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Data in, lures out: designing selective lures against fruit pests

Sebastian Larsson Herrera

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Faculty of Landscape Architecture, Horticulture and Crop Protection science

Department of Plant Protection Biology

Alnarp

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Cover: Mango trees and the ghostly fly
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Swedish University of Agricultural Sciences, Department of Plant Protection Biology, Alnarp, Sweden

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Abstract

With the push for rebuilding ecosystem resilience and the aim to lower the footprint of agriculture, there is an urgent need for novel, sustainable tools for managing pests. Insect olfaction is a good target to designing such novel tools. However, the rate at which odor-based insect control innovations are churned out is underwhelming. This thesis aims to accelerate the identification of lures using pests of wine and tropical fruits as models.

Volatiles from microorganisms, hosts or other ecological relevant substrates, can be used to construct attractive lures. Such a lure was designed for *Lobesia botrana*, a severe pest in wine. First, volatiles emitted by microbe inoculated grapes were identified using GC-MS. A limited set of shared volatiles was attractive in the field, and further tailored to find a balance between attractiveness and selectivity. The best lure for *L. botrana* also caught more of other species such as important natural enemies, impacting ecosystem services provided in the vineyard. Hence the most attractive lure is not always the best.

A group of invasive pests in the true fruit fly family, Tephritidae, was used as model organisms to design a novel workflow from primary research to lure design. Ranges of olfactory responses, olfactomes, of fruit flies that differ in ecology and phylogeny served as input to a database. Custom tools were developed that allowed for mining this database for ecological as well as evolutionary signals. In the selected pests, ecology overrode phylogeny in the electrophysiological response profile across both olfactory organs, antennae and palps, as well as different substrates. Further, a set of compounds was found that formed a preadaptive bridge between fruits, and a subset links the flies ancestral saprophily to their derived frugivory. Candidate lures, more attractive than fruits, were also tested in a novel six-choice olfactometer.

The work shows that selective lures can be designed from generic volatiles and that this process can be strongly accelerated through comparative olfactomics.

Keywords: GC-EAD, olfactomics, *L. botrana*, Tephritidae, lures, sustainability

Data in, lockbeten ut: utvecklandet av selektiva lockbeten mot fruktskadegörare

Abstrakt

Med målet att återbygga resiliensen i ekosystem och att minska det agrikulturella avtrycket, följer ett akut behov av hållbara verktyg för att kontrollera skadegörare. Insekters doftsinne kan nyttjas när man designar sådana nya verktyg. Dock så är hastigheten som vilka doft-baserade innovationer för insektskontroll skapas alldeles för långsam. Denna avhandling siktar på att påskynda utvecklandet av lockbeten genom att använda skadegörare på vin och tropisk frukt som modeller.

Flyktiga ämnen från mikroorganismer, värdar eller andra relevanta substrat kan användas för att konstruera lockbeten. Ett sådant designades för *Lobesia botrana*, en allvarlig skadegörare på vindruvor. Först identifierades flyktiga ämnen från druvor som inokulerats med mikroorganismer med hjälp av GC-MS. Några av dessa var attraktiva i fält, och vidareutvecklades för att hitta en balans mellan attraktivitet och selektivitet. Det bästa lockbetet för *L. botrana* fångade även mer av andra arter såsom viktiga naturliga fiender, vilket påverkar ekosystemtjänster i vinodlingen. Det mest attraktiva lockbetet är inte alltid det bästa.

En grupp av invasiva skadegörare i borrflugefamiljen, Tephritidae, användes som modellorganismer för att skapa ett nytt arbetsflöde från primär forskningsdata till nya lockbeten. Vidden av doftsinnesresponser, olfaktom, hos borrflugor som skiljde sig åt i ekologi och fylogeni användes för att konstruera en databas. Anpassade verktyg utvecklades som tillät utvinning av information från denna databas av såväl ekologiska som evolutionära signaler. Hos dessa skadegörarna överskuggade ekologin fylogenin i den elekrofysiologiska responsprofilen från olika doftorgan, såväl som från olika substrat. En grupp ämnen skapade en preadaptiv brygga mellan frukter, och en del av dessa länkar ihop saprofylin hos flugornas förfäder med dess attraktion till frukter. Tentativa lockbeten, mer attraktiva än frukter, testades också i en ny sex-arms olfaktometer. Detta arbete visar att selektiva lockbeten kan designas från generiska flyktiga ämnen och att denna process kan accelereras med hjälp av komparativ olfaktomik.

Nyckelord: GC-EAD, olfaktomik, *L. botrana*, Tephritidae, lockbeten, hållbarhet

Dedication

To my little sunshine Frej, Mimmi, friends and cats

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Tasin M, **Larsson Herrera S**, Knight AL, Barros-Parada W, Fuentes Contreras E and Pertot I, Volatiles of grape inoculated with microorganisms: Modulation of grapevine moth oviposition and field attraction (2018), Microbial ecology; 76(3): 751–761

II. **Larsson Herrera S**, Rikk P, Köblös G, Szelényi MO, Molnár BP, Dekker T and Tasin M, Designing a species-selective lure based on microbial volatiles to target *Lobesia botrana* (2020), Scientific Reports; 6512

III. Biasazin TD, **Larsson Herrera S**, Kimbokota F and Dekker T, Translating olfactomes into attractants: shared volatiles provide attractive bridges for polyphagy in fruit flies (2019), Ecology letters; Jan; 22(1):108-118

IV. **Larsson Herrera S**, Kimbokota F, Biasazin TD, Ahmad S, Heise K and Dekker T Maxillary palps of Tephritidae are tuned to food volatiles and diverge on ecology rather than phylogeny, manuscript

V. **Larsson Herrera S**, Candia IF, Biasazin TD, Walker III W, Cha D, Tasin M and Dekker T, Comparative olfactomics links ancestral saprophily to derived frugivory in tephritid fruit flies, manuscript

The contribution of Sebastian Larsson Herrera to the papers included in this thesis was as follows:

I. Analyzed the headspace data and field catch data. Assisted MT in writing the manuscript

II. Planned the experiment together with MT, assisted in the field experiments, analyzed the data, wrote the paper together with TD and MT

III. Conceived the idea with TD and TDB, assisted TDB and FK in gathering primary data, analyzed the data with TDB and TD, assisted TDB and TD in writing the paper

IV. Conceived the study with FK, TDB and TD, did the SEM together with TDB, led the data analysis with assistance of TD, wrote the manuscript with TD and FK

V. Conceived the study with TD, led the data analysis with assistance of TD, wrote the manuscript with TD

1. Introduction

Popular demand to mitigate climate change and enhance ecosystem resilience is putting pressure on policy makers, and puts agri- and horticultural practices in a transformative spotlight. Today, the narrative describing the ideal farmer is shifting. Farmers should provide food sustainably and locally, and shift from chemically dependent, annual monocultures to more perennial systems that strengthen the ecosystem by using structures that provide ecosystem services. At the same time farmers across the globe are under an unprecedented and increasing pressure of invasive species. Some of these invasive species have the potential to become or are already severe threats to agri- and horticultural crops, impacting the livelihoods of farmers, who resort to abandoning crops or doubling down on the use of pesticides and large scale practices to control pests and perform cost-effective agriculture. Science needs to support the transition to sustainable agriculture by developing innovations that provide practical and targeted solutions to pests with minimal spill-over on the environment.

In this thesis the focus is on two problematic groups of invasive insects that are causing considerable problems in horticultural production across the globe, Tortricidae and Tephritidae. The first, exemplified by *Lobesia botrana* (Denis & Schiffermüller), and the second by several Dacini species and *Ceratitis capitata* (Wiedemann), Ceratitidini. The first part centers around developing a species-selective lure based on generic volatiles from microbial activity on grapes, and testing the resulting lures in the field. The second part focuses on mapping the olfactory breath, the olfactome, of Tephritidae and generating a data pipeline that permits compiling and then extracting information from electrophysiological data. One of the main goals

of the resulting database is to generate leads on novel lures.

2. Background

Pesticides, once hailed as the solution for farming, have been questioned since Rachel Carson and the release of her book Silent spring. The focus of the book was on the dramatic effect of DDT and other pesticides on birds and human health, which led to the ban of DDT use in agriculture across the globe. Today, we are witnessing an unprecedented erosion of life on earth, frequently referred to as the sixth mass extinction (Ceballos et al., 2017; Hallmann et al., 2017). At the core of this is humanity, exerting what has been called the "death by a thousand cuts" (Wagner et al., 2021), where the impact from climate change, huge monocultures, deforestation, pesticide use, loss of habitat, invasive species and much more are hard to detangle from each other as they reinforce each other in threatening life on the planet. A key issue is the usage of insecticides in both organic and non-organic horticulture, in conjunction with monocultural practices. These kinds of practices accelerate the collapse of insect species communities, with far-reaching impacts and unimaginable cascading effects on nature. The dependency on agrochemicals is a legacy from the green revolution in the mid 20th century when agriculture underwent dramatic changes, with increased yields by novel varieties but also saw dramatic increases of inputs such as fertilizers, pesticides and mechanization. To change these unsustainable production patterns requires at least equally strong effort and incentives as those that brought them about, a new green revolution.

2.1 Lack of tools in pest management

While it is generally accepted that we need to change unsustainable practices in agriculture, measures aimed at correcting the underlying patterns may at

times have opposite effects. For instance, as a response to the negative impact of insecticides on, amongst other insects, bees, the EU banned some neonicotinoids, a group of compounds that systemically control plant-eating insects for a long period. They are often applied to seeds, and the removal of these compounds on the market for plant protection almost doubled the number of pesticide applications in oil seed production in England (Scott & Bilsborrow, 2019). It seems like the backside of decisions to ban some insecticides is forcing farmers to tick up machine hours on their machinery, having to reapply contact-acting insecticides in both organic and conventional farming practices. Pyrethrins are such insecticides, they are relatively safe for human health, but less effective and not specific. Spraying more frequently with fewer insecticide types is an obvious risk for amplifying resistance genes in insect populations. The other backside is that non-specific insecticides risk having severe knock-out effects on the insect communities far beyond the target pest. The farmers risk impacting those species that provide functional ecosystem services such as predation of pests and pollination.

Despite the urgent need to find novel tools to handle problematic pests, the tempo is slow and is further hindered by invasive species. Removed from the forces from their natural habitats that kept these pests in check, their new habitats offer unbridled expansion. With agricultural systems taking on the brute force of extreme weather and climate change, a transition towards sustainability is far away but has never been more urgent. What is really needed are novel tools to monitor and selectively control native and invasive pest species, while having a minimal impact on ecosystems and human health.

2.2 Insect olfaction and Chemical ecology - Developing novel tools to control pests with semiochemicals

Insect chemical ecology as a field of study centers around how insects utilize chemical signals to effectively steer their behavior and favor their survival and reproduction. Topics could include for instance plants that, under the attack of herbivorous insects, are sending chemical ‘cry for help’ signals to which natural enemies orient and ‘come to the plant’s aid’ (Dicke, 2009), or, orientation of many species of insects towards nitrogen-rich sources of food to sexually mature and produce offspring (Candia et al., 2019), or keying in to defined sets of plant volatiles in the search for a suitable host for their offspring (Tasin et al., 2010). Insect chemical ecology thus merges chemical,

sensory physiological, molecular biological, behavioral and ecological sciences to study insect behavior. It has a long track-record of mapping out the semiochemicals underlying the behavior of insects, with the start some 60 years ago marked by the identification of the first insect pheromones. Pheromones are in the most general of cases a species-specific combination of one, two or more, often elaborate semiochemicals, that are produced by either females or males to attract the other sex. They can be used to directly control pests, through mass-trapping (Oehlschlager et al., 2002), attract-and-kill, or indirectly through mating disruption (Ioriatti & Lucchi, 2016), or can be used in an integrated manner as monitoring tools for one or several pests at once (Porcel et al., 2015). They can even be employed to detect the presence of rare species and identify rare and vulnerable habitats to support nature conservation efforts (Molander et al., 2019). The focus of pheromones for use in pest control have primarily been on lepidopterans and some coleopterans (Witzgall et al., 2010), this is mainly due to biological restraints, as not all species utilize sex pheromones to attract conspecifics as clear and strong as in lepidopterans. The usage of pheromones has the advantage of being sustainable, highly selective and useful on large scales, where entire communities of growers can work together and completely shut down a pest over large areas. Techniques that utilize plants as factories for pheromones give the possibility of producing pheromones with a low environmental footprint and at sufficiently low costs to make it ultimately affordable for even small-scale farmers (Ding et al., 2014).

While most of the successful applications of pheromones are from Lepidoptera species, most species do not use pheromones in such a way that their use can be readily translated into pest control. For instance, the use of pheromones may not be effective when thousands of gravid females from the neighboring areas may migrate into the crop. In other species, such as many dipterans, the pheromones described might not be attract from a long-range and can be part of a more complex mating ritual that can even include auditory signals (Wicker-Thomas, 2007), hence leaving them less suitable for pest management purposes in the field.

The use of other semiochemicals, derived from oviposition or food hosts, have their own limitations. For instance blends of generic compounds might not be selective, as many insects might tune into the same type of compounds. For instance, acetic acid, a microbial derived compound, that alone is not very attractive but when combined with other compounds synergises: with pear ester it is attractive for *Cydia pomonella* (L.) (Knight et al., 2019; Preti et al., 2021); with 2-phenylacetaldehyde and/or 2-phenyl ethanol it is known

to be attractive for lacewings (Badra et al., 2021; Tóth et al., 2009); with linalool oxide pyranoid it is attractive to *Enarmonia formosana* (Scopoli); and a combination of 2-phenyl ethanol and pear ester is attractive for *Pandemis heparana* (Denis & Schiffermüller) and *Choristoneura rosaceana* (Knight et al., 2017; Larsson Herrera et al., 2020).

The problem with generic volatiles is that they are not only produced in the plant of importance for a single pest. They can often also be produced by many different sources, such as fungi, bacteria or other plants. A yeast lure for instance will catch unwanted species, and in worse case beneficials or pollinators. For a non-pheromonal semiochemical to work on a large scale, minimal spill-over on other non-pest species is, for sustainability, needed. There might be practical solutions if no selective lure can be found, such as designing traps that take advantage of a certain trait, such as cheap trap-bottles that utilize the behavior associated with positive phototaxis of *C. capitata* (Candia et al., 2019). These kinds of practical solutions are still overshadowed by the potential of lures that are selectively attractive to the target species, making it possible to for example manage pests using attract and kill in orchards (Klick et al., 2019).

2.3 *Lobesia botrana*: from pheromones to kairomones

The european grapevine moth, *L. botrana*, is of European origin and a severe pest in vineyards, to the concern of wine regions across the globe. Its larvae feed on grape clusters making it susceptible to *Botrytis cinerea* (Pers.) infection, which causes rotting damage during post-harvest (Ioriatti et al., 2011). It is present in Chile and Argentina in South America (Ioriatti et al., 2011). In 2009 *L. botrana* was detected in California, USA (Gilligan et al., 2011). It led to one of the few successful, outside islands, eradication programs based on widespread pheromone trap monitoring and area-wide mating disruption (Simmons et al., 2021). If farmers are able to organize across large areas, area-wide management using pheromone works. Alternatives such as spraying overwintering pupae with *Bacillus thuringiensis* (Berliner) and entomopathogenic fungi shows some promise (Altimira et al., 2021; Ifoulis & Savopoulou-Soultani, 2004), but require motivated growers. For smaller isolated farms and for those that lack the opportunity of area-wide intervention a functional non-pheromonal semiochemical lure would be very useful for both control and monitoring of females, the damaging sex. Beyond control and intervention measures non-pheromonal semiochemicals are also a valuable tool to monitor inflying

females in fields treated with pheromones.

