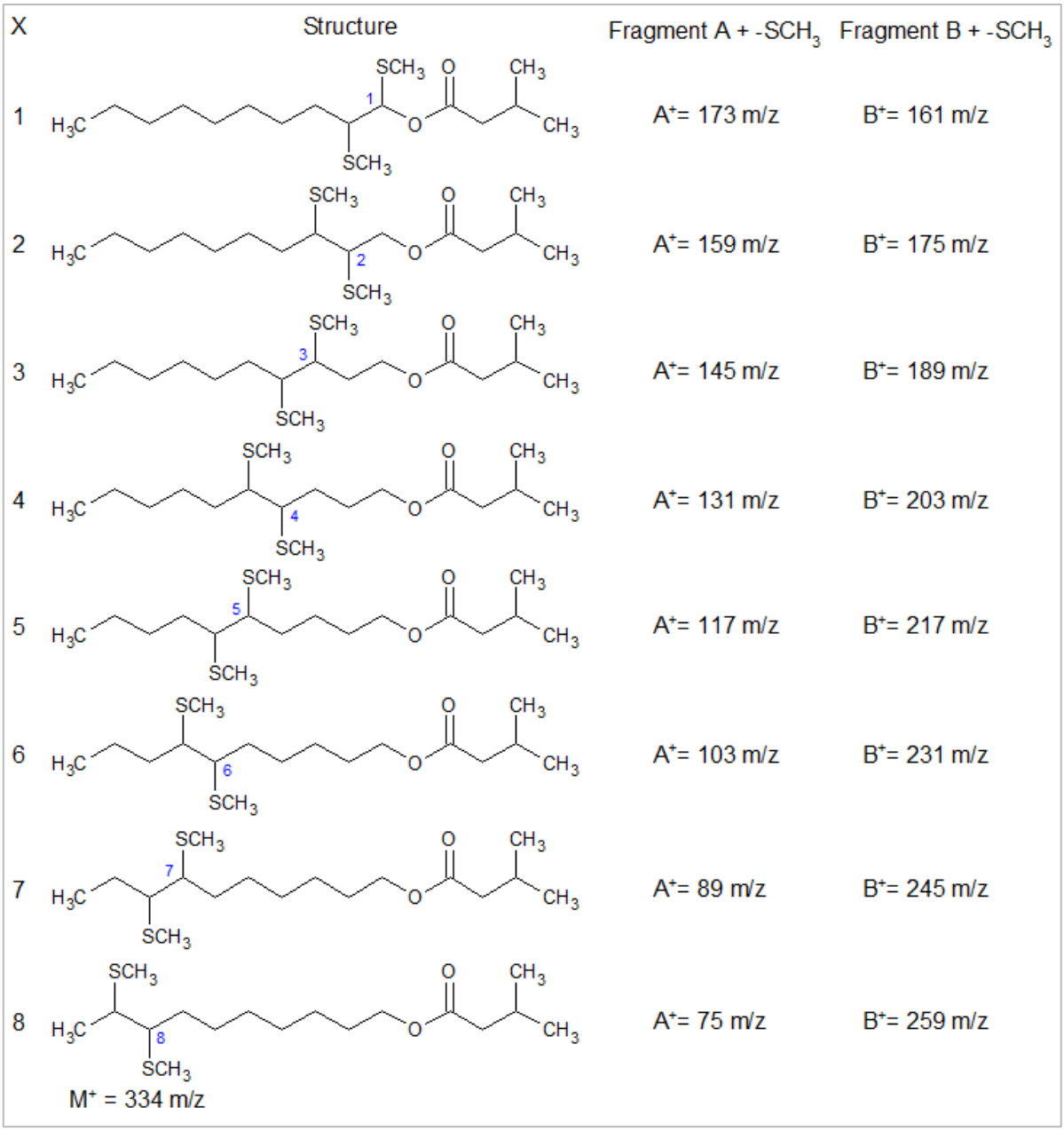


Figure B.1: Proposed treated pheromone compound structures (left), and their characteristic expected fragments after DMDS derivatization and fragmentation in the GC-MS ion source (right). X is the placement of the double bond as indicated in blue numbers on the figure, from Z-dec-x-en-1-yl 3-methylbutanoate parent compounds.

Methods and materials

The same methods were used as reported previously (Buser et al. 1983), except that incubation proceeded for 90 hours at a temperature of 60°C.

Results

Our results confirmed that the pheromone was, in fact, derivatized successfully, as the elution time of the treated pheromone on the DBWax column was much later than the untreated pheromone, and no detectable peak is observed at the original elution time (Figure B.2). This shows that the reaction proceeded to completion. One

of the four components in the synthesized standard was expected to be the saturated derivative of the synthetic Z-dec-5-en-1-yl-3-methylbutanoate based on previous results and is expected not to react with the DMDS reactant. This expected result was confirmed upon identification of only three distinguishable peaks, and not four from treated samples (Figure B.2 and B.3, n = 2).

Chromatographic peaks were expected to elute in all treated samples (and blanks) due to impurities in the synthesized standard mixture, and introduced impurities from the solvent and DMDS-derivatizing mixture. Therefore, the elution profiles of untreated and treated blanks were included for clarification of peaks as much as possible (Figure B.3).

The peaks of the DMDS-derivatized compounds eluted at 13.26 min, 13.39 min and 13.44 min (Figure B.3). The associated Kovat's retention Indexes (I_K) of these peaks are I_K (1) = 2739, I_K (2) = 2756 and I_K (3) = 2762 on the DBWax column. The untreated pheromone components eluted at (Rt (1): 8.204 min; Rt (2): 8.314 min; Rt (3): 8.366 min and Rt (4): 8.389 min (major)) when the same GC-parameters were used. Because the structure of the expected major pheromone component is known, we hypothesized fragmentation ions that could result from the reacted derivative (Figure B.4).