2.4 Tephritidae from male lures to female attractants

Among the true fruit flies, Tephritidae, there are many severe pests that ovi-posit in fruits and vegetables. Here, the larvae develop inside the fruit, well protected from external threats. Several of them are polyphagous and have many alternate hosts and have a spread well beyond their center of origin; The mediterranean fruit fly, *C. capitata* has wreaked havoc in South America; the oriental fruit fly, *Bactrocera dorsalis* (Hendel) invaded Sub-Saharan Africa over the last two decades; and the melon fruit fly *Zeugodacus cucurbitae* (Coquillett) with similar invasive patterns in Africa and the Pacific region. While these species originate in tropical and sub-tropical climates, there are tephritid pests of the genus *Rhagoletis* that thrive in temperate regions. Some of these are indigenously present in Europe (eg. *R. cerasi* [L.] and *R. batava* [Hering]), while others constitute serious invasive threats such as the North American species, *R. pomonella* (Walsh) that attack apples and *R. mendax* (Curran) with blueberries as hosts.

Tephritid chemical ecology has perhaps been most well known for a group of compounds that attract males and can be used in male annihilation (Vargas et al., 2010). These compounds, of which the origins of attraction are obscure, are called parapheromones. These compounds are extremely attractive to male flies, with methyl eugenol for *B. dorsalis*, cue lure/raspberry ketone for *Z. cucurbitae* readily deployed as pest control. They are excellent lures in integrated pest management systems, but only when deployed on larger areas. Because in smallholder settings, the dispersal rate of the flies is many times the size of small scale orchards (Biasazin et al., 2021), they require other techniques such as those that augment parasitoids in cropping systems (Deguine et al., 2011), and integration with more generic lures such as protein baits and sanitation techniques (Piñero et al., 2009). There are examples of pheromones that have been used in the field, such as olean for *Bactrocera oleae* (Rossi), but they are not efficient enough for full control and are often complemented with other attractants (Broumas et al., 2002).

With the lack of available tools to control Tephritidae, there has been an increasing focus on understanding what host volatiles are detected by the olfactory system (Biasazin et al., 2014; Cha et al., 2017; Malo et al., 2005; Siderhurst & Jang, 2006; Siderhurst & Jang, 2010; Zhang et al., 1999). At the core of these efforts is the goal of designing a lure that is attractive for

females, which would be a valuable tool in pest management. Despite a number of such studies, none of the studies have managed to be translated into a lure that has been deployed in pest management.

2.5 Understanding the sense of smell using GC-EAD to deliver novel lures

While thus there have been many efforts to create non-pheromonal lures for use in monitoring and control of pest insects, progress is slow. One of the reasons is that much of these efforts are singular and ill-connected to each other. There is a lack of fundamental understanding on how insect olfaction is shaped through evolution and ecology. This despite considerable efforts to develop tools for mapping out the neural response pathway from detection of ligands by olfactory receptors, to the interpretation in the higher brain. While the 'interpretation of the code' may not be very accessible to study outside model organisms (Seki et al., 2017), the basic input that determines what is ultimately attractive, is already present in the peripheral organs. For instance, the relative number of sensory neurons, their relative sensitivity and their relative axonal diameter appears correlated to 'attractiveness' (Dekker et al., 2006; Dekker et al., 2015; Kárpáti et al., 2008; Koutroumpa et al., 2014). These external correlates of attractiveness are readily accessible using peripheral recordings from the organs that detect odors, the antennae and the maxillary palps. Thus by studying the sensitivity of peripheral organs to odors, and shifts therein in comparison with other species, important hints toward the significance of the odors in attraction can be surfaced.

A decades-old technique that has been used for studying peripheral sensitivities is coupled gas chromatography - electroantennographic detection, or short GC-EAD. A historical survey of over 1000 articles on GC-EAD shows that after a steady flow of studies on insect pheromones, many studies have been focusing on non-pheromonal semiochemicals (see figure. 1). Despite a later onset of studies focusing on non-pheromonal semiochemicals, the rate of GC-EAD studies investigating pheromones was similar to that of non-pheromone papers. However, in the last 10 years the rate of new papers focusing on pheromone is declining, while the number of non-pheromone papers are steadily increasing.

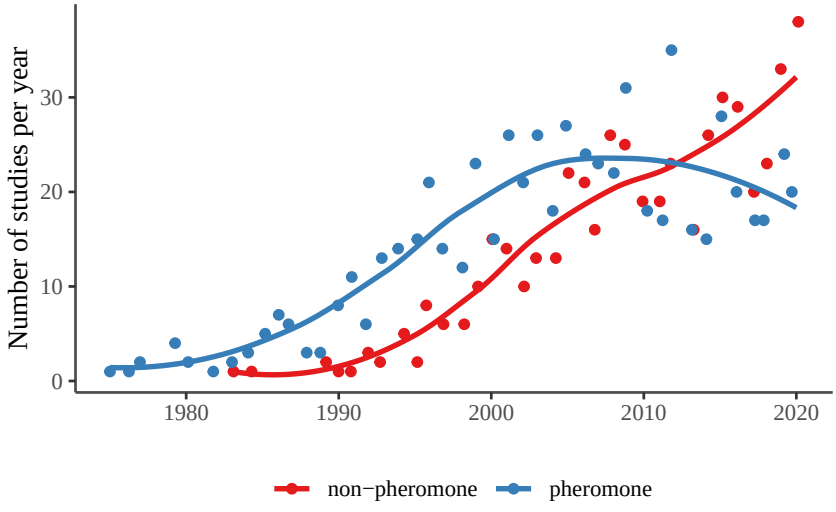

Figure 1: Number of studies have been published that have used GC-EAD to either investigate pheromones, in blue, or non-pheromonal semiochemicals in red. Dots, with a little bit of horizontal jitter to avoid overlap, are the total number of studies for each year and the line is a fitted local regression using the data underlying the points.

In the published literature, much of the research focus has been on families that contain prominent pest species, such as: the bark beetles *Ips typographus* (L.) and *Dendroctonus frontalis* (Zimmerman) (Curculionidae:Coleoptera); Tortricidae (Lepidoptera) a family that contains several problematic pests such as *L. botrana*, the apple pest *C. pomonella* and the oriental fruit moth *Grapholita molesta*; and also Tephritidae. Most of the studies on non-pheromonal semiochemicals unfortunately lack any type of standardization, only around 8 of the articles provide millivolt data to the electrophysiological responses. Many of these studies also lack proper chemical annotation, with no retention indices and questionable identification. In contrast, if the data would have been annotated according to a standard, a comparative database of olfactory responses across several hundreds of insect species would have been available. Such a database would not only allow for understanding how olfaction has been shaped by evolution and ecology but would also permit generating many leads for novel lures.

2.6 Concerted efforts - DoOR and pherobase

There have been only two concentrated efforts to provide searchable databases for compounds that are relevant for insects, one is the DoOR database (Galizia et al., 2010; Münch & Galizia, 2016), a database that is publicly available as an R package as well as on their homepage. It has gathered published data on the electrophysiological responses in *Drosophila melanogaster* (Meigen), the model species par excellence, on a receptor/neuron level. One of the main caveats of the DoOR database is that the input data is based on single sensilla recordings (SSR) without the use of a GC, and often at ecological irrelevant high concentrations. The other drawback is that it only contains a single species making it hard to refer to similarities in binding affinities with receptors found outside its closest phylogenetic relatives. The other considerable effort that has been used to map out compounds of ecological relevance for insects, beyond electrophysiological active ones, is pherobase: a private run database that is only available to users by a web interface. Data from this database is, however, not designed for analysis outside the webpage, and the database is not publicly available.

3. Aim and objectives

The overarching aim of this thesis was to accelerate the development of sustainable and specific-selective lures for use in the monitoring and control of pests of fruits.

The first objective was to construct a species-selective lure for *L. botrana* from volatiles derived from attractive sources. The lure was based on volatiles from microbially infestes grapes, and throughout field trials continuously improved in terms of attractiveness and specificity.

The second objective was to construct a comparative database of olfactory responses across fruit flies, using diverse odor sources, including fruits and fermentation sources, and use the data to extract meaningful evolutionary-ecological analysis and compounds of interest for behavioral trials.

4. Main approaches and Methods

In this section, the approaches of gathering and analyzing data will be presented. It will not describe the fine methodological details, which can be found in the articles, appended as separate chapters to this thesis. Below two workflows are highlighted, estimating specificity of lures used in the *L. botrana* studies, and the olfactomics workflow used in comparing the olfactory sensitivities of tephritid.

4.1 Field trial - investigating specificity

Field trials with the goal of developing species-selective lure for *L. botrana* was key in paper II. The trial consisted of a randomized line of traps with different lures (treatment), and investigated the attractiveness of the lures, as well as the specificity of the lure. For calculating the specificity of the lure, to which taxonomic level of field collections needed to be identified is relevant, sorting field-caught insects is labor intensive. To speed up the process the sticky bottoms were compared to photographs of previously caught and identified insects. This approach can be limited in the taxonomic resolution that can be reached. For example lacewings were only identified to *Chrysoperla*, in total 25 taxonomic groups were assigned (see article II). Although some measures of specificity are dependent on the taxonomic resolution, all treatments received similar resolution and were thus comparable.

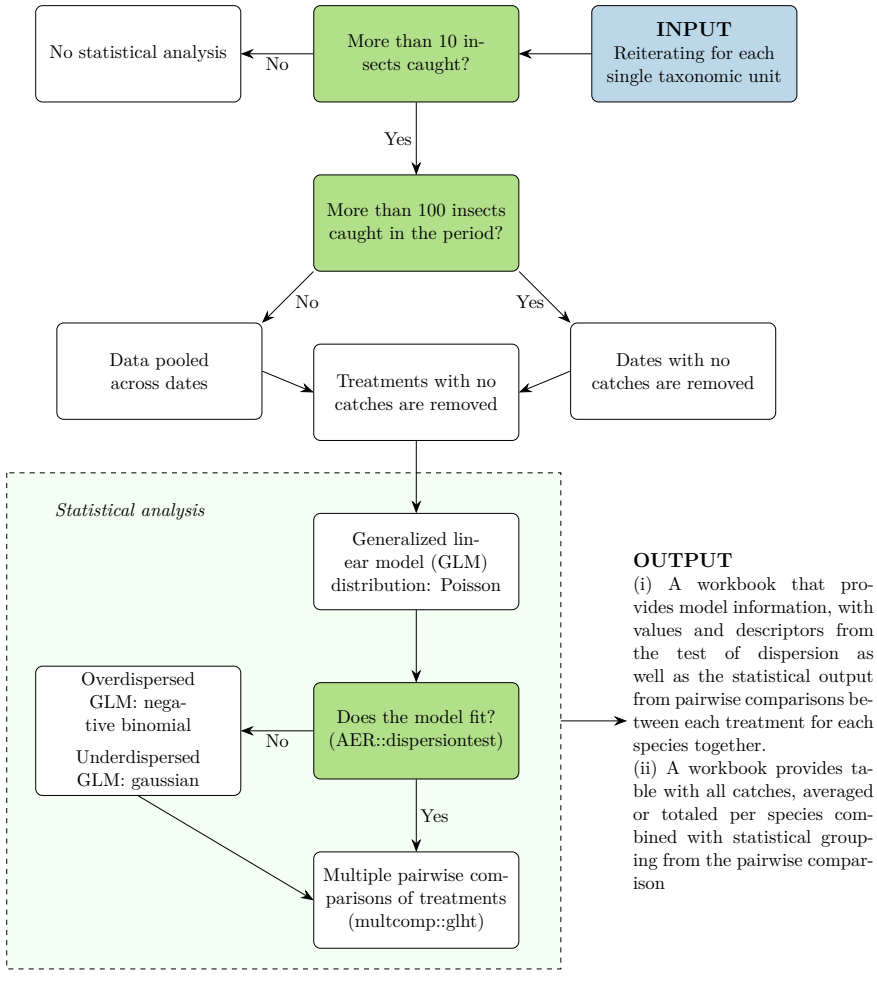

Figure 2: The statistical workflow used to analyze field data. The idea is to use a number of rules (in green), that guides the workflow. The input is the field catches of one species or taxonomic group, outputted from a reiterating loop. The first rule sets the limit to any kind of statistical analysis at all. The second rule determines if the data should be grouped or pooled across dates. Data groups that have zero variation, e.g. no catches, are removed from the analysis. The model is first fitted with a generalized linear model with a poisson data distribution. Then the model is tested for under/overdispersion using the package AER, and if underdispersed is fitted with a gaussian distribution, and if overdispersed it is fitted with a negative binomial.

The data was read into R for analysis, with a dedicated template. A workflow (fig. 2) had to be created to be able to analyze the catches of target and non target species. It followed the following set of rules; (1) If less than 10 insects were caught across all treatments, no stats was performed, (2) If the number of insects caught for a species was less than 100 in each fight period, the catches were pooled across dates, (3) for species with more than 100 catches, dates with no insect of a given species in any of the treatments were filtered out. Treatments with no catches were omitted from the analysis, and data were subsequently fitted to a Poisson generalized linear model (glm) and tested for overdispersion. If the data were significantly overdispersed the Poisson model was replaced by the corresponding negative binomial. This allowed for comparing all treatments for all species, providing valuable information on generic volatiles and what other insect species that can be caught with the compounds.

4.2 Olfactomics combining analytical chemistry with electrophysiology across studies

In this section the workflow depicted in figure 3 will be further explained. Gas chromatography or GC is simply a technique of separating molecules depending on their size or chemical configuration. On the input side of the GC is a heated inlet that evaporates whatever is injected. In article I and V, volatiles were sampled using a solid-phase-microextraction (SPME) fiber. These fibers' coating absorb volatile compounds, which are thermally desorbed when heated in the inlet of a GC. In article III and VI, samples were instead collected with a filter connected to air pumps. The filter consists of an absorbent filled PTFE tubing through which air from the sample of interest passes. Absorbed volatiles are then extracted using a solvent, such as hexane, see also; Biasazin et al., 2018. The advantage of using a solvent-based method is repeatability allowing for multiple injections with identical composition. The downside is the need of a solvent, which creates large solvent peaks that can blur the presence and hamper identification of more volatile compounds that elute at the same time as the solvent.

On a GC-MS, the GC is connected to a mass-spectrometer (MS), an analytical machine that ionizes the injected compounds and produces mass spectra of their ions. These mass spectra are then used for identification purposes by comparing them against a spectral library. This is done by an algorithm, often packaged in software such as Masshunter.