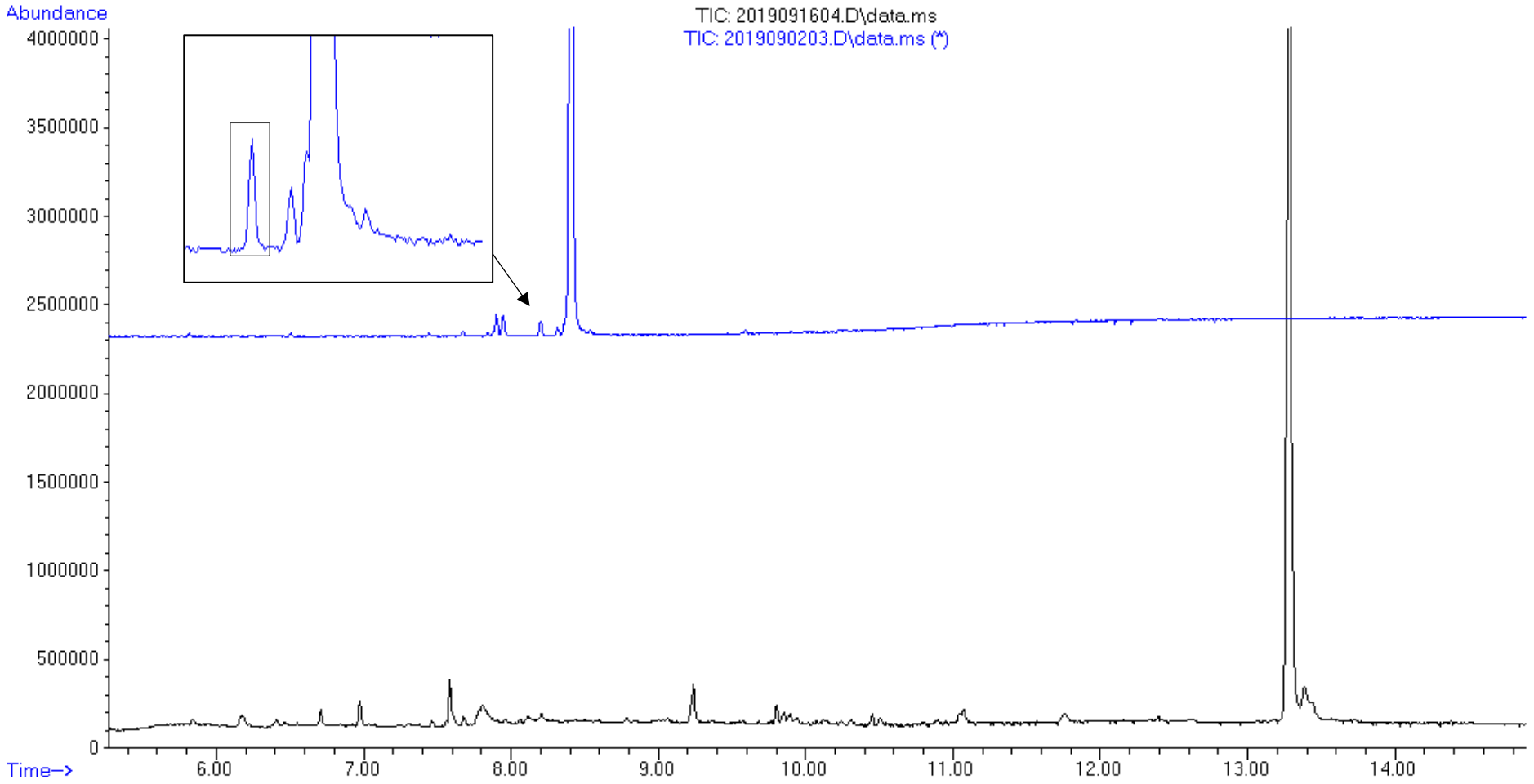


Figure B.2: The chromatographic elution profiles of the untreated (blue) and treated (black) synthesized pheromone, indicating the distinct difference in retention time between reagent and treated product as described in Buser et al. 1983. The indicated peak, eluting at 8.21 min, did not react in the DMDS derivatization due to its saturated structure, as expected.