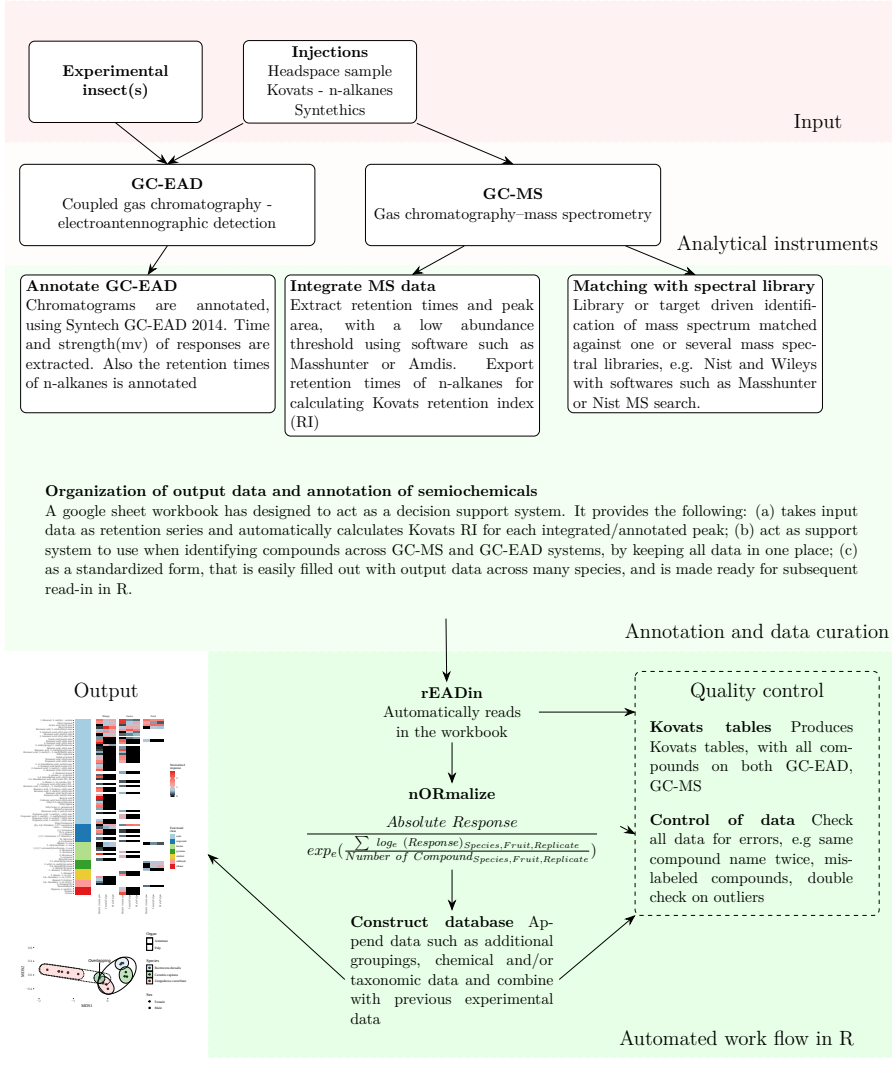

Figure 3: A schematic, simplified version of the major steps in the workflow of the current olfactomics pipeline that is used to analyze, annotate and assemble databases for analysis. The underlying factors determining sampling, setting up analytical machinery are not shown. Particularly useful is the google sheet that provides a sharable template for annotating volatiles and the corresponding olfactory answer. Bottom green box: functions executed by R (left), with the intermediate alternative to produce Kovats tables and perform quality controls of the data (right). There are several different outputs that can be had, of which the depicted heatmap and a NMDS analysis are examples.

GC-EAD analyses combine analytical chemistry with insect sensory physiology. In a GC-EAD half of the GC effluent goes to a flame ionization detector and produces a chromatogram. The other half of the effluent flows over an insect olfactory organ, connected between a recording electrode, typically placed on the tip of the sensory organ, and a reference electrode attached to the head. When a compound that is electrophysiological active elutes this can be seen as co-occurring depolarization and a FID peak.

Annotation of chemicals in the headspaces was simplified by a standardized Google sheet. The idea with such a sheet is to organize all data, in a way that is easily translatable from both the GC-EAD and GC-MS output into one sheet. It also allows automatic calculation of Kovats RI of all peaks, and is to be used as support throughout the annotation process helping to compare the chromatograms from the GC-EAD and GC-MS with each other.

Kovats retention indices are used in support of the library-driven identification of mass spectra. A custom built tool, a kovats extractor allowed for scraping the homepage of Nist, and giving the highest, lowest and mean of reported kovats for the column of interests. By producing boxplots of the output it is easy to see the spread of values and if the kovats of a tentatively ID:ed compound fit or not.

After the initial annotation has been done, the data is imported into a data analysis workflow (package is in preparation) that is written in R. The processes in the workflow are to a large degree automated, making it relatively easy to analyze and organize headspace and electrophysiological data. The user needs to look at the quality control output; printed tables for each compound with each calculated retention index; checks the CAS/INCHI identifiers for duplicates; and retention indices that are outliers either to published or to other samples/recordings are double checked in the input. Before analysis and visualization, data can be appended with descriptors e.g treatment grouping, chemical grouping and renaming, taxonomic info or other relevant information. At this stage the data can also be combined with earlier data output to form larger datasets or databases.

In the articles (I, III, VI, V) multidimensional reduction techniques such as PCA and NMDS were useful to visualize data groupings. Additionally, data can also be collapsed into dendrograms, an intuitive way of comparing electrophysiological data with transcriptome data.

All analyses were done in R (R Core Team, 2020), using the ‘tidyverse’ (Wickham et al., 2019) for data manipulation and visualization. Examples of packages used are vegan for dissimilarity indices and nmds (Oksanen et al.,

2019), AER for testing for overdispersion (Kleiber & Zeileis, 2008), MASS for negative binomial (Venables & Ripley, 2002) and emmans or multcomp for pairwise testing (Hothorn et al., 2015).

5. Main results and discussions

5.1 Using generic plant-microbe volatiles to design species specific lures for *Lobesia botrana* (Paper I & II)

The headspace of inoculated grapes was collected through SPME. The grapes were inoculated with *Botrytis cinerea* (Pers.), a pathogenic fungi; a combination of five different yeasts; a solution of two "sour rot" bacteria; and these three in all possible combinations (for detailed information see paper I). Although compounds were mapped, it was hard to correlate composition with attraction. A number of compounds were emitted irrespective of the treatment on the grapes. Three compounds, i.e. ethanol, 3-methyl-1-butanol and ethyl acetate were emitted by all treatments and at the highest amount. Therefore, we named them as the major fermentation volatiles (Fig 4A). The same grapes that had undergone the different inoculation treatments were used in oviposition tests. Here the number of eggs that *L. botrana* females laid in a two choice egg laying assay was counted. The egg-laying female got to choose between either a "sterile" grape, the control, or an inoculated grape and the results were compared to the oviposition rate in a control-control experiment. Surprisingly, all but one of the treatments led to lowered oviposition rates compared to control, first when a grape was inoculated with a combination of all the three inoculates, that the egg-laying increased, although not significantly (Figure 4B).

Field trials, using sticky traps, were performed in Chile to test the attractiveness of compounds identified in the grape headspace to the European grapevine moth. Unfortunately, the samples shared too many compounds and no clear associations between the chemical analysis from the

SPME-GC-MS and the oviposition behavior could be found. It seemed reasonable to assume that the most abundant, major fermentation volatiles, could be worth testing in combination with acetic acid (AA) and 2-phenyl ethanol; both present in the headspace analysis and known to in combination attract several insects (Badra et al., 2021; Giacomuzzi et al., 2016; Knight et al., 2017; Larsson Herrera et al., 2020). While the major fermentation compounds caught more than acetic acid and 2-phenyl ethanol alone, the combination of acetic acid and 2-phenyl ethanol synergised and caught more, whereas the major fermentation compounds didn't change the attractiveness of the lure (Figure 4A).

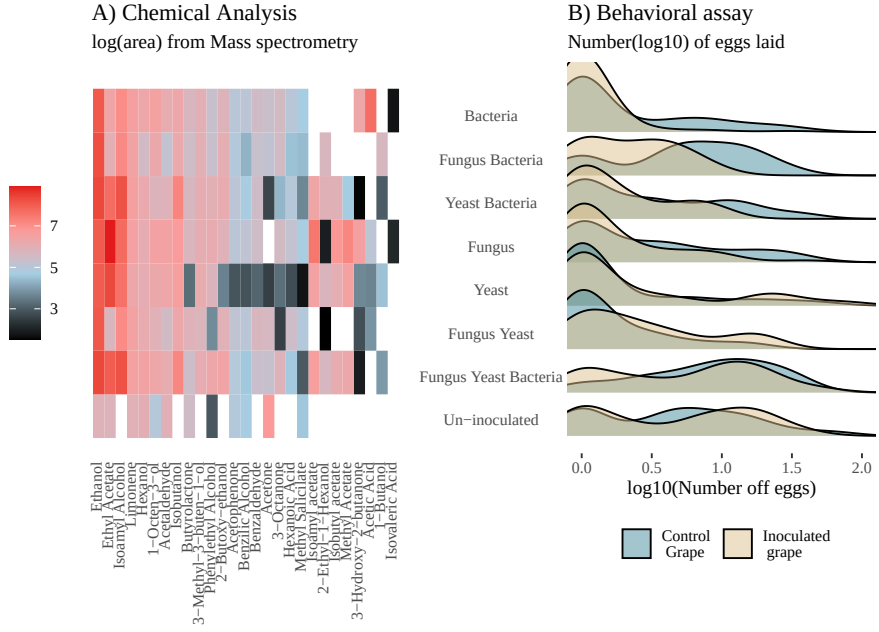

Figure 4: From paper I. The heatmap (A) on the left side shows SPME sampled headspace of the grapes inoculated with different microorganisms. The joy plot (B) on the right shows: Density distribution of *L. botrana* egg in a laboratory dual-choice experiment with uninoculated or microorganism inoculated grapes. The area delimited by each ridgeline is equal to 1.

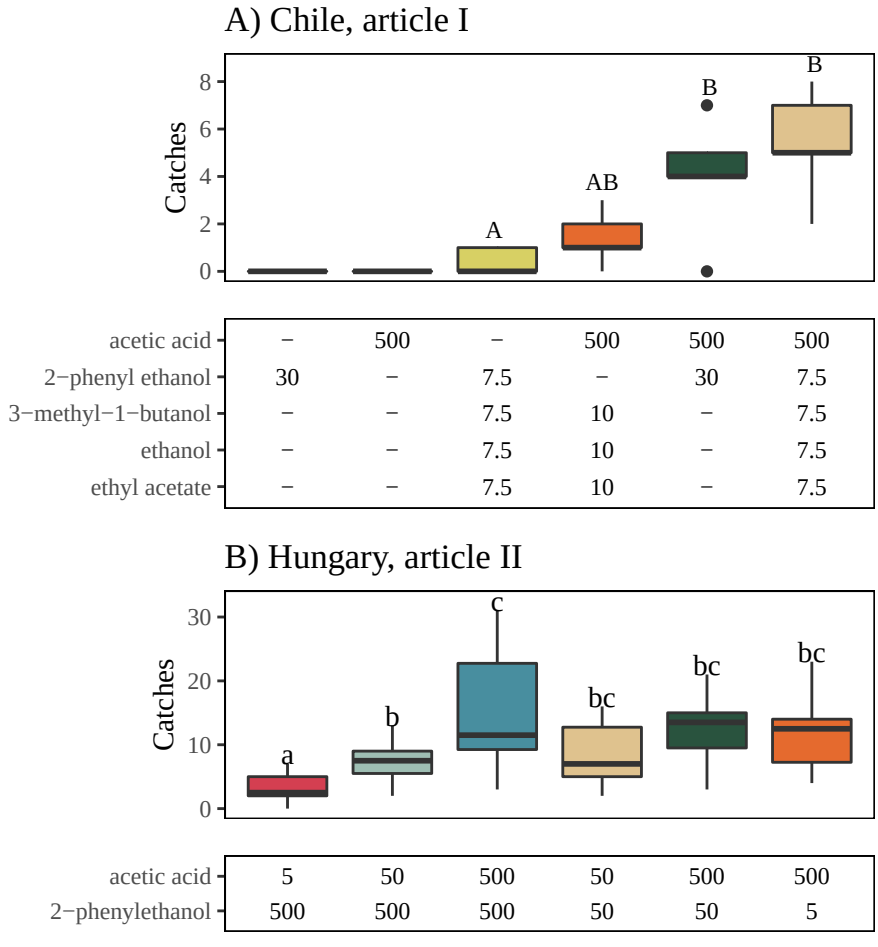

Figure 5: The boxplot shows how many females, of *Lobesia botrana*, were caught in A) Chile in 2017 (paper I); and b) Hungary in 2018 (paper II). On the bottom part of the graphs are the quantities in mg, for each compound that were used to construct the lures. Blank caught no insects during either trial.

In a larger field trial in Hungary, the base lure of AA and 2-phenyl ethanol was tested in combinations with other sets of compounds and at different ratios. Both catches of *L. botrana* and other non-targeted insects were counted. Adding a large blend of microbial volatiles improved the lure, but caught many insects of several other species (see article). Interestingly, the lure was equally attractive to *L. botrana* independently of the amount of 2-phenyl ethanol added to AA. Instead lowering the AA load led to a decrease in attraction (Figure 5B). High amounts of 2-phenyl ethanol in

combination with AA attracted lacewings (*Chrysoperla*), however, the lowest amount of 2-phenyl ethanol still attracted *L. botrana* with no lacewings showing an impressive 97% specificity in the third flight (Figure 6).

For sustainability reasons the best lure is not always the good lure.

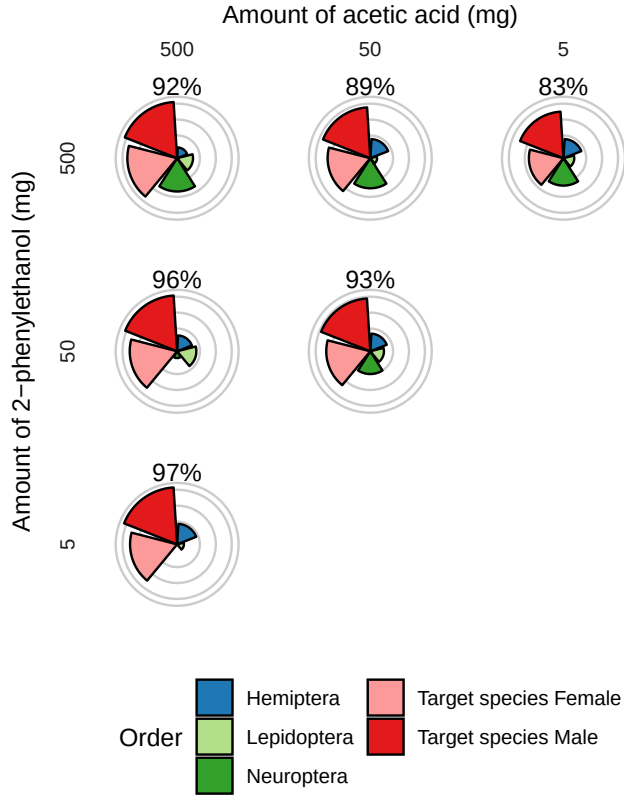

Figure 6: From paper II. Total captures (square root transformed) of *Lobesia botrana* (target species) and other species during the third flight in traps baited with different loads of AA and 2-PET. Concentric lines indicate 10, 50, 250 and 500 insects caught. The percentage of target species caught is indicated at the top of each radial plot.

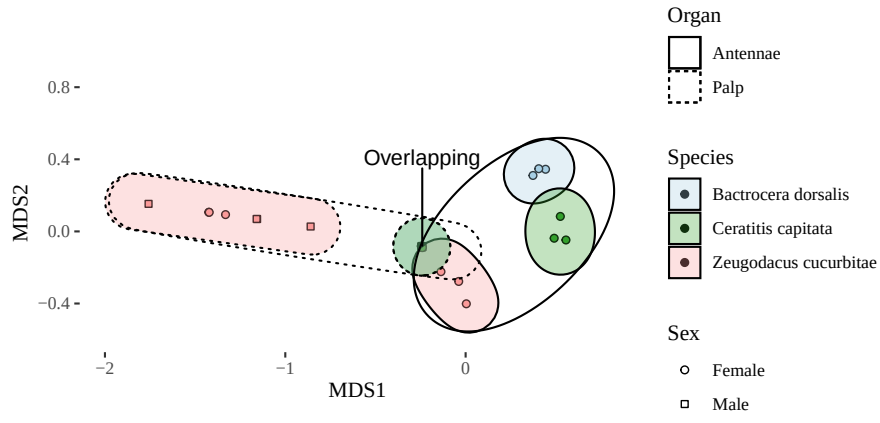

Figure 7: From paper V, supplemental material. A non-metric multidimensional scaling (NMDS), with filled colors representing the different species (*B. dorsalis*, *C. capitata* and *Z. cucurbitae*) used in the analysis. Filled and dashed lines represent the antennae and the palps, respectively. While the recordings on the antennae were only done on females, recordings in the palps were done in both males (square dots) and females (circles). No differences in sensitivity were observed between sexes.

5.2 Constructing olfactomics databases mapping the sense of smell in Tephritidae (Paper III, IV and V)

In paper III, IV anv V we created fruit odor volatilomes (database of volatiles from substrates) and fruit fly olfactomes (database of olfactory responses). These were mined to find patterns of volatile release and sensitivities related to the ecology or phylogeny of species, and used to rationally design attractants for use in pest control.