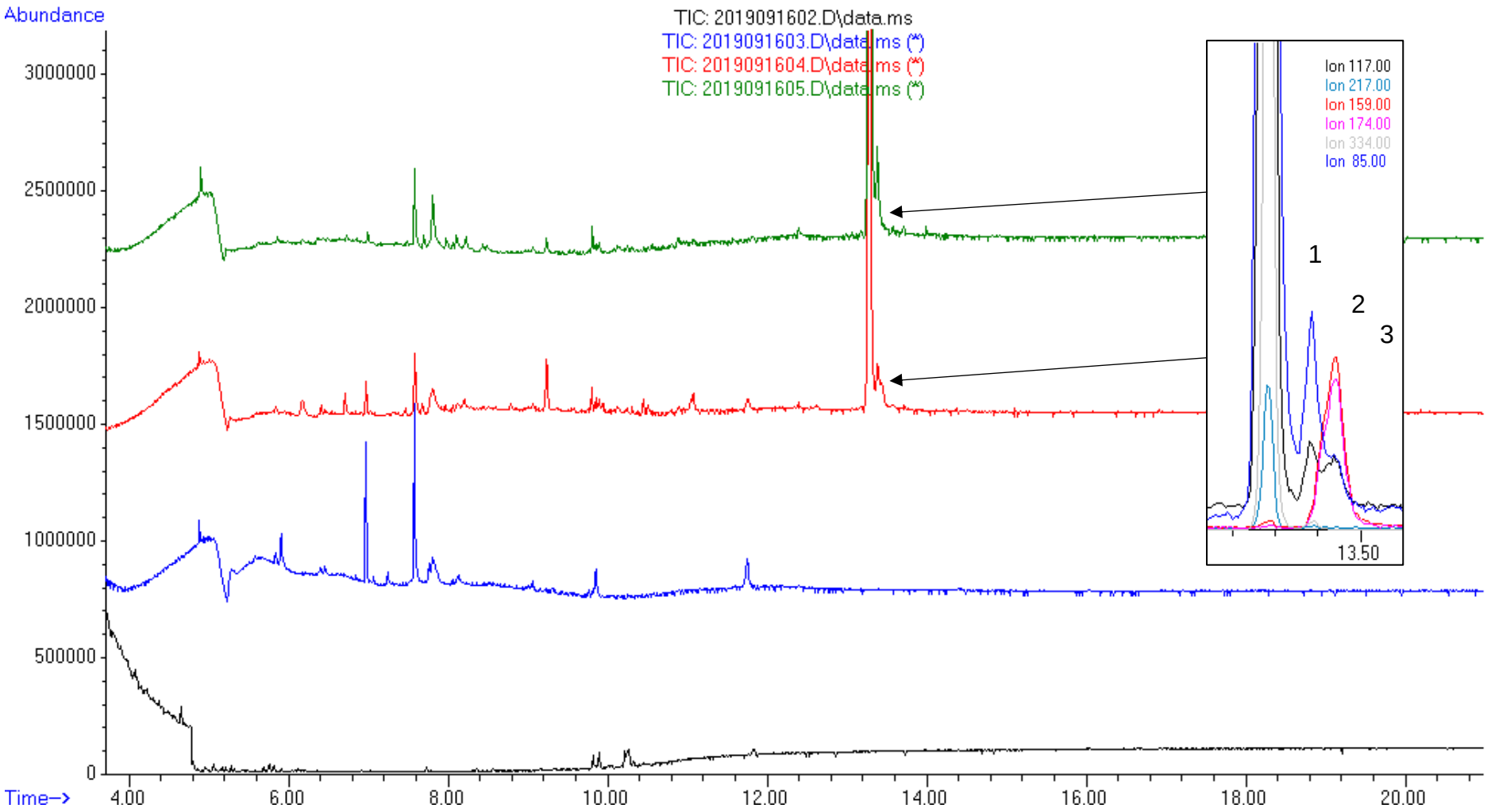


Figure B.3: Overlaid chromatographic elution profiles of an untreated n-hexane blank (black), treated blank (blue), treated pheromone samples 1 (red) and 2 (green). The suspected peaks of the treated pheromone components are indicated with arrows, and enlarged to show three distinguishable peaks, with overlapping distinct ions of peak 3 over peak 2.

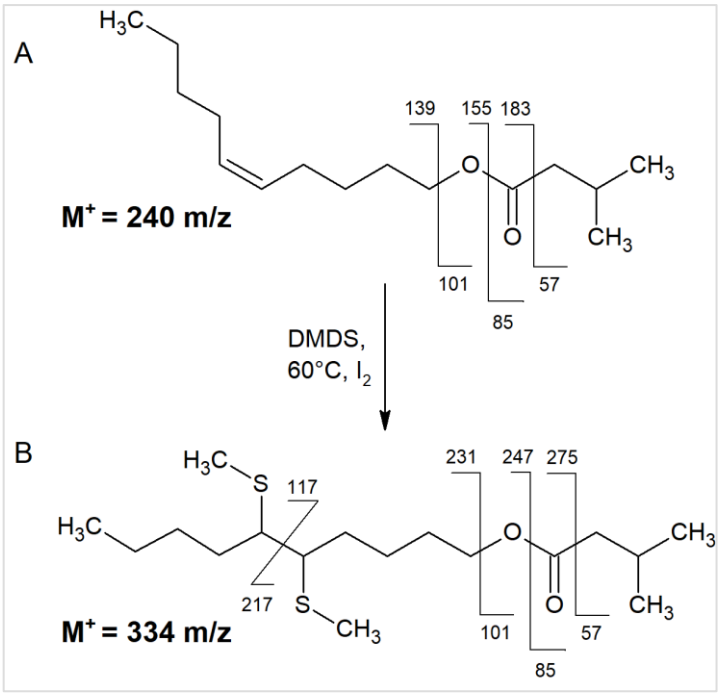
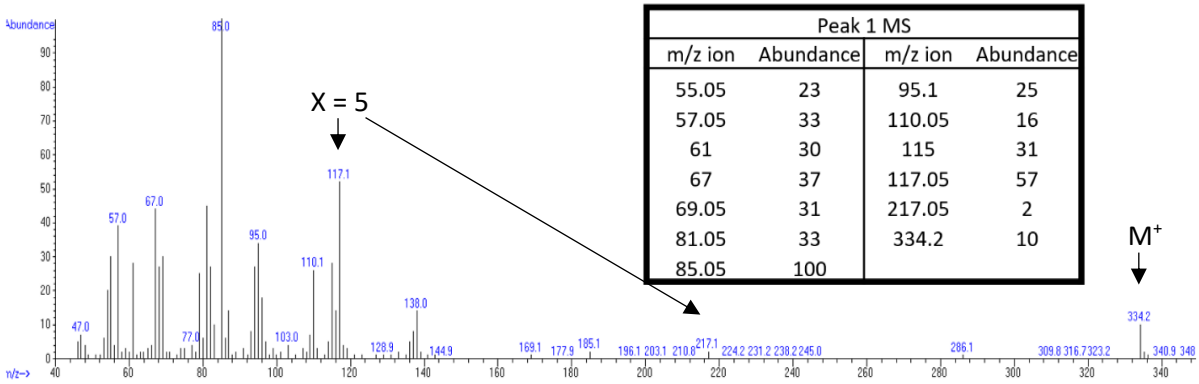
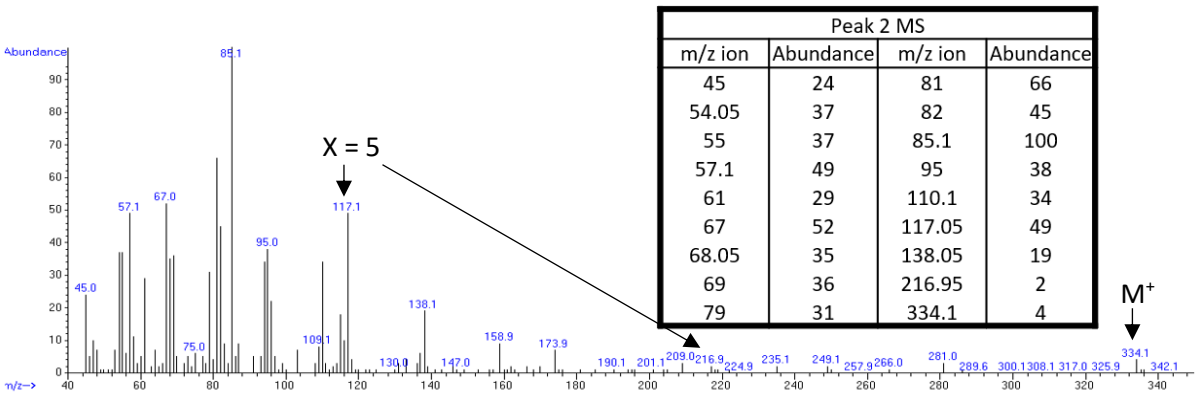


Figure B.4: The expected reaction scheme from the structure of the previously identified pheromone, Z-dec-5-en-1-yl-3-methylbutanoate (A), to its derivatized form after treatment with DMDS (B). Expected mass-to-charge ratios are 117 and 217 m/z, as shown on B.



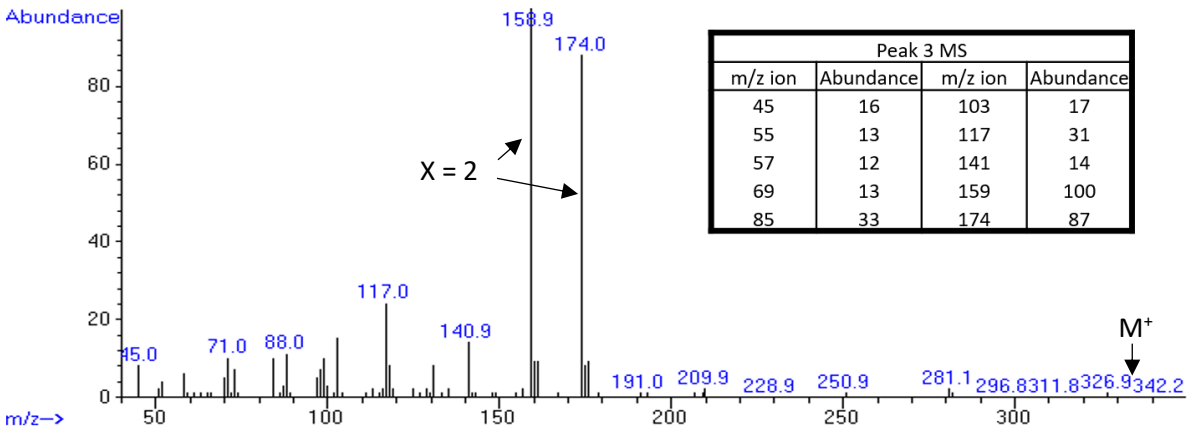
Peak 1 MS			
m/z ion	Abundance	m/z ion	Abundance
55.05	23	95.1	25
57.05	33	110.05	16
61	30	115	31
67	37	117.05	57
69.05	31	217.05	2
81.05	33	334.2	10
85.05	100		

Figure B.5: The MS fragmentation pattern of peak 1 (I_K (1) = 2739). The characteristic ions, 117 and 217 m/z are shown with arrows. The molecular ion at 334 m/z is also indicated.



Peak 2 MS			
m/z ion	Abundance	m/z ion	Abundance
45	24	81	66
54.05	37	82	45
55	37	85.1	100
57.1	49	95	38
61	29	110.1	34
67	52	117.05	49
68.05	35	138.05	19
69	36	216.95	2
79	31	334.1	4

Figure B.6: MS fragmentation pattern of peak 2 (I_K (2) = 2756). Characteristic ions, 117 and 217 m/z are present and pointed out with an arrow. Other ions, including 159 and 174 m/z (Figure B.4), are also present, but these are due to a coelution with peak 3 (Figure B.3). The molecular ion, 334 m/z is also indicated with an arrow.



Peak 3 MS			
m/z ion	Abundance	m/z ion	Abundance
45	16	103	17
55	13	117	31
57	12	141	14
69	13	159	100
85	33	174	87

Figure B.7: MS fragmentation pattern of peak 3 (I_K (3) = 2762). The 159 and 174 m/z fragments are shown with arrows, and are indicative of sulfur-containing fragments due to characteristic m/z + 1 and m/z + 2 isotopic ions in similar abundances for both fragments. The molecular ion, 334 m/z, is present in very low abundance (0.25%) in this fragmentation pattern.