The antenna detected several times more fruit volatiles than the palps, while the palps of *B. dorsalis* detected a similar number of food related volatiles as the antennae (Figure 7 & 8). It seems like the antennae is tuned to fruit odors, while the palp is tuned to food related volatiles. In addition, the palps uniquely detected several compounds. That palps detect a unique subset of compounds is further supported by a number of recent studies showing that pheromones and male lures are detected by the palps (Noushini et al., 2020; Park et al., 2018; Verschut et al., 2018). This is despite the fact that the palps have a projected six different receptor neurons types.

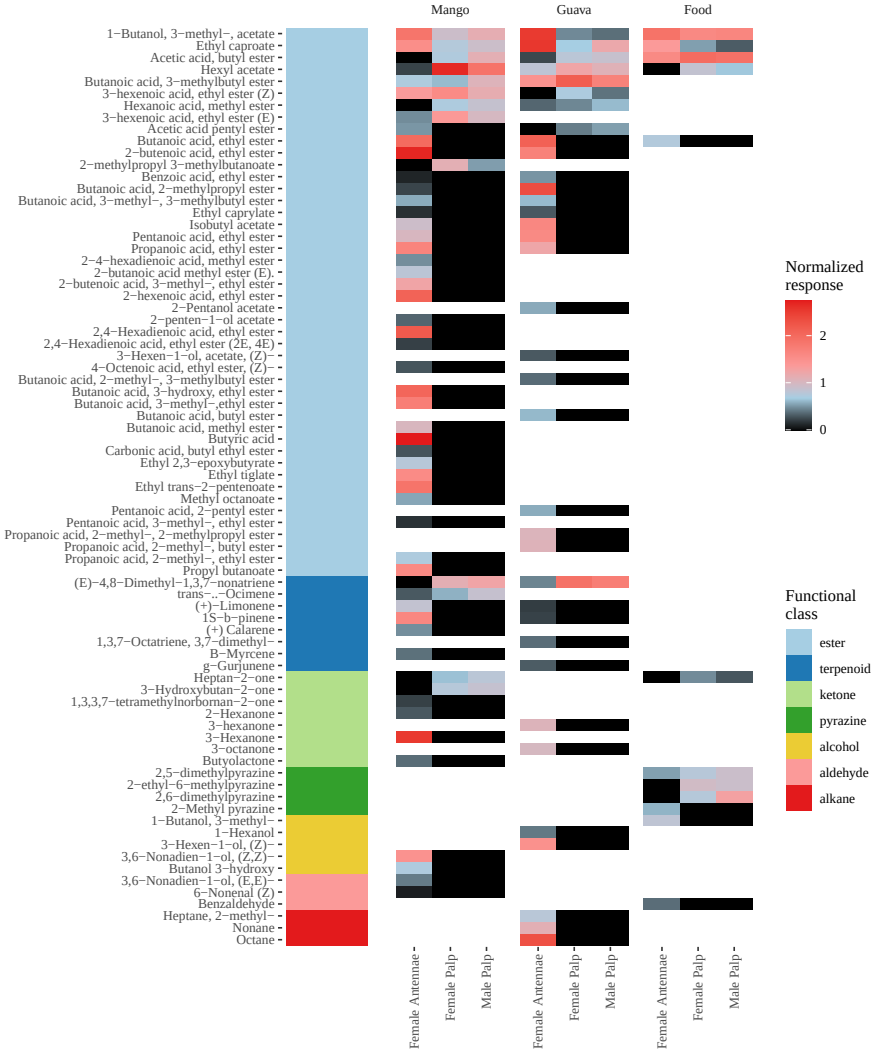

Figure 8: From paper IV. A heatmap depicting antennal and palpal responses using fruit (guava and mango) and food headspace extracts (yeast and protein baits). From left to right: name of the compound identified, functional classes they belong to, responses from female antenna, and female and male palps of *B. dorsalis*. The compounds are organized according to functional class and within these in decreasing order of detection frequency. The response strength from 0 (black) to 3 dark red is first normalized within a run, before being averaged across runs. The strength of a response is therefore always relative to the average responsiveness of the organ to all compounds within the same column. Color intensity coding can therefore only be compared within a column and not across and do not represent absolute values.

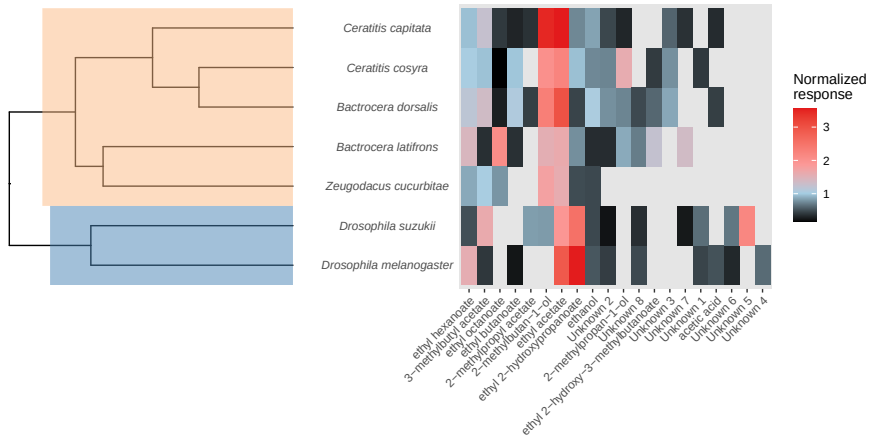

Figure 9: From article V. Right: a heatmap over 20 compounds eliciting a response in five species of Tephritidae (*Ceratitis capitata*, *Ceratitis cosyra*, *Bactrocera dorsalis*, *Bactrocera latifrons* and *Zeugodacus cucurbitae*) and two species of *Drosophila* (*D. suzukii* and *D. melanogaster*). The name of the compounds can be found on the lower axis including eight compounds that were minor and could not be identified with certainty. The heatmap reflects normalized responses ranging from low values (black) to high (red). Left: a dendrogram from normalized responses in the heatmap and using a NMDS model fitted with Jaccard dissimilarity index. Tephritidae are in an orange background and drosophilids in blue.

Already in the first article on tephritidae (paper III), when comparing responses across evolutionary divides it was clear that the electrophysiological response patterns lacked a clear phylogenetic signal. Instead, by collapsing response data using dissimilarity indices and multidimensional reduction (PCA, NMDS, see figure 9) across both organs, we found that ecological niche is a more determinant factor than phylogenetic distances (papers III, IV and V). It seems that the olfactory sensitivities are convergent for species with similar ecological niches, in spite of phylogenetic distance. In figure 9, this is clearly visualized, were the polyphagous *B. dorsalis* groups with the two species of *Ceratitis*, and the species that prefers solanaceous plants, *B. latifrons*, grouped closer to the cucurbit specialist *Z. cucurbitae*. The two species of *Drosophila* grouped by themselves.

We utilized data gathered from the first paper (paper III) as a baseline for the database, and for each additional research question the data generated was appended to this dataset. This provided an unique comparative approach, being able to further understand the sense of smell in Tephritidae by building

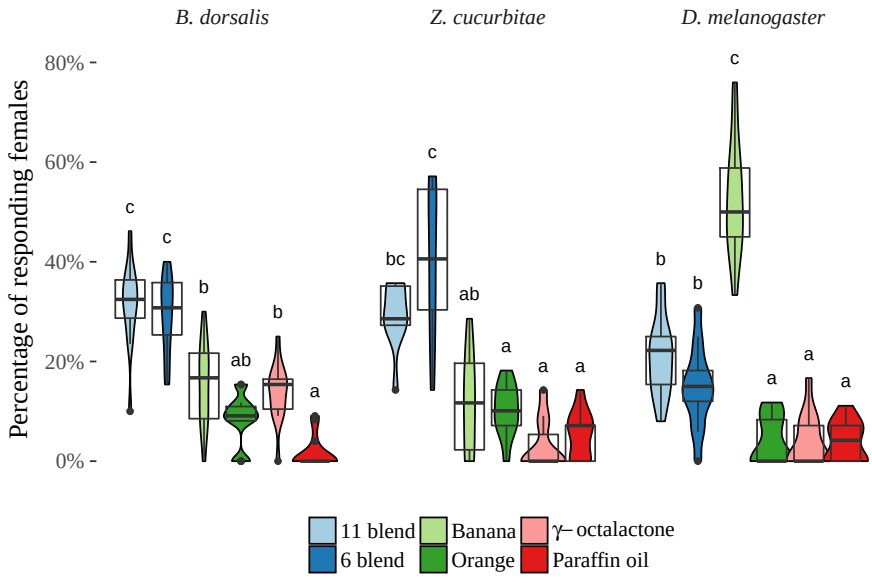

Figure 10: From paper III. Captures of *Bactrocera dorsalis*, *Zeugodacus cucurbitae* and *Drosophila melanogaster* in a six-choice olfactometer assay after 30 min assay. Treatments in each test included the 11-component blend (shared across all flies and 3 fruits, each of the blends in mango headspace ratios), the 6-component blend (shared across all flies and fruits), orange, banana, c-octalactone and paraffin oil. Graphs are plotted using a combination of boxplot and violin plot. The box plot indicates the interquartile ranges, whereas the violin plot indicates the distribution of the data.

on previous knowledge. In paper III, a set of six compounds were found to be shared across fruits and detected across fruit flies. The level of sharedness of volatiles between fruits dramatically increased the chance of detection in flies. These shared compounds could provide an attractive bridge between fruits, explaining part of the polyphagous behavior of fruit flies. Behavioral trials on *Z. cucurbitae*, *B. dorsalis* and *D. melanogaster* indeed supported this idea, with an 11 blend (shared across fruits, mango, guava and orange) and a six blend, a subset of the 11 compounds that was also found in banana, being more attractive to *Z. cucurbitae* (the eleven blend was not significantly more attractive than banana) and *B. dorsalis* but not for *D. melanogaster* (figure 10).

In article V, data from electrophysiological recordings using volatiles from chicha, an attractive fermentation based lure for *C. capitata* (Candia et al., 2019), were appended to the existing database of fruit-odor responses. Out of

71 compounds detected in only one of the fruit species, only one was present in chicha. In contrast, out of the eleven compounds that were shared across fruits, five of these electrophysiological active compounds were also present in chicha. The hypothesis is that these compounds form a bridge from the ancestral saprophytic behavior in Tephritidae to derived frugivory. We also screened these compounds for behavior and hypothesized that an equal mix, in 1:1 ratio, of these compounds would be more attractive than mimicking their amount from chicha. Indeed, these six compounds at a one:one ratio were almost as attractive as chicha.

Further details into the receptors underlying olfactory responses was given by sequencing antennae and annotating olfactory receptors. Phylogenetic analysis of the receptors gave 30 quadruplets, across *B. dorsalis*, *B. latifrons*, *Z. cucurbitae* and *C. capitata*. These quadruplets were orthologous to each other. Out of the other orthologous groups, where quadruplets across all species could not be found, it was most of the time, with very few exceptions, the sequence for *C. capitata* that was missing. Despite the divergent responses depending on their ecology, the sequences strictly seem to follow phylogeny (Virgilio et al., 2015), see figure 11, making it hard to deduce which are the likely receptors underlying the conserved responses to shared chicha and fruit volatiles. Constructing a tree of olfactory receptors across Tephritidae and *Drosophila* and superimposing responses from the doOR database (Münch & Galizia, 2016) for *D. melanogaster* revealed that the compounds that links saprophily to frugivory, are detected by a wide range of receptors in *D. melanogaster*. If this would be true also for Tephritidae, single receptors may perhaps evolve sensitivity to compounds in their new ecological niche, while as a whole the fruit fly still retains the ability to detect compounds that form preadaptive bridges between substrates from the cohort of receptors that detects these.

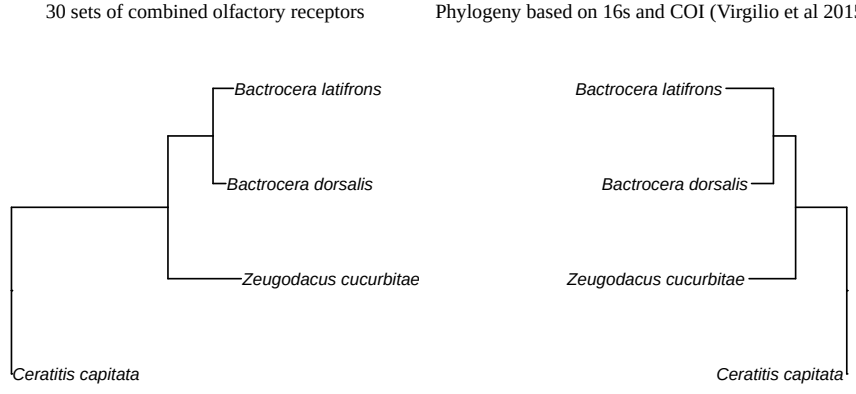

Figure 11: From paper V. On the left; a tree based on concatenation of 30 olfactory receptors quadruplets into a single tree for four Tephritidae species (*C. capitata*, *B. dorsalis*, *B. latifrons* and *Z. cucurbitae*). On the right a tree for the same species are plotted using phylogenetic data concatenated for 16s and COI on a subset of data from (Virgilio et al., 2015)

6. Concluding remarks and perspectives

It is difficult to find non-pheromonal semiochemicals that are effective in pest management. This is because most volatiles associated with hosts are diverse and ubiquitous, which is matched by a highly multidimensional olfactory circuitry on the insect side. It thus comes down to which of the volatiles are important and in which ratios they are needed to induce attraction, which is fundamentally still a puzzle. The lure developed for *L. botrana*, shows that attractive and selective lures can be developed even using generic volatiles from microbial activity. However, this required testing and finetuning of the ratios in the blend but also extensive identification of bycatches. Unfortunately, species besides the target are seldomly, if ever reported, whereas it is from a sustainability perspective pivotal to record such bycatches to determine the selectivity of a lure, and hence minimize the spillover effect on ecosystem functions. An additional perk of reporting such information is generating leads beyond single studies, this could help in designing lures for species that may be pests in other cropping systems, or shed light on which combinations should be avoided. A step forward to simplify the acquisition of such data, could for example be the use of intelligent traps that can either by photography or wingbeat frequencies determine what insect species are caught Chen et al., 2014; Lima et al., 2020. Such monitoring tools would also allow for larger field trials screening many combinations of semiochemicals, while minimizing the labor needed to identify all species caught.

To develop lures for a species, there are a number of techniques that can be used, such as taking fractions of headspace samples to detect attractive compounds and using chemometrics to evaluate differences between samples with different attractivity. However, most studies focus on a single

species at the time, often with non-standardized approaches. Although this can provide valuable insights, such data is generally incomparable across studies. Yet, we demonstrate that comparative approaches are highly informative and can readily lead to lure development. Therefore we propose olfactomics, a generalized framework for collecting chemical and electrophysiological data, that permits data to be appended to a database. Such comparable data across studies and species accelerates the understanding of the sense of smell across insect species in an evolutionary-ecological context, and provides leads for behavioral work and identification of attractants, even through an entirely in-silico approach. In addition, when the database is populated with a sufficient number of data points, it could also assist in validating, or correcting mis-identified responses to compounds across studies. Such an olfactomics framework can also support fundamental research on insect olfactory receptors; it can assist in deorphanization studies providing rational leads for ligands of olfactory receptors in non-model species.

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Popular science summary

Insects are essential for life on earth as we know it. They are integrated in food-chains, and provide invaluable ecosystem services, such as pollination, biological control and much more. But some of them have plagued humans since before the advent of agriculture. Today's monocultural agriculture is heavily mechanized and relies on agrochemicals, such as insecticides to handle problematic pests. This, together with human caused problems such as habitat loss and climate change, is the source of an unprecedented loss of insect biodiversity across the globe. Ironically, farmers thus risk eroding the ecosystem services that are much needed, whilst targeting the pest damaging their crop. Novel tools are urgently needed that harmonize ecosystem health with pest control.