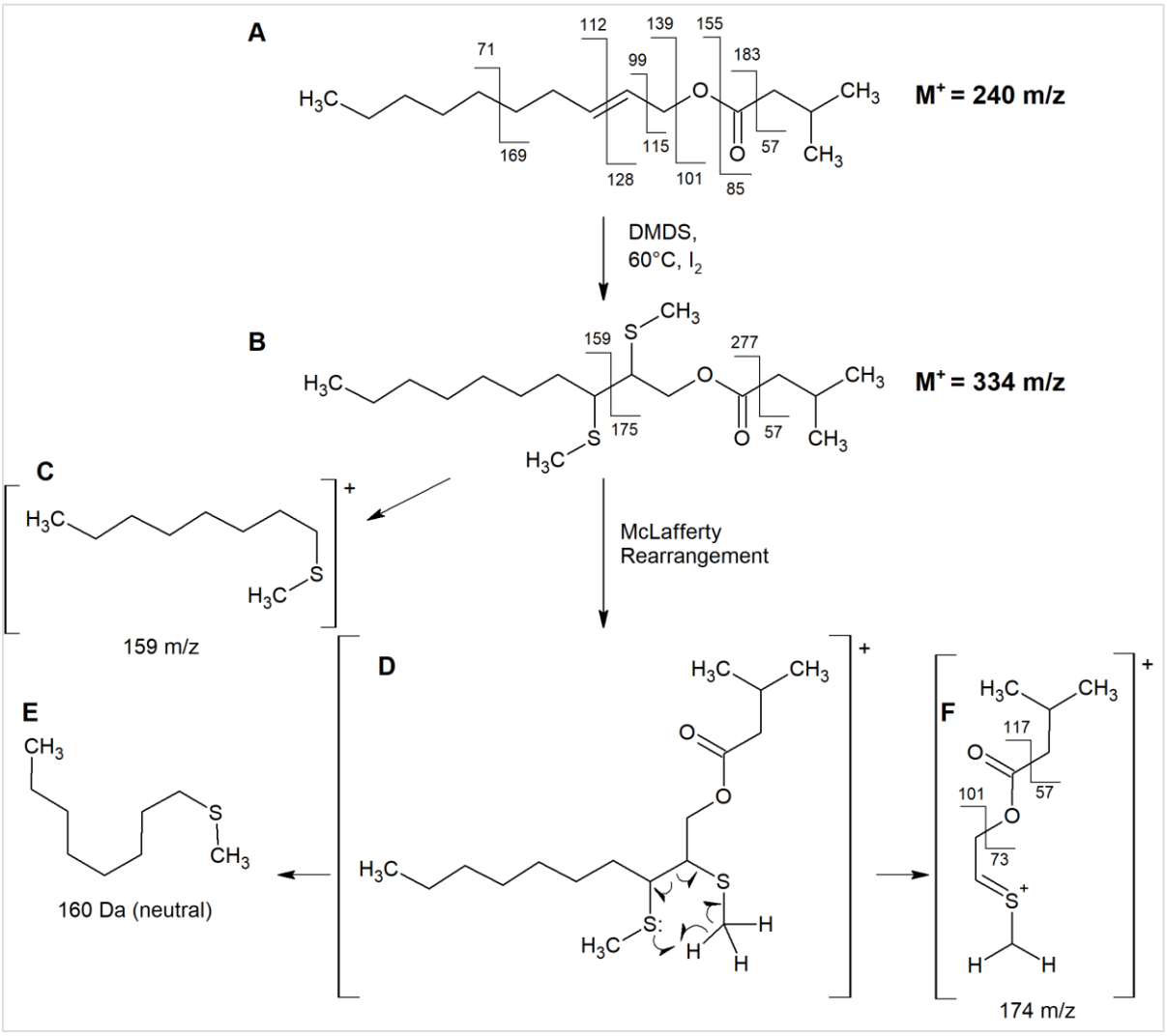


Figure B.8: A hypothesized reaction scheme of 2-decen-1-yl-3-methylbutanoate (A) after derivatization with DMDS (B). The expected characteristic ions, C and F, are shown. We propose a mechanism of a McLafferty rearrangement of B to result in the formation of a neutral fragment (E) and the prominent 174 m/z-ion (F) seen in Figure B.7.

Discussion

The expected molecular ion of the reacted pheromone (334 m/z, Figure B.1) is present in the mass fragmentation profile of the major component, peak 1 (Figure B.3). This indicates that the DMDS reaction has indeed taken place. Both indicative fragments, 117 and 217 m/z are present, indicating the X = 5-position of the double bond on the correct structure (Figure B.1 and B.4). The base peak is 85 m/z, indicating the presence from the isovalerate moiety of the structure (B, Figure B.4). The major pheromone component is thus confirmed as Z-dec-5-en-1-yl-3-methylbutanoate.

Peak 1 and peak 2's fragmentation patterns are similar (Figures B.5 and B.6), and have the presence of the same molecular ion (334 m/z, Figure B.1). The only difference is the presence of ions 159 m/z and 174 m/z, but these ions are due to a coelution of peak 3 (Figure B.3). Peak 2 was distinguishable due to the fact that fragments including 57, 67, 81, 95 and 110 m/z were present only in peak 2 and not peak 3 (Figure B.3). The tentative identity of peak 2 is expected to be the trans-isomer of dec-5-en-1-yl-3-methylbutanoate. The presence of the same characteristic ions, 117 and 217 m/z-in this peak's elution do not contradict the expected results, even though co-elution hinders a clear result. The hypothesis that this compound identity is E-dec-5-en-1-yl-3-methylbutanoate, is not rejected. All other possible double bond positions were excluded based on the absence of other expected indicative fragments (Figure B.1). Analysis of the fragmentation pattern and retention index of a pure form of this compound is necessary to confirm this tentative identification.

Peak 3 has a much different fragmentation pattern than the other peaks, and the expected molecular ion is present in very low abundance in the mass spectral profile (334 m/z, Figure B.7). The presence of the m/z+1 and m/z+2 isotopic peaks in similar abundances in both 159 m/z and 174 m/z suggest that both these fragments contain a sulfur-molecule, as expected for DMDS-treated fragments. These ions are also the two most abundant ions and correspond to at least one of the expected fragments if X = 2 (Figure B.1 and B.8). A 175 m/z fragment was expected (Figure B.1), but the results showed only the fragment of 174 m/z. We propose that this fragment may have been formed in a McLafferty rearrangement, where a single hydrogen is lost to a nucleophilic sulfur after the DMDS reaction (D, E and F, Figure B.8). Thus, 159 m/z and 174 m/z-ions (C, F, Figure B.8) indicate that the parent component could be dec-2-en-1-yl-3-methylbutanoate (A, Figure B.8). This work is part of the investigation of

the pheromone of the pine emperor moth, that can aid in the sustainable management of this pine plantation pest.

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Addendum C

Poster

Feromone van die Denneboom Pou-oogmot

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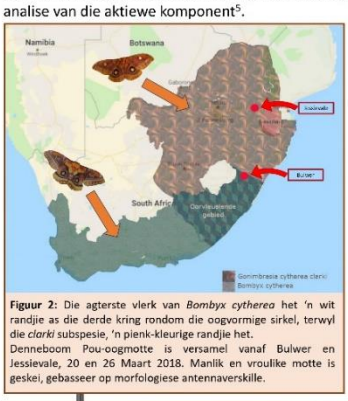
Inleiding

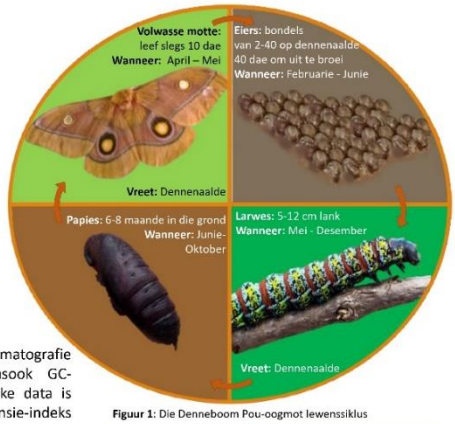
Die Denneboom Pou-oogmot, *Gonimbrasia cytherea* (Fabricius) (Lepidoptera: Saturniidae) het twee subspesies in Suid Afrika. Plaagbeheer van dié endemiese motte word vereis in denneplantasies, aangesien sporadiese populasie-uitbarstings plantasies beskadig[3,4], en vorige beheermaatreëls nie effektief was nie[3]. 'n Enkele feromoon-komponent is voorheen ontrafel as cis-dek-5-eniel 3-metielbutanoaat, deurdat die ekstrakte van 35 000 vroulike motte gekombineer, gekonsentreer en chromatografies geskei is vir struktuur-analise van die aktiewe komponent[5].



Figuur 2: Die agterste vlerk van *Bombyx cytherea* het 'n wit randjie as die derde kring rondom die oogvormige sirkel, terwyl die *clarki* subspesie, 'n pienk-kleurige randjie het. Denneboom Pou-oogmotte is versamel vanaf Bulwer en Jessievale, 20 en 26 Maart 2018. Manlik en vroulike motte is geskei, gebasseer op morfologiese antennaverskille.

Doel van die studie:

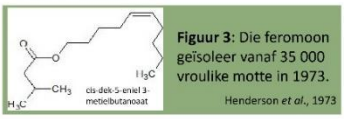
- Bepaal of die feromoonsamestelling volledig beskryf is in die 1970's.
- Peil of die motte wat vir die studie gebruik is, verskillende subspesies verteenwoordig of nie.



Figuur 1: Die Denneboom Pou-oogmot lewenssiklus

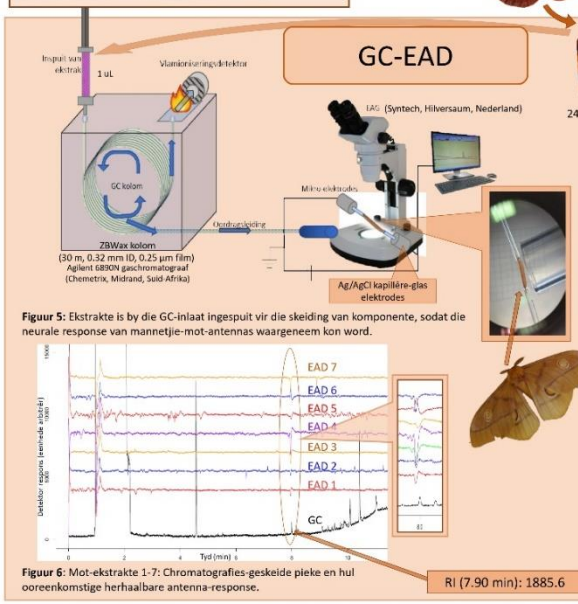
Metodes en Resultate

Mot-ekstrakte is geanaliseer met behulp van gaschromatografie (GC) gekoppel aan elektro-antennografie (EAG), asook GC-gekoppelde massaspektrometrie (GC-MS). Die toepaslike data is versamel en vergelyk met literatuur-gegewens soos retensie-indeks en fragmentasieprofiel.



Figuur 3: Die feromoon geïsoleer vanaf 35 000 vroulike motte in 1973. Henderson *et al.*, 1973

Figuur 4: 24 Vroulike motte se abdominale segmente is geamputeer en individueel in gedistilleerde heksaan geëkstraheer.



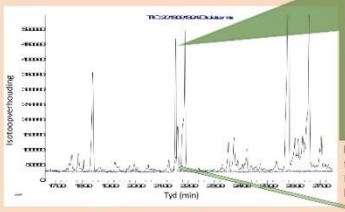
Figuur 5: Ekstrakte is by die GC-inlaat ingespuit vir die skeiding van komponente, sodat die neurale response van mannetjie-mot-antennas waargeneem kon word.