One such species is the European grapevine moth, *Lobesia botrana*, a severe pest on grapes that is spreading around the globe. In this thesis a lure is developed from generic microbial volatiles focusing on sustainability by not only analyzing the catches of the target species, but also that of other species. Our results show that the best lure for the pest are not necessarily the most sustainable ones. However, by changing the composition and ratio, the selectivity and thus sustainability of the lure increased.

The other major focus of the thesis is to improve the flow of information from basic research to the development of novel lures using a family of problematic pests, the Tephritidae, as models. A novel framework, olfactomics, based on electrophysiological responses in the olfactory organs, the antennae and palps, was designed. This framework permits for the extraction and analysis of data across many species and odor sources, and mine the resulting database for evolutionary ecological analyses, as well as for finding new behaviorally attractive volatile blends. Using olfactomics, a set of compounds were found that was shared across fruits and several Tephritidae, explaining in part how flies can utilize many different fruits as hosts. A subset of these compounds also linked the attractiveness of fruits, to ancestral traits of feeding on fermenting resources.

Taken together, the thesis highlights the opportunities that do exist in developing sustainable odor-based alternatives to control pests. To churn out such innovations at the rate needed, concerted research efforts are a must.

Populärvetenskaplig sammanfattning

Insekter är nödvändiga för livet på jorden såsom vi känner till den. De är integrerade i näringskedjor och ger ovärderliga ekosystemtjänster såsom pollinering, biologisk kontroll och mycket mer. Men några av dessa har varit problematiska för människan sedan innan jordbruket startade. Dagens monokulturella jordbruk är tungt mekaniserat och beroende av agrokemikalier, såsom användandet av insekticider, för att hantera problematiska skadeinsekter. Detta tillsammans med problem orsakade av människan såsom habitatförlust och klimatförändringar är källan till en aldrig tidigare skådad förlust av insektsbiodiversitet över hela jorden. Ironiskt nog riskerar bönder att erodera de ekosystemtjänsterna de så väl behöver, när de kontrollerar skadeinsekterna som förstör deras grödor.

En sådan art är vinskottvecklaren, *Lobesia botrana*, en allvarlig skadegörare på druvor som sprider sig jorden runt. I den första delen av denna avhandling utvecklades lockbeten baserat på generiska ämnen från druvor infekterade med mikroorganismer. Detta med ett fokus på hållbarhet, genom att inte bara analysera fångsten av skadeinsekten, utan också av andra insektsarter. Resultat visar att det bästa lockbetet för skadeinsekten inte nödvändigtvis är det mest hållbara. Däremeot genom att ändra komposition och förhållande mellan ämnen, så ökade selektiviteten och således hållbarheten av lockbetet.

Det andra huvudsakliga fokuset av denna avhandling är att förbättra informationsflödet från grundläggande forskning till utvecklandet av nya lockbeten med hjälp av en problematisk grupp av skadegörare, borrflugorna, som modell. Ett nytt ramverk, olfaktomik, som baseras på elektrofysiologiska responser i doftorganen, antenn och palper, utvecklades. Detta ramverk tillåter extraktion och analys av data för många arter och doftkällor, samt utvinning av information från den konstruerade databasen för evolutionär-ekologisk analys, men tillåter även att hitta nya attraktiva doftblandningar. Genom att använda olfaktomik, kunde en grupp ämnen identifieras som delades mellan frukter och flera borrflugor, vilket till en del kan förklara hur flugor kan använda många frukter som värdar. En undergrupp av dessa ämnen länkade också attraktiviteten hos frukter, till dess förfäders preferens för fermenterad mat.

Allt sammanslaget, så påvisar denna avhandling de möjligheter som finns i att utveckla hållbara doftbaserade alternativ för att kontrollera skadeinsekter. För att kunna nå ut med sådana innovationer i den hastighet som behövs så måste forskare arbeta mot gemensamma mål.

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I

Paper I - Volatiles of Grape Inoculated with Microorganisms: Modulation of Grapevine Moth Oviposition and Field Attraction

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PLANT MICROBE INTERACTIONS

Volatiles of Grape Inoculated with Microorganisms: Modulation of Grapevine Moth Oviposition and Field Attraction

Marco Tasin[1,2] · Sebastian Larsson Herrera[1] · Alan L. Knight[3] · Wilson Barros-Parada[4,5] · Eduardo Fuentes Contreras[4] · Ilaria Pertot[2,6]

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Abstract

Semiochemicals released by plant-microbe associations are used by herbivorous insects to access and evaluate food resources and oviposition sites. Adult insects may utilize microbial-derived nutrients to prolong their lifespan, promote egg development, and offer a high nutritional substrate to their offspring. Here, we examined the behavioral role of semiochemicals from grape-microbe interactions on oviposition and field attraction of the grapevine moth *Lobesia botrana* (Denis & Schiffermüller). The volatile constituents released by grape inoculated with yeasts (*Hanseniaspora uvarum* (Niehaus), *Metschnikowia pulcherrima* (Pitt.) M.W. Miller, *Pichia anomala*, *Saccharomyces cerevisiae* Meyen ex E.C. Hansen, and *Zygosaccharomyces rouxii* (Boutroux) Yarrow), sour rot bacteria (*Acetobacter aceti* (Pasteur) Beijerinck and *Gluconobacter oxydans* (Henneberg) De Ley), and a fungal pathogen (*Botrytis cinerea* Pers.) all endemic of the vineyard were sampled by solid-phase microextraction and analyzed by gas-chromatography coupled with mass spectrometry. Ethanol, 3-methyl-1-butanol, and ethyl acetate were the most common volatiles released from all microbe-inoculated grapes. In addition, acetic acid was released at a substantial amount following bacteria inoculation and in a three-way inoculation with yeasts and the fungus. 2-phenylethanol, a compound reported to attract tortricid moths when used in combination with acetic acid, was found at a relatively low level in all microbial combinations as well as in the control grape. While grapes inoculated with a consortium of yeasts stimulated oviposition in comparison with uninoculated berries, the phytopathogenic fungus deterred egg-laying. Nonetheless, the highest preference to lay eggs was measured when the yeasts were co-inoculated with the fungus. The lowest preference was obtained when grapes were inoculated with sour rot bacteria and their binary co-inoculation with yeasts and the fungus. Interestingly, oviposition on berries simultaneously inoculated with all the three microbial groups was unaffected. Lures loaded with either acetic acid or 2-phenylethanol were not attractive when placed in traps as single component in vineyards, but a binary blend attracted both sexes of grapevine moth in significant numbers. Further addition of the three most common volatiles released by infected berries (ethanol, 3-methyl-1-butanol, and ethyl acetate) did not significantly increase moth catch with this binary blend. The ecological implications of the grape-microorganism and grapevine moth interaction as well as the possibility to develop a pest monitoring system based on microbial volatiles are discussed.

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✉ Marco Tasin
marco.tasin@slu.se

[1] Integrated Plant Protection Unit, Department of Plant Protection Biology, Swedish University of Agricultural Science, 23053 Alnarp, Sweden

[2] Department of Sustainable Agro-Ecosystems and Bioresources, Research and Innovation Centre, Fondazione Edmund Mach (FEM), San Michele all'Adige, Italy

[3] USDA, Agricultural Research Service, 5230 Konnowac Pass Rd, Wapato, WA 98951, USA

[4] Millennium Nucleus Center in Molecular Ecology and Evolutionary Applications in the Agroecosystems (CEM), Facultad de Ciencias Agrarias, Universidad de Talca, Casilla 747, Talca, Chile

[5] Escuela de Agronomìa, Facultad de Ciencias Agrarias y de los Alimentos, Pontificia Universidad Católica de Valparaíso, Casilla 4-D, Quillota, Chile

[6] Center Agriculture Food Environment (C3A), University of Trento, San Michele all'Adige, Italy

Keywords *Lobesia botrana* · Acetic acid · 2-phenylethanol · Dual sex attractant · Pest monitoring

Introduction

Olfactory cues emitted by plant-microbe associations are utilized by a number of insects to locate food resources [1]. In comparison with other sensory cues such as visual or tactile stimuli, olfactory cues can be sensed over large distances and are likely to play an ecological role within the triple plant-microbe-herbivore interaction. In herbivorous insects with plant-feeding larval stages and a non-feeding adult stage, the quality of the food consumed during pre-imaginal stages settles the reproductive output of the adults. Microorganisms can affect such performance by changing the nutritional value of the plant on which they grow. This process is accompanied by a simultaneous shift in the volatile profile of the plant, which will carry not only plant compounds but also de-novo synthetized microbial components.

Microbial compounds can attract insects to infected plant with an increased content of vitamins, protein, and other nutrients, which adult insects utilize to prolong their lifespan, to increase their resistance against parasitoids, to promote egg development, and to offer a high nutritional substrate to the offspring [2, 3]. The ecological function of microbial food-signaling volatiles has been studied, but the utility of these compounds as attractant to monitor or mass trap insect pests has been explored for only a few species [1, 4–8].

Several studies have evaluated the use of microbial volatiles from fermenting baits to survey moths, and noctuids have consistently been the most common species group collected [9–11]. However, more recent studies have focused on the attraction of various tortricids to microbial volatiles, including key horticultural pests, such as the codling moth *Cydia pomonella* (L.) and the summer fruit tortrix *Adoxophyes orana* (Fischer von Röslerstamm) [12, 13]. Less information is available for a number of pests of other economically important crops such as grapevine.

In this study, we examined the effect of microbial volatiles on the grapevine moth *Lobesia botrana* (Denis & Schiffenmüller). *Lobesia botrana* is a polyphagous herbivore associated with grapevine *Vitis vinifera* (L.). While oviposition, larval and wind tunnel attraction of grapevine moth to host plant volatiles, and their physiological response were established and confirmed through several studies [14–17], the response to microbial volatile metabolites has been the object of more recent investigations. In vineyards, due to a diverse range of microorganisms that may infect the grapes, *L. botrana* larvae and adults are attracted to berries with a highly variable nutritional value. Both oviposition and larval fitness were substantially affected by these microorganisms [18, 19], with larvae being involved in spreading a fungal pathogen of grape [20].

A large variation among the volatile composition of single microorganism headspace and their effect on moth oviposition was measured. While yeasts (*Hanseniaspora uvarum* (Niehaus), *Metschnikowia pulcherrima* (Pitt.) M.W. Miller, *Pichia anomala*, *Saccharomyces cerevisiae* Meyen ex E.C. Hansen, and *Zygosaccharomyces rouxii* (Boutroux) Yarrow) were found to stimulate egg deposition, the phytopathogenic fungus *Botrytis cinerea* Pers. and the bacteria associated with grape rot (*Acetobacter aceti* (Pasteur) Beijerinck and *Gluconobacter oxydans* (Henneberg) De Ley) triggered the opposite effect [18]. In vineyards, microorganisms such as fungi, yeasts, and bacteria co-occur often at the grape surface [21]. However, the possible effect of combinations of these microorganisms on the behavior of the herbivore has not previously been considered. Similarly, the volatile profile of berries in the field exposed to a diverse microbial inoculation has not previously been characterized.

Here, we identify the volatiles released by grape berries infected with different combinations of the abovementioned microorganisms endemic of the vineyard using solid-phase microextraction (SPME) coupled to gas-chromatography and mass spectrometry (GC-MS). Second, we compared the level of oviposition on infested berries in a laboratory choice test against uninoculated and sterilized berries. Third, we evaluated the potential attractiveness of various volatile blends to *L. botrana* in a field setting.

Material and Method

Insects and Microorganisms

Lobesia botrana was originally collected in Italy and maintained in the laboratory on a semi-artificial diet at 25 °C, 70% relative humidity, and under a 17:1:6 h light/dusk/dark photoperiod. Field-collected larvae were grown to adulthood and the following offspring have been added to this colony each year to minimize an inbreeding effect [18]. The microorganisms used in this study were isolated from untreated vineyards in Trento (Italy) as described in an earlier study [18]. We tested a consortium of five yeasts (*S. cerevisiae*, *Z. rouxii*, *M. pulcherrima*, *H.uvarum*, and *P. anomala*) commonly present on ripe berries; two species of bacteria (*G. oxydans* and *A. aceti*) commonly isolated from berries showing sour rot symptoms; and *B. cinerea*, the phytopathogenic fungus causing gray rot. Ripe grapes (*V. vinifera* cv. Pinot gris) were

randomly collected from an untreated vineyard in Trento (Italy). Five replicates of ten berries each were washed by dipping for 10 min in 50 ml of sterile water with 0.01% Tween 80 (polyoxyethylene sorbitan monooleate, Acros Organics, Geel, Belgium). The suspensions were then serially diluted and plated on potato dextrose agar (PDA; Oxoid, Milan, Italy). Morphologically different colonies were selected and identified at specie level based on morphological, biochemical, physiological, and molecular approaches [22, 23]. One isolate for each of the yeast species found (*H. uvarum*, *M. pulcherrima*, *P. anomala*, *S. cerevisiae*, and *Z. rouxii*) was selected and maintained on PDA at 5 °C until use. Isolates of two species of acetic acid bacteria (*G. oxydans* and *A. aceti*) were selected and maintained on LPGA (Oxoid). *Botrytis cinerea* was isolated from grapes (*V. vinifera* cv. Cabernet Sauvignon) with gray mold in the same vineyard and maintained on PDA at 5 °C until use.

Grape Inoculation

The inoculation of berries was carried out at FEM (Italy) following a published protocol [18]. Briefly, 100 intact ripe berries cv. Waltham were surface-sterilized with sodium hypochlorite (1%; Sigma-Aldrich, Milan, Italy) for 5 min and thereafter washed twice in sterile water. Five evenly distributed wounds (~2.0 mm) were inflicted on the longitude of each berry with a sterile scalpel. The abovementioned isolates were grown on the respective media in Petri dishes for 5 to 7 days at 25 °C. Suspensions of cells were collected with 5 mL of sterile distilled water, and cell concentration was adjusted to 1×10^6/mL for yeasts and 1×10^7/mL for bacteria by dilution, after counting the yeast cells under the microscope in a Thoma cell and by estimating the bacterial cells by reading the optical density (OD_{600}) with the spectrophotometer. The adjusted suspensions were then mixed in equal proportion to obtain two suspensions (consortia of the yeasts and the bacteria) Berries were then inoculated by placing a drop (5 μL) of each microbial suspension. The following combination of suspensions were carried out: consortium of yeasts, consortium of bacteria, *B. cinerea*, consortium of yeasts + consortium of bacteria, consortium of yeasts + *B. cinerea*, consortium of bacteria + *B. cinerea*, consortium of yeasts + consortium of bacteria + *B. cinerea*. Berries wounded and treated with a drop of sterile distilled water served as untreated control. For *B. cinerea*, a small portion of mycelium was placed on the wounds. Inoculated and control berries were placed separately in sterile Petri dishes on wet filter paper (three berries per dish), covered by a pierced plastic cup, sealed with parafilm, and incubated for 16 h at 22 °C and 99% RH. At the end of the incubation, berries were used in the oviposition bioassay as odor stimulus. Plastic cups (61 mm base diameter × 88 mm top diameter × 130 mm high) served as oviposition devices and were assembled to avoid any physical contact of the insect with the berry. Cups and all materials used for experiments were glove-handled to avoid any contamination and disposed after each single use.

Analysis of the Odor Profile

Following the incubation time described above, volatiles emitted from uninoculated berries and from berries inoculated with *B. cinerea*, yeasts, acetic bacteria and their binary and ternary combinations were collected by solid-phase microextraction (SPME). Six berries with visible successful inoculations were randomly selected from each batch and placed into a 100-ml glass jar, with an opening closed by a single layer of parafilm© for each collection assay. Following an equilibration time of 30 min, volatiles in the jar were adsorbed by a SPME fiber previously conditioned at 250 °C for 5 min in a gas-chromatograph injection port (triphasic fiber SPME, 2 cm length, film thickness 50/30 μm, coating divinylbenzene/carboxen/polydimethylsiloxane; Supelco, USA). After a collection time of 60 min, volatiles collected on the fiber were desorbed and injected in a gas-chromatograph coupled to a mass spectrometer (GC-MS, Clarus 500, Perkin Elmer, Waltham, USA) equipped with an Innowax column (30 m × 0.32 mm × 0.5 μm, Agilent, Palo Alto, USA). The SPME fiber was desorbed in splitless mode for 5 min in the GC injector port at 250 °C. The GC oven was programmed at 40 °C for 3 min, raised from 40 to 180 at 4 °C min^{-1}, 180 °C for 4 min, raised from 180 to 220 at 10 °C min^{-1}, and held at 220 °C for 10 min. Helium was used as carrier gas with a constant flow of 1.5 mL min^{-1}. The temperature of the transfer line was set at 250 °C. The mass spectrometer operated in electron ionization mode (EI, internal ionization source; 70 eV) with a scan range between m/z 30 and 300. A calibration of the SPME collection efficiency was carried out for the compounds ethanol and ethyl acetate by using synthetic standards (Anfora et al. 2005). Results were used to calculate the amount release by each treatment (Fig. 1). The GC-MS database were analyzed using the Agilent MS software version 4.1 (Agilent, Santa Clara, USA). Compounds were identified by comparing their spectra with those of Wiley library as well as by comparing their Kovats retention indices with those published in literature. Kovats index of compounds was based on retention times of a blend of reference hydrocarbons. All identified compounds were injected as synthetics to calculate their Kovats index.

Oviposition Bioassay

Oviposition preferences of *L. botrana* females were conducted at FEM (Italy) with each of the seven types of inoculated versus uninoculated *V. vinifera* grapes in a series of choice assays conducted in cylindrical net-cages (25 cm diameter, 50 cm long, 1.5 mm mesh). Following emergence, a male and a female were

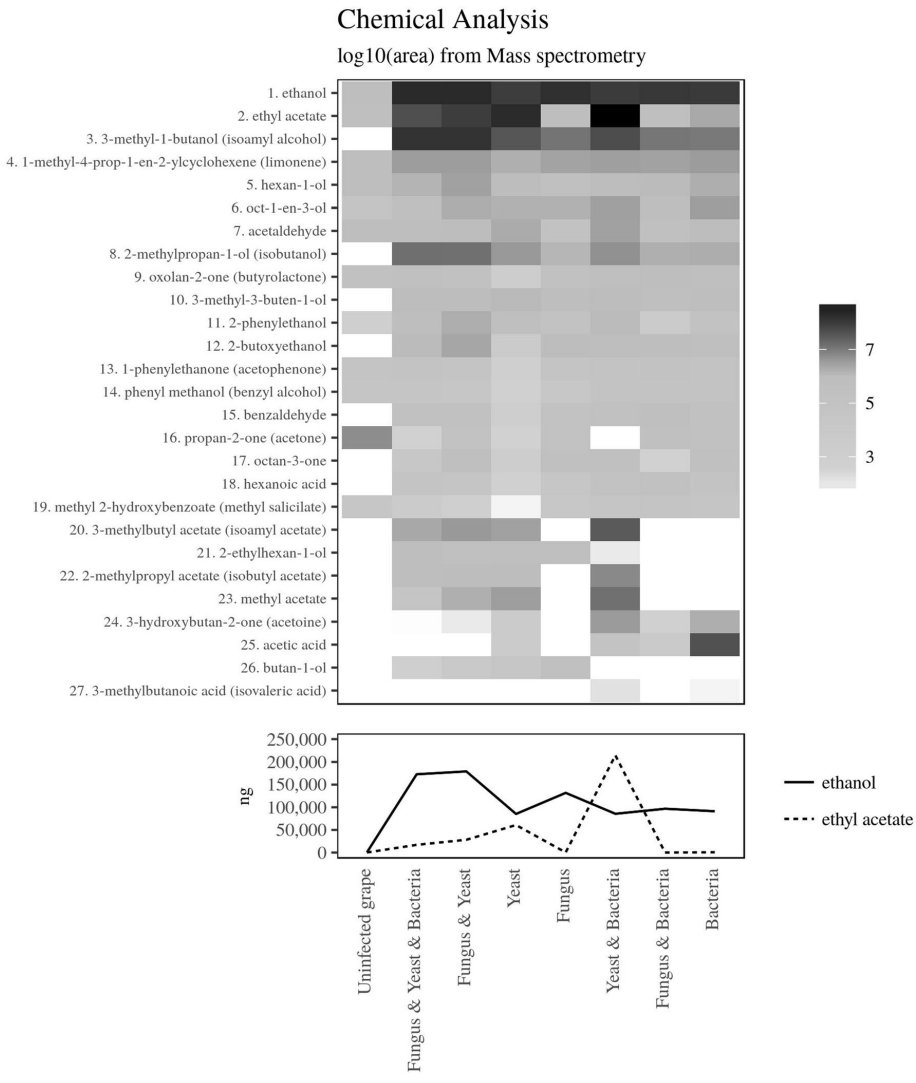

Fig. 1 Heat map representing the chemical analysis of volatile compounds emitted by single or multiple microorganisms inoculated on grapes. Compounds were identified via SPME-GC-MS. The scale of the heat map represents a log 10 value of the compound abundance. The calibration of the SPME efficiency is shown in the graph at the bottom

confined for 24 h into a plastic container to mate. Only 1–2-day-old females that laid eggs were used in bioassays. Oviposition assays were conducted under the same climatic conditions of the rearing. A 2-day-old mated female was released into the center of each cage. Mated females were allowed to choose between two oviposition substrates confined into a cage at a distance of 30 cm. After 72 h, moths were removed and laid eggs counted. The replication of each oviposition choice experiment is presented in Fig. 2.

Field-Trapping Experiment

Through an exploratory experiment carried out in a vineyard in Verona (Italy) with a moderate population of grapevine moth, we found that a lure releasing ethyl acetate, 3-methyl-1-butanol, ethanol, 2-phenylethanol, and acetic acid attracted more moths than a blank trap. Although this result was not supported by a statistical significance, we decided to further challenge the potential

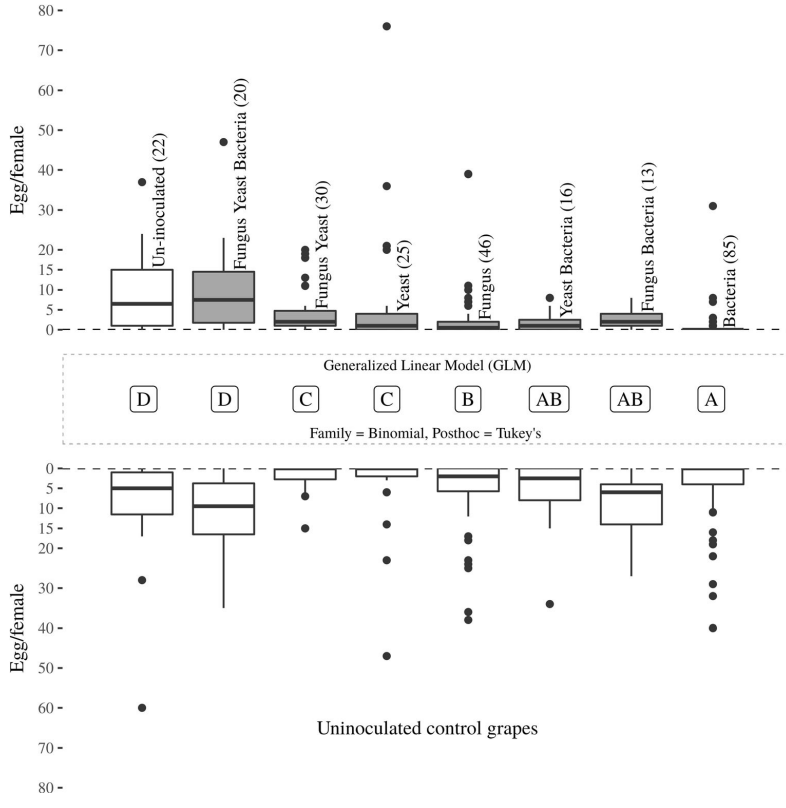

Fig. 2 Boxplot representing the number of eggs laid by *L. botrana* females a laboratory dual-choice experiment with uninoculated or microorganism inoculated grapes. Choice experiments were done in net-cages. Non-respondent insects were included in the statistical model. The boxplot includes the median line (tick line inside the box), the interquartile range (lower and upper box limits), the variability outside the interquartile range (whisker), and the outliers (points). Letter in the middle box indicates significant difference based on the number of eggs laid at each side of the bioassay and their ratio

of these compounds in a larger field-trapping test with a higher population of the target pest. Our attention focused on the major common volatiles (ethyl acetate, 3-methyl-1-butanol, ethanol, and acetic acid) and on 2-phenylethanol, a microbial and plant volatile reported in literature as moth attractant [24–27]. A field test in the Maule Region (Chile) was therefore conducted during February and March 2017 in a "Cabernet Sauvignon" vineyard situated near Molina (35° 04′ 14.29″ S, 71° 15′ 17.92″ W). Vines were planted at a density of 1110 plants ha^{-1} with a "tendone" trained 2.3 m tall canopy. The vineyard was managed with mating disruption for *L. botrana* using Isonet L (Shinetzu, Tokyo, Japan) at 500 dispensers ha^{-1}. No insecticides were sprayed during the experiment. Orange delta traps (Süsbin, Mendoza, Argentine) with hot melt pressure adhesive liners (Alphascent, West Linn, OR, USA) were used to monitor *L. botrana*. Volatile compounds were loaded in 1.5 mL microcentrifuge plastic tubes (Sorenson BioSciences, Salt Lake City, UT, USA), termed from now on "lures," with a 1-mm perforation hole in the lid, which contained also a dental cotton wick to adsorb the solution. Blends of volatile compounds (Fig. 4) were kept cold on ice during lure loading to prevent evaporation. Volatiles were loaded as single compound or as a blend within a single lure, except for acetic acid, which was loaded in a different lure to prevent esterification of the alcohols present in the blends. Due to the particularly high volatility of the compounds, we increased the load of the lure in comparison to the exploratory trial. In accordance with data from literature [25, 28, 29], we chose a 500-mg load for acetic acid and a 7.5–30-mg load for the other compound (Fig. 4). After loading the cotton wick with the compound(s), 30 μl of mineral oil (Sigma-Aldrich, Saint Louis, MO, USA) was added on top of the volatile(s) and the cotton wick to slow down the evaporation rate (Knudsen et al. 2015). For acetic acid, 500 mg was loaded in the lure and no mineral oil was added. Lures were hung from the roof of the delta traps with a clip. Five trap replicates were randomly located in the canopy with a spacing distance of approximately 20 m on January 31, 2017. Lures were replaced weekly or every 2 weeks (acetic acid). Liners were inspected weekly, and trap location was rotated on each sample date until March 24, 2017.

Statistics

Statistical analyses were carried out using R software [30] and results are presented in Table 1. Cook's distance was used to investigate influential points as possible outliers in the chemical dataset. When a single data point deviated more than three times from the respective mean, it was counted as an outlier and removed from the dataset. The composition of the microbial odors is graphically presented as a heat map (Fig. 1 and Table S1 in the additional data). The quantification of ethyl acetate and ethanol in each microbial headspace was calculated using a linear model based on the correlation between area count from injections of

Table 1 Output from the statistical analyses

Model		Distribution[a]	Dispersion	Estimate	SE	z	*P* value
Oviposition treatment vs control							
Uninoculated (control)		Negative binomial (0.569)	0.938	− 0.026	0.400	− 0.065	0.948
Fungus yeast bacteria		Negative binomial (0.861)	0.828	− 0.213	0.322	− 0.661	0.508
Fungus yeast		Negative binomial (0.429)	0.976	0.820	0.417	1.965	0.049
Yeast		Negative binomial (0.188)	0.978	0.604	0.656	0.922	0.357
Fungus		Negative binomial (0.302)	1.128	− 0.841	0.419	− 2.006	0.045
Yeast bacteria		Negative binomial (0.378)	0.720	− 0.995	0.517	− 1.927	0.054
Fungus bacteria		Negative binomial (1.051)	0.670	− 1.136	0.351	− 3.233	0.001
Bacteria		Negative binomial (0.133)	(1.368)	− 1.598	0.516	− 3.096	0.002
Oviposition pairwise comparison[b]		Binomial, cbind()	1				
Fungus yeast vs control				0.846	0.182	4.640	< 0.001
Yeast vs control				0.630	0.158	3.992	0.002
Fungus vs control				− 0.815	0.150	− 5.453	< 0.001
Yeast bacteria vs control				− 0.970	0.225	− 4.310	< 0.001
Fungus bacteria vs control				− 1.110	0.219	− 5.063	< 0.001
Bacteria vs control				− 1.572	0.167	− 9.391	< 0.001
Fungus yeast vs fungus yeast bacteria				1.033	0.179	5.773	< 0.001
Yeast vs fungus yeast bacteria				0.817	0.154	5.307	< 0.001
Fungus vs fungus yeast bacteria				− 0.628	0.146	− 4.319	< 0.001
Yeast bacteria vs fungus yeast bacteria				− 0.782	0.222	− 3.520	0.010
Fungus bacteria vs fungus yeast bacteria				− 0.923	0.217	− 4.263	< 0.001
Bacteria vs fungus yeast bacteria				− 1.385	0.164	− 8.456	< 0.001
Fungus vs fungus yeast				− 1.661	0.187	− 8.892	< 0.001
Yeast bacteria vs fungus yeast				− 1.815	0.251	− 7.223	< 0.001
Fungus bacteria vs fungus yeast				− 1.958	0.246	− 7.944	< 0.001
Bacteria vs fungus yeast			–	− 2.417	0.201	− 12.01	< 0.001
Fungus vs yeast				− 1.446	0.163	− 8.862	< 0.001
Yeast bacteria vs yeast				− 1.600	0.234	− 6.830	< 0.001
Fungus bacteria vs yeast				− 1.740	0.229	− 7.608	< 0.001
Bacteria vs yeast				− 2.202	0.180	− 12.25	< 0.001
Bacteria vs fungus				− 0.756	0.172	− 4.388	< 0.001
Multicomparison of field catches[b]							
Males	Blend 7 vs blend 2/3/4	Negative binomial (1.099)	0.661	2.944	0.968	3.043	0.019
	Blend 8 vs blend 2/3/4			2.708	0.972	2.785	0.040
Females	Blend 7 vs Blend 4	Negative binomial (0.809)	0.409	2.996	0.795	3.769	0.001
	Blend 8 vs blend 4			3.296	0.791	4.164	0.001
	Blend 7 vs blend 5			2.303	0.654	3.523	0.004
	Blend 8 vs blend 5			2.603	0.650	4.007	< 0.001

[a] Theta parameter for negative binomial distribution

[b] Only significant comparisons are shown

synthetic amounts and SPME collections ($R^2 = 0.97$ and 0.98 for ethanol and ethyl acetate, respectively).

A density plot representing the number of laid eggs in the oviposition choice experiment was produced using the R package ggjoy 2.10 (Fig. 3). We used a density plot in order to avoid the stipulation of the data in bin width, which may lead to a skewed picture due to differences in replication. In the density plot, the overall area of each "ridgeline" is equal to 1. This gives the reader a direct understanding of the differences between egg distributions in each treatment. The whole dataset was used in this analysis, including non-responding insects.

In addition, oviposition choice data were also analyzed using a binomial generalized linear model with a cbind function. Through this analysis, it is possible to compare treatments with each other taking into consideration not only the amount of eggs laid at the inoculated side but also the ratio of eggs between the two choices. Data are presented as a box plot including outliers. Tukey's post hoc test was used to discriminate between treatments (Fig. 2).

The field dataset distributed according to a negative binomial family and was analyzed using the function glm.nb (library MASS). Because of our dataset did not fit into a zero-inflated model, treatments with no variance, i.e., with no catches, were excluded from the analyses. This allowed us to fit the data to a more accurate model. Treatments were separated by Tukey contrasts (Fig. 4).