Figuur 7: Die GC-temperatuurprofiele gebruik op die GC-EAD en GC-MS tydens die eluering van alle monsters op beide instrumente.

Figuur 8: Na analise met die GC-EAD is dieselfde ekstrakte deur die GC-MS geanaliseer, gevolg deur die gesintetiseerde feromoon-standaard.

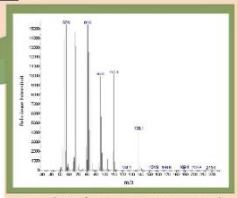
Figuur 6: Mot-ekstrakte 1-7: Chromatografies-geskeide pieke en hul ooreenkomstige herhaalbare antenna-response.

RI (7.90 min): 1885.6



Figuur 9: Gaschromatogram (teen 5°C/min) van 'n mot-ekstrak (**Swart**) en gesintetiseerde feromoon-standaard (**Blou**).

RI (21.50 min): 1885.7



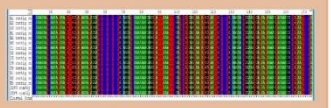
Figuur 10: Die fragmentasie-patroon van die feromoon-standaard, gegenereer deur die MS, presies soortgelyk aan die feromoon beskryf deur Henderson *et al.*, 1973.

Bepaling van die mitochondriale COI-basispaarvolgorde

Figuur 11: **A:** Motbene gepreserveer in 70% Etanol, voordat dit gevriesdroog is vir DNA-ekstraksies. **B:** Fenol-Chloroform DNA-ekstraksie.

DNA-ekstraksies (Fenol-chloroform) → COI-geen amplifikasies (Lep F en Lep R voorlopers) → Volgorde- bepaling (HiSec-Illumina) → Belyning en wysigings aan DNA basispaar volgordes

Figuur 12: Die volgorde van gebeure tydens basispaar belyning en die spesifieke metodes benut vir elke stap.



Figuur 13: Basispaar-belynings van die onderskeie motte se DNA materiaal. Geen variasie is gesien in die basispaarvolgorde van die volle 680 basispare nie.

Bespreking

Die herhaalbare GC-EAD-data gee besliste bewyse van 'n reaksie op die mot-ekstrakte, presies ooreenstemmend met die retensie-indeks van die feromoonstandaard. As kwaliteitsbeheer, is die fragmentasiepatroon van die feromoon vanuit die GC-MS vergelyk met die MS-spektrum wat deur Henderson (1973) gerapporteer is. Aangesien daar voorlopige bewyse is van 'n (laer konsentrasie) tweede komponent, word hierdie saak verder ondersoek. Verdere analise van enige addisionele komponente sal ook gedoen word op 'n nie-polêre GC-kolom. Die presiese basispaar-belyning tussen manlike en vroulike motte van die onderskeie versamelingspunte, gee voorlopige bewyse dat die motte vanuit Bulwer, KZN en Jessievale, Mpumalanga, een subspesie is. Dit is egter onbekend watter subspesie, aangesien geen vergelykbare molekulêre data van die mot beskikbaar is nie. Die morfologie gee wel 'n indikase, aangesien die oogsirkel op die agtervlerk van die versamelde motte pienkerig was. Dit stem ooreen met die gerapporteerde Noordoostelike verspreiding van die *G. cytherea clarki* subspesie in Suid-Afrika.

Bedankings Dr. Jan Bello word bedank vir die sintese van die mot-feromoonkomponent, Dr. Gudrun Dittrich-Schröder vir haar hulp met die geen-analise en Dr. Martin Kruger vir sy kundigheid in die motspesie-benamings. Die NRF, TPCP, FABI en Universiteit van Pretoria word bedank vir hul finansiële bydrae.

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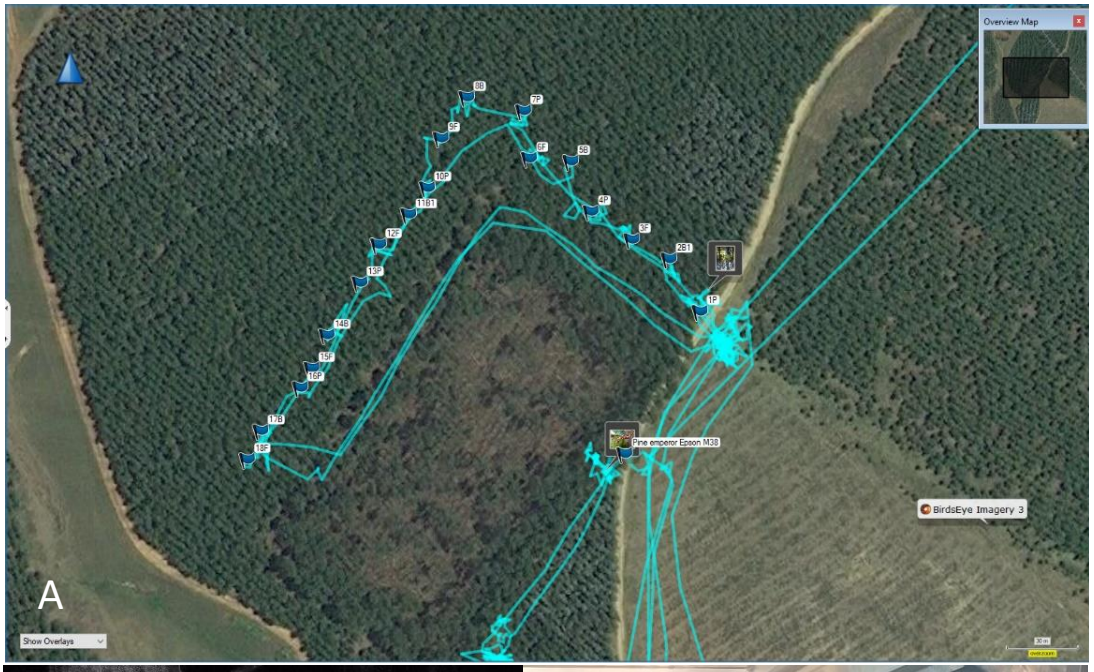
Prepared for presentation at the SAWK symposium (Suid-Afrikaanse Wetenskap en Kunste simposium, translated as South African Technology and Arts Symposium), 25-26 Oct 2018.

Addendum D

Pictures

Addendum D – Pictures



(Cont.)

- **Figure C.A:** The layout of the field trial for trapping *Nudaurelia clarki* in *Pinus patula* plantations in Bulwer, KZN. Traps (flags in the picture) were placed on the edge of the plantation plot next to a plot with young *P. patula* trees. Blue lines represent movement of researchers in open areas between trees.
- **Figure C.B:** Camera footage of captured *N. clarki* male moths in a pheromone-baited trap in a *P. patula* plantation during the night. Most moths were captured successfully, but a few moths lingered outside of this trap.
- **Figure C.C:** Custom traps were foldable to enable easy transportation of 20 traps from Pretoria to KZN to test whether synthesized pheromone, Z-dec-5-en-1-yl-3-methylbutanoate, is attractive to *N. clarki*. in a field trial.
- **Figure C.D:** Comparison of an expanded and collapsed custom-built trap, used for trapping *N. clarki* in field trials.
- **Figure C.E:** One of six traps in which a field-collected female *N. clarki* moth was put in a separate, closed compartment in a female-baited trap during field trial with the synthesized pheromone, Z-dec-5-en-1-yl-3-methylbutanoate.
- **Figure C.F:** A view through the entrance hole of a trap used for capturing male *N. clarki* moths. The attachment of a polydimethylsiloxane (PDMS) lure dispenser in a pheromone-baited trap is shown. No lure dispenser was attached to the wire on the inside of the blank traps, and in other treatments, different lure dispensers (shown in bottom right corner: PDMS, RR and PE from left) were attached to the internal wire in the trap.
- **Figure C.G:** Preparation of sampling chambers in the dynamic headspace sampling facility. Sampling chambers contain reared virgin female *N. clarki* moths. This photo was taken before PoropakQ adsorbent was attached for use during sampling.
- **Figure C.H:** The connection of a *N.clarki* female antenna to glass capillaries with an electrolyte solution attaching the antennal tip to the grounding electrode and the base of the antenna to the working electrode. This antennal preparation was used for male and female antennae during GC-EAD screening and puffing experiments.
- **Figure C.I:** The antennal preparation of a *Gonipterus* sp. 2 weevil antenna for GC-EAD screening. The antennal tip was removed, as well as the pedicel, before attachment to pulled glass capillaries filled with electrolyte solution surrounding silver wires on each side.
- **Figure C.J:** Dynamic headspace sampling of *Gonipterus* sp. 2 weevils with purified air entering through the tube and volatile-filled air exiting and being adsorbed onto the PoropakQ adsorbent. The paper towel in the Consol glass sampling chamber gave large numbers of sampled weevils grip during the sampling period.
- **Figure C.K:** A fully developed larva of *N. clarki* on *P. patula* needles. Larvae like these are the defoliators of pine needles in plantation plots.
- **Figure C.L:** An adult *Gonipterus* sp. 2, firmly gripping the *Eucalyptus* branch where it has fed on the terminal leaves in one of the collection sites in the field.
- **Figure C.M:** A few visible *Gonipterus* sp. 2 larvae burrowing into the sterile soil in a clear polypropylene pupation chamber (250 mL). Groups of five to 15 larvae were

initially reared in these chambers to obtain virgin weevils to use for sampling experiments.

- **Figure C.N:** Small pupation chambers (30 mm ID, 37 mm) were part of twelve-well plates. Individual larvae of *Gonipterus* sp. 2 were reared in these chambers to ensure that adult weevils were kept virgin after they had emerged.
- **Figure C.O:** Pupae of *N. clarki* were collected from the soil in the undercarriage of a pine plantation in Bulwer, KZN, together with the ground in which pupation had naturally started.

Summary

In this study, we investigated the pheromones of *Gonipterus* species 2 (Coleoptera: Curculionidae) and *Nudaurelia clarki* (Lepidoptera: Saturniidae). These insects are two South African plantation pests that have caused the forestry industry tremendous economic losses due to defoliation. The development of pheromone-based management tools for these pests can ensure sustainable and effective pest control without detrimental effects to surrounding environments.

Our results showed that *Gonipterus* sp. 2 produces at least cis- and trans-verbenol, which were described as putative pheromone components for *G. platensis*. Female headspace samples contained larger concentrations of the same volatiles than male samples. Furthermore, topical application of Juvenile hormone III does not induce pheromone production in *Gonipterus sp. 2*.

The described pheromone of *N. cytherea*, Z-dec-5-en-1-yl-3-methylbutanoate, was shown to be effective for trapping *N. clarki* in KZN. The synthesized pheromone consisted of four electroantennographically active compounds, and attracted numerous *N. clarki* male moths species-specifically. We report on the first instance of female autodetection in the Saturniidae family, because female *N. clarki* antennae also detect the pheromone. The attractiveness of the same pheromone to both *N. cytherea* and *N. clarki*, together with preliminary genetic evidence that shows identical cytochrome oxidase I sequences between these species, reveal that these two species might actually be one. Future development of pheromone-based pest management strategies requires confirmation of the attractiveness of cis- and trans-verbenol to *Gonipterus* sp. 2, and optimizing *N. clarki* pheromone-based trapping for use in mass-trapping or mating disruption mechanisms.