Results

Analysis of Odor Profile

Volatiles released by grapes inoculated with microorganisms belong to the chemical classes of aldehydes, ketones, alcohols, acids, esters, lactones, terpenoids, and benzenoids (Fig. 1 and Table S1). The composition of the headspace showed a high variability among microorganisms. Ethanol and 3-methyl-1-butanol were identified as main components in all three categories of microorganisms. Ethanol, 3-methyl-1-butanol, and limonene were the major compounds identified in the headspace from grapes inoculated with the fungus. Ethyl acetate, along with ethanol and 3-methyl-1-butanol, was the major component released by the yeasts. Ethanol, acetic acid, and 3-methyl-1-butanol were the major volatiles from grape inoculated with the sour rot bacteria. Co-inoculating yeasts with the fungus resulted in a relative increase in 3-methyl-1-butanol, a reduction of ethyl acetate, and a total inhibition of acetic acid emission compared to the release of yeasts and the fungus alone. An increase

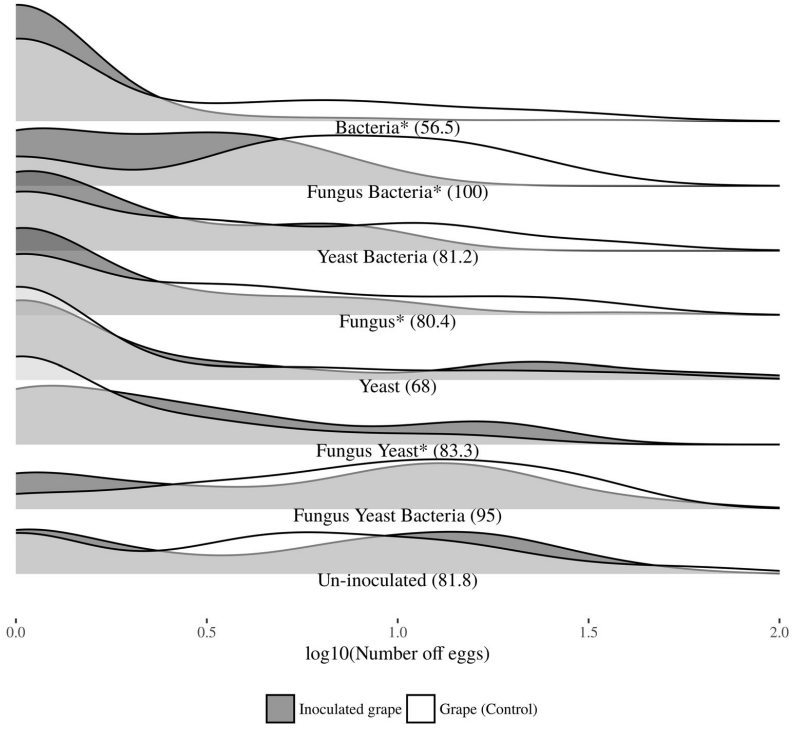

Fig. 3 Density distribution of *L. botrana* egg in a laboratory dual-choice experiment with uninoculated or microorganism-inoculated grapes. The experiment was done in net-cages. Percentage of responding female is shown in parenthesis. The asterisk indicates a significant choice for one of the two treatments. The area delimited by each ridgeline is equal to 1

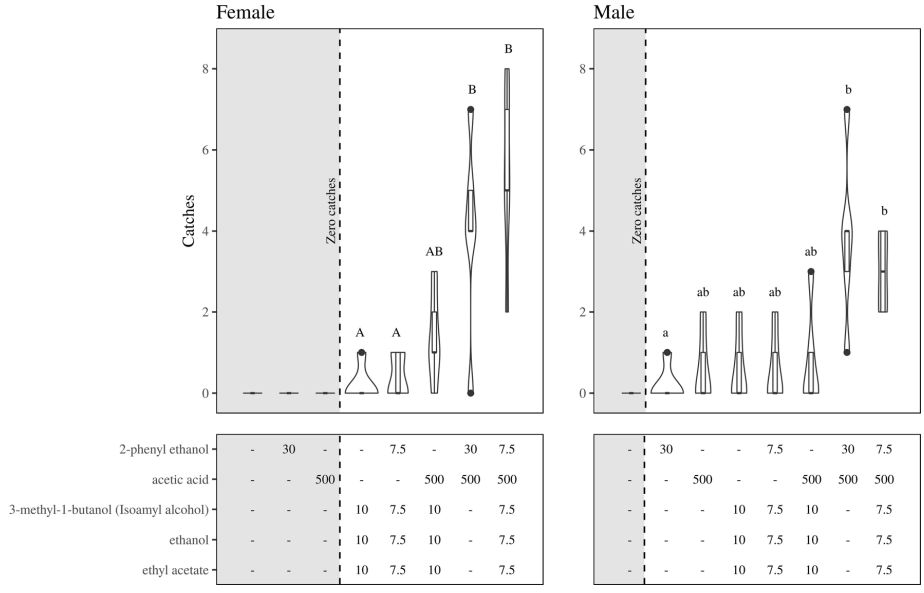

Fig. 4 Boxplot with field catches of both sexes of *L. botrana* from a vineyard in Chile during 2017. A total of 57 females and 48 males were caught. The boxplot includes the median line, the 25 and 75% range (lower and upper box limits), and the outliers. The thickness of the bar mirrors the density of the catch at a given level. Treatments capped with the same letter do not differ significantly in the number of caught moths

in acetic acid emission was observed when the bacteria were added to the fungus, while the release of its precursor, ethanol, diminished. When bacteria were inoculated with yeasts, release of ethanol and acetic acid decreased while their corresponding ester ethyl acetate increased. The ternary combination showed a higher release of 3-methyl-1-butanol compared to each of the single microbial categories. Although released by the entire range of tested microbes, a higher proportion of 2-phenylethanol was measured in the headspace of yeasts and both binary and ternary combinations. While the bacteria and yeast co-inoculation released the highest absolute amount of ethyl acetate (214 ng per sample), the fungus and yeast co-inoculation followed by their combination with the bacteria emitted the highest quantity of ethanol (192 and 181 ng per sample, respectively). Uninfected wounded grapes release a number of plant volatiles such as hexan-1-ol, limonene, 1-octen-3-ol, benzyl alcohol, methyl salicylate, and 2-phenylethanol. Although to a much limited extent than infected grapes, compounds possibly associated with the wounding process such as acetone, acetaldehyde, ethanol, ethyl acetate, butyrolactone, and acetophenone were also released by the uninoculated grapes.

Oviposition Bioassay

In Fig. 3, it is presented the egg density measured in each dual choice experiment. While grapes inoculated with the yeasts stimulated oviposition, the fungus deterred egg-laying. However, the highest choice to lay eggs was measured when the fungus was co-inoculated with the yeasts. This co-inoculation triggered a significantly higher number of eggs than the control grape. Repellence was observed when grapes were inoculated with sour rot bacteria or their combination with yeasts or the fungus. Grapes inoculated with all the three microbe categories were neither repellent nor attractive to grapevine moth females (see Table 1 for further details).

When comparing the different dual-choice experiments with each other through a GLM, it is possible to appreciate that the treatments including the bacteria and the one including the fungus alone triggered a significantly lower amount of eggs in comparison to the yeast and the yeast + fungus. These last two treatments stimulated a lower egg-laying than the three-way inoculum or the uninoculated grapes (Fig. 2). The higher number of eggs released at the side of the arena with the microbe-inoculated grape was measured for the ternary inoculation (9.7 eggs female^{-1}), followed by the yeast consortium (7.8 eggs female^{-1}). A lower number of eggs was laid when fungus plus yeasts were co-inoculated (4.8 eggs female^{-1}) or at the stimuli with the sour rot bacteria and their combination with the yeasts or the fungus (2.1 and 2.8 eggs per female^{-1}). Similarly, the fungus alone elicited a low oviposition (2.3 eggs per female).

Field-Trapping Experiment

Blank traps did not catch any moth. While females were not attracted to traps baited with single components (acetic acid or 2-phenylethanol), a small number of males responded to those components (Fig. 4). When these two volatiles were presented in a unique blend, the response of both sexes increased, with a stronger effect in females. Although both sexes showed some attraction to a three-component blend of 3-methyl-1-butanol, ethanol, and ethyl acetate, no synergy occurred when acetic acid or/and 2-phenylethanol were added to this blend.

Discussion

The chemical signals produced by the interactions of the grapes and microorganisms can be characterized by a set of major volatiles, including ethanol, ethyl acetate, acetic acid, and 3-methyl-1-butanol. However, the blends of these volatiles differ widely among the three groups of microorganism and are altered by the various binary and ternary combinations. Importantly, our laboratory oviposition assays demonstrate that these volatile bouquets have a strong behavioral effect impacting the utilization of the host plant resource by female *L. botrana*. Our preliminary field trial demonstrates that specific blends of microbial volatiles may be key cues used by both male and female moths to orient to the host plant.

Interestingly, a relatively minor but common volatile 2-phenylethanol when presented in combination with acetic acid was attractive to both sexes of moths. In addition, when presented with all of the major volatiles, this blend retained its attractiveness.

A change in host quality induced by a microbial infection may trigger a variation in volatile emission, which is sensed by herbivorous insect [31, 32]. An attempt to correlate food quality with attraction to food volatiles was done by Tasin et al. [18] for *L. botrana*. In particular, eggs laid on a yeast-containing medium developed towards a hgiher fitness in comparison to a blank medium or to one with gray rot. When the acetic acid bacteria were added to the medium, a similar fitness to the yeast-containing medium was measured.

While we have no information on the relation between attraction to single compound and larval fitness, it is intriguing that in the present study, gravid females were trapped with a binary blend of ubiquitous microbial compounds released either by all microbial combinations (2-phenylethanol) or by yeast and single or co-inoculated bacteria (acetic acid). Because this component was emitted with the highest amount by the repellent bacteria, it would be intuitive to exclude this compound from the candidate volatiles for field attraction. In fact, its attraction in the field as single components was not different from the blank. Similarly, 2-phenylethanol was inactive when presented alone. Although released at a very little amount in comparison with the major compounds, 2-phenylethanol may play a major behavioral role, as reported for other minor components [33].

While the emission of acetic acid from the yeasts was totally inhibited by the fungus in their co-inoculation, 3-methyl-1-butanol emerged as the second most abundant volatile after ethanol. According to these data, we may expect a stimulating effect of 3-methyl-1-butanol when co-occurring at a higher dose with other compounds such as ethyl acetate. The attractive properties of this alcohol are known for a number of insects [29, 34, 35]. When in the present study 3-methyl-1-butanol was presented in the field in combination with ethanol and ethyl acetate, no significant attraction was scored. However, although not significant, the ternary blend could have an additive effect on female captures when added on the top of 2-phenylethanol and acetic acid (Fig. 4). In the study of Tasin et al. (2012), the response of the grapevine moth to grapes with *B. cinerea* shifted from attraction to repulsion according to the time from inoculation. In the same study, 3-methyl-1-butanol was found to be repellent at a high dose while attractive at a low dose. We observed here that *L. botrana* females were not repelled when a blend of 30 mg of 3-methyl-1-butanol, ethyl acetate, and ethanol was added to the attractive binary mixture of acetic acid and 2-phenylethanol. From our result, the role of 3-methyl-1-butanol seems to be context-dependent on the presence of other constituents. The detrimental effect observed in Tasin et al. (2012) could have been reversed into an attractive stimulus by the addition of other volatiles. The new blend may represent to the insect a yeast related odor, which, according to the literature, should provide a higher fitness food to the offspring. The generalist feeding habit of *L. botrana* with populations interplaying between cultivated grape and other wild or cultivated plants adds further complexity to the observed yeast/fungus preference on grape.

Perhaps different volatiles are involved in triggering different behavioral functions, but the synergy between them is fundamental to elicit field attraction from a distance. While 2-phenylethanol could be relevant for both attraction and oviposition, acetic acid may elicit a rather longer-range attraction, because of its higher emission and potential to travel further from the source. While in the headspace from the inoculated berries the ratio between acetic acid and 2-phenylethanol ranged from 0.7 (yeasts) to 67 (bacteria), an intermediate ratio of 16 (load of the field lure in this study) was attractive in the field experiment. Although promising, our data form a preliminary base towards the identification of multicomponent field attractants, because a large number of minor compounds identified in the microbial headspace remain to be tested.

Recently, both acetic acid and 2-phenylethanol were scored in the headspace of damaged plants by different tortricid species as caterpillar induced volatiles [25]. These compounds were field attractive to conspecific adults across a range of moths, including *Pandemis* spp. and other tortricids [26]. It is intriguing that acetic acid and 2-phenylethanol were identified as behaviorally active both as microbial and caterpillar-induced plant volatiles. We

speculate here that such a behavioral activity on a broad range of species may reflect a conserved behavioral pattern in Tortricidae, as shown for other olfactory traits in moths [36, 37]. According to the preference-performance hypothesis, it is predicted that herbivorous insects will evolve to lay eggs on hosts that will elicit the best performance in the offspring [38, 39]. Perhaps both microbial and caterpillar-induced volatiles are perceived by a searching insect as oviposition cues carrying an ecologically shared message, i.e., a nutritious substrate for the offspring.

Although plant volatiles were released in the oviposition arena, our laboratory experiment may be biased by the lower background of grapevine volatiles in comparison with a field situation. Accordingly, the preference observed in the laboratory may be shaped in a different way when the same experiment would be moved in a vineyard. The effect of grapevine volatiles on attraction and oviposition was earlier examined by Anfora et al. [40] in a semi-field setting through a release and recapture assay with gravid females. While green grapes were removed from the plants to eliminate the competition between the trapping odors and the fruits, only a small proportion of the released females were recaptured, with higher numbers in a synthetic grape mimic compared to a grape cluster [40].

In the same study, the synthetic mimic stimulated a higher oviposition on shoots surrounding the traps in comparison with the grape cluster. Overall, synthetic volatiles identified from the cultivated *V. vinifera* were not highly attractive to *L. botrana* females, probably due to a high degree of similarity with the background odor of the vineyard. *L. botrana* female may instead be attracted by an odor with a lower degree of similarity to grapevine, such as that released by other host plant or by microorganisms. While *L. botrana* wind tunnel response to artificial plant volatile mixtures with a higher attraction to *Daphne gnidium* compared to *V. vinifera* was examined, it is currently unknown whether or not such laboratory active compounds may play a role in a field setting [41]. Recently, a grapevine genotype with a distorted ratio of two terpenoids was created to show the effect of plant volatile ratio on grapevine moth attraction [42]. Such a result highlighted the importance of considering the ratio between volatiles when testing multicomponent blends in the field.

The potential role of microbial volatiles in overtaking the volatile background of the crop was demonstrated earlier in *L. botrana*. Field attraction of grapevine moth to fermenting apple juice was reported by Thiery and co-workers as a valuable tool to predict oviposition [43]. However, the fermentation of the initial product induced by air-borne microorganisms may lead to a large and unpredictable variation in the emission of volatiles over time. In addition, the attraction to water, which cannot be distinguished from the effect of volatiles, adds further variation to the efficacy of such a lure. Accordingly, the optimization of food lures through the identification of their volatile components seems to be a prerequisite to improve the reliability of such monitoring tool.

This study paved the way for the identification of field attracting volatiles for male and female grapevine moth. We showed here that a combination of major and minor volatile constituents is essential to reach this goal. In particular, a blend of a compound commonly released during microbial fermentation (acetic acid) with a volatile emitted by a number of flowering plant as well as by microbial activity (2-phenylethanol) encoded field attraction for the studied pest. The practical need to identify bisexual food attractants in this species was highlighted during its recent invasion of America along with its range expansion to new host species [44]. The identification of a kairomone for field monitoring is a relevant tool to facilitate the implementation of insecticide-free method and move towards an advanced integrated pest management of vineyards.

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Paper I supplementary table - Volatiles of Grape Inoculated with Microorganisms

Compound[b]	KI[c]	UG[d]	Treatments[a]						
			FYB	FB	FY	Y	F	YB	B
			Relative area in (%)						
Acetaldehyde	792	9.13	0.20	0.17	0.25	0.63	0.08	0.27	0.50
Acetone	815	54.07	0.02	0.50	0.04	0.41	0.08		0.08
Methyl Acetate	819		0.26		0.37	1.03		1.31	
Ethyl Acetate	842	6.04	12.96	0.29	19.04	54.36	0.53	68.72	0.91
Ethanol	877	7.18	46.43	63.70	42.61	26.89	86.84	9.66	44.68
Isobutyl acetate	940		0.19		0.24	0.30		0.59	
Isobutanol	1036		3.36	1.00	2.75	1.03	0.84	0.45	0.83
Isoamyl acetate	1053		0.59		0.73	1.06		2.87	
1-Butanol	1099		0.11		0.11	0.10	0.24		
Limonene	1137	8.16	0.76	1.56	0.69	0.58	1.45	0.32	1.41
Isoamyl Alcohol	1170		32.88	6.44	28.92	11.25	6.84	4.71	4.23
3-Methyl-3-buten-1-ol	1221		0.26	0.30	0.22	0.35	0.49	0.10	0.37
3-Octanone	1228		0.10	0.04	0.13	0.05	0.18	0.04	0.14
3-Hydroxy-2-butanone	1266		0.09	0.14	0.04	0.20		0.41	0.99
Hexanol	1349	9.81	0.38	0.79	0.57	0.36	0.18	0.11	0.91
2-Butoxy-ethanol	1411		0.33	0.45	2.26	0.16	0.60	0.07	0.26
1-Octen-3-ol	1466	0.88	0.21	0.51	0.44	0.57	0.95	0.30	1.29
Acetic Acid	1510			22.85		0.17		9.73	42.85
2-Ethyl-1-Hexanol	1512		0.21	0.16	0.09	0.17	0.27	0.02	
Benzaldehyde	1552		0.09	0.28	0.04	0.03	0.10	0.03	0.12
Butyrolactone	1668	2.90	0.14	0.42	0.05	0.04	0.15	0.05	0.19
Acetophenone	1693	0.73	0.04	0.08	0.02	0.01	0.04	0.01	0.04
Isovaleric Acid	1734							0.07	0.02
Methyl Salicilate	1825	0.36	0.03	0.06			0.01		0.02
Hexanoic Acid	1921		0.06	0.17	0.01	0.01	0.02	0.01	0.04
Benzilic Alcohol	1924	0.59	0.02	0.07	0.01	0.01	0.01	0.01	0.05
Phenylethyl Alcohol	1958	0.16	0.29	0.03	0.38	0.21	0.11	0.12	0.08

[a] B. cinerea (F), S. cerevisiae + Z. rouxii + M. pulcherrima + K. apiculata + H. anomala (Y), A. aceti + G. oxydans (B), FY (coinoculum of F+Y), FB (coinoculum of A+F), YB (coinoculum of Y+A), FYB (coinoculum of F+Y+A).

[b] Compound identified by correlation with mass spectra (Wiley library) and Kovats index.

[c] Kovats index on a Innowax (30m x 0.32mm x 0.5 Îijm) fused silica column.

[d] Uninoculated Grape, control

II

Paper II - Designing a species-selective lure based on microbial volatiles to target Lobesia botrana

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Designing a species-selective lure based on microbial volatiles to target *Lobesia botrana*

Sebastian Larsson Herrera[1*], Péter Rikk[2], Gabriella Köblös[2], Magdolna Olívia Szelényi[2], Béla Péter Molnár[2], Teun Dekker[1] & Marco Tasin[1]

Sustainable, low impact control methods, including mating disruption and microbial insecticides against *L. botrana* have been available for decades. Yet, successful implementation has been restricted to only a few grapevine districts in the world. A limiting factor is the lack of a female attractant to either monitor or control the damaging sex. Volatile attractants for both female and male insects can be used to assess when *L. botrana* populations exceed economic thresholds, and to decrease the use of synthetic pesticides within both conventional and pheromone programs. Rather than using host-plant volatiles, which are readily masked by background volatiles released by the main crop, we tested the attractiveness of volatiles that signify microbial breakdown and more likely stand out against the background odour. A two-component blend of 2-phenylethanol (2-PET) and acetic acid (AA) caught significant numbers of both sexes. Catches increased with AA and, to a minimal extent, 2-PET loads. However, a higher load of 2-PET also increased bycatches, especially of Lepidoptera and Neuroptera. Major (ethanol, ethyl acetate, 3-methyl-1-butanol) or minor (esters, aldehydes, alcohols and a ketone) fermentation volatiles, did surprisingly not improve the attraction of *L. botrana* compared to the binary blend of 2-PET and AA alone, but strongly increased bycatches. The most attractive lure may thus not be the best choice in terms of specificity. We suggest that future research papers always disclose all bycatches to permit evaluation of lures in terms of sustainability.

The replacement of synthetic pesticides with selective, low-impact innovations is an important prerequisite to develop more sustainable agricultural production systems at the landscape level[1,2]. The challenge is particularly significant in cultivated monocultures such as orchards and vineyards, which represent generous 'invitations' to pests, while disfavoring natural control mechanisms[3].

In vineyards, the grapevine moth *Lobesia botrana* (Denis & Schiffermüller) is among the most important pests and requires regular insecticide applications[4]. Although the technology of mating disruption has been available for *L. botrana* for almost three decades, implementation is only achieved on a restricted number of viticultural districts in the world[5]. Factors that limited the spread of this environmentally friendly technology are among others, the challenge to involve a critical number of motivated stakeholders to reach an area-wide approach, and the lack of reliable attractants to monitor pest populations within a pheromone permeated crop[6]. Similarly, the use of microbial agents with a lower consistency than conventional insecticides requires meticulous monitoring to assess the efficacy, and thus are adopted only by either motivated growers or wine districts with advanced extension services[7].

Availability of a monitoring tool to forewarn growers and advisors when the population of the grapevine moth exceeds damage threshold would facilitate the implementation of both mating disruption and biocontrol application. Whereas effective monitoring tools are already identified for several other tortricid pests[8–10], further investigations are needed in *L. botrana*. Previous studies showed attraction of both sexes of *L. botrana* to volatiles emitted by host plants, including grapevine *Vitis vinifera* and flax-leaved daphne *Daphne gnidium*[11–13]. Although promising, these laboratory and semi-field results were not mirrored by trap catches in the field, due possibly to a suboptimal release of single compounds and blend ratios from dispensers, suboptimal trap properties, and the competition with the background volatiles emitted by the crop[14].

The issue of host plant background odor masking the lure may be circumvented by instead using volatiles that stand out against the background odors, such as volatiles associated with microbial breakdown[15]. Recently,

[1]SLU, Department of Plant Protection Biology, 230 53, Alnarp, Sweden. [2]Plant Protection Institute, Centre for Agricultural Research, Budapest, Hungary. *email: sebastian.larsson.herrera@slu.se

microbial volatiles identified from grapes were screened in behavioural experiments in South American vineyards and a blend of two microbial compounds, acetic acid (AA) and 2-phenylethanol (2-PET), was identified as attractive for both sexes of *L. botrana*[16]. Whereas the field attraction of this two component blend was further corroborated by El Sayed *et al*. 2019[17], the importance of the component ratio in the same blend remains, to the best of our knowledge, to be investigated. We hypothesized that a ratio skewed toward AA would increase the trap attraction range for the grapevine moth, while a 1:1 ratio would instead decrease the lure specificity without augmenting *L. botrana* catches. To test this hypothesis, we measured field attraction towards traps baited with six different loads of AA/2-PET (5:500, 50:500, 500:500, 500:50, 500:5 and 50:50). Beside testing for the first time the importance of ratio and load of these two components in conventionally managed European vineyards, we also investigated the significance of additional microbial compounds to further enhance attraction. Because *L. botrana* responded to volatiles released by grapes inoculated with microorganisms such as yeasts (*Hanseniaspora uvarum, Metschnikowia pulcherrima, Pichia anomala*, *Saccharomyces cerevisiae*) or sour rot bacteria (*Acetobacter aceti*, *Gluconobacter oxydans*)[16], we hypothesized that a more complete blend mimicking microbial release would enhance trap catches in comparison to the reference two-component blend. Finally, we evaluated the selectivity of the lure, a hallmark of sustainable pest control innovation, by carefully analysing catches of non-target species.

Material and Methods

Vineyards. Trapping tests were carried out during 2018 in two commercial vineyards in the Eger wine region in North-Eastern Hungary in the municipality of Maklár. Vineyards (6 and 7 hectares, respectively) were planted at a density of 4000 vine ha^{-1}. Grapevine plants were planted at 2.5 × 1 m and belonged to the variety 'Merlot', 'Kékfrankos', 'Turán', 'Cabernet franc'. An integrated pest management program[18] was applied all along the season to control pests and diseases. To control *L. botrana*, Avaunt (Indoxacarb, 150 g/l) and Actara SC (Thiametoxam, 240 g/l) were applied on May 17 and on July 14, respectively. Although sprayed with insecticides, we selected these fields due to the very high pest population reported in the previous season.

Volatile compounds. Major microbial volatiles emanating from inoculated grapes[16] were tested on their attractiveness for *L. botrana*. These were added to an existing 2-component blend consisting of AA and 2-PET. Microbial volatiles were formulated in polyethylene Eppendorf vials[16]. Synthetic volatiles included acetic acid (AA, 99.8%; VWR Chemicals, Belgium), 2-phenylethanol (2-PET, 99%; Acros Organics, China), ethanol (96%; VWR Chemicals, France), 3-methyl-1-butanol (99%; Acros Organics, Germany), ethyl acetate (99.5%; Riedel-de Haën, Germany), isobutanol (99.75%; Fisher Chemical, England), 3-methyl-3-buten-1-ol (97%; Acros Organics, Germany), isoamyl acetate (99.5%; Fisher Chemical, England), isobutyl acetate (98%; Acros Organics, Germany), methyl acetate (99%; Acros Organics, Belgium), acetaldehyde (99%; Fisher Chemical, England), benzaldehyde (99.5%; Sigma-Aldrich, USA), 3-hydroxy-2-butanone (acetoin) (95%; Sigma-Aldrich, China). Except for AA and 2-PET, all other chemicals were pipetted into the vial onto a dental cotton plug as neat compounds at 100 mg each. To test whether or not blending AA and 2-PET in a single vial would affect moth attraction, AA/2-PET field performance was evaluated with the two compounds loaded either in the same or in two different vials (S and D in Tables 1–3). In order to more evenly release the compounds and over a longer time, 100 mg of paraffin was added onto the cotton plug (see Tables 1–3 for description of attractants). Vials were hung at the centre of a transparent plastic delta trap with a replaceable sticky insert of 160 × 100 mm (Csalomon, Budapest, Hungary). Along the rows in the vineyard(s), traps were placed in randomized lines, with 4 rows of vine (12.5 m) between each trap line and 20 m between traps. Traps were inspected two or three times per week and inserts with captures were stored at +5 °C for later identification using a stereomicroscope. Trapping experiments were carried out in 2018 during May 3–17 (first generation), June 14-July 5 (second generation) and August 3–22 (third generation). Pheromone traps loaded with 0.3 mg of E7,Z9-12:Ac (Csalomon, Budapest, Hungary) were installed in an adjacent plot to monitor seasonal activity of males of the pest.

Statistical analysis. R was used for statistical analyses and visualisations[19]. A function was developed using the 'tidyverse'[20] to analyze the catches of target and non target species using the following workflow and criterias; (1) If less than 10 insects were caught across all treatments, no stats was performed, (2) If the number of insects caught for a species was less than 100 in each flight period, the catches were pooled across dates, (3) for species with more than 100 catches, dates with no insect of a given species in any of the treatments were filtered out. Data was subsequently fitted to a Poisson generalized linear model (glm) and tested for overdispersion using the package AER[21]. If the data were significantly overdispersed ($p < 0.05$), the Poisson model was replaced by the correspondent negative binomial, setting the maximum likelihood "theta" as extracted with library MASS[22]. Treatments in the model were compared pairwise using the package multcomp[23]. Treatments with no catches were omitted from the analysis. Specificity was calculated as the number of catches of target species divided by the total number of catches.

Results

***Lobesia botrana* captures.** In the first flight a total of 49 females and 38 males were captured in 140 traps. Captures across the 15 treatments are summarized in Table 1. Due to the low population level, no differences between treatments were found. In the second generation a total of 163 female and 98 male *L. botrana* were caught (Table 2). Similarly to the first flight, catches were too low to permit comparison among treatments. In the third generation (Figs. 1–4, Table 3) a much higher population level was present and a total of 1158 females and 2763 males were caught. On average 12.1 females and 28.5 males per trap were caught in traps baited with 500 mg AA and 50 mg 2-PET. Changing the load of 2-PET to 500 or 5 did not affect trap catches of either sex (Fig. 1). However, a 100-fold reduction of the AA load halved the catch of *L. botrana* compared to 500:500 (Fig. 2). A similar ratio of males vs females were caught in all traps baited with any AA:2-PET load (Fig. 3). The number of

Compound		Chemical class					A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15
Vial for AA and 2-PET (D = different, S = Same)							D	S	D	D	D	D	D	D	D	D	D	—	S	S	—
acetic acid (AA)		acid					500	500	500	500	500	500	500	500	500	500	500	—	500	500	—
2-phenylethanol (2-PET)		benzene and subs. der.					50	50	50	50	50	50	50	50	50	50	50	—	50	50	—
ethanol		alcohol					—	—	100	100	—	100	—	—	—	—	—	100	—	—	—
3-methyl-1-butanol		alcohol					—	—	100	100	—	—	100	—	—	—	—	100	100	—	—
ethyl acetate		ester					—	—	100	100	—	—	—	100	—	—	—	100	—	—	—
isoamyl acetate		ester					—	—	100	—	—	—	—	—	—	100	—	—	—	—	—
isobutyl acetate		ester					—	—	100	—	—	—	—	—	—	100	—	—	—	—	—
methyl acetate		ester					—	—	100	—	—	—	—	—	—	100	—	—	—	—	—
isobutanol		alcohol					—	—	100	—	—	—	—	—	100	—	—	—	—	100	—
3-methyl-3-buten-1-ol		alcohol					—	—	100	—	—	—	—	—	100	—	—	—	—	100	—
acetaldehyde		aldehyde					—	—	100	—	—	—	—	—	—	—	100	—	—	—	—
benzaldehyde		aldehyde					—	—	100	—	—	—	—	—	—	—	100	—	—	—	—
acetoin		acyloins					—	—	—	—	100	—	—	—	—	—	—	—	—		—
Order	**Family**	**Species**	**Stat**	**p-val**	**χ2**	**P χ**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**	**A11**	**A12**	**A13**	**A14**	**A15**
Lepidoptera	Tortricidae	*Lobesia botrana* (female)	P	0.927	18.9	0.927	5 a	10 a	9 a	2 a	5 a	4 a	6 a	1 a	6 a	1 a	—	—	—	—	—
Lepidoptera	Tortricidae	*Lobesia botrana* (male)	P	0.588	12.4	0.588	1 a	6 a	5 a	4 a	6 a	3 a	2 a	1 a	4 a	2 a	3 a	—	1 a	—	—
Lepidoptera	Tortricidae	*Lobesia botrana* (total)	P	0.204	36.2	0.204	6 a	16 a	14 a	6 a	11 a	7 a	8 a	2 a	10 a	3 a	3 a	—	1 a	—	—
Coleoptera	Coccinellidae	*Coccinellidae*	—				—	1	—	—	2	—	—	—	1	—	—	—	—	—	—
Diptera	Muscidae	*Musca spp.*	NB	0.000	333.1	0.000	11 ab	6 a	175 ef	122 def	20 ab	77 ce	10 ab	40 bc	174 ef	20 ab	5 a	18 ac	8 acd	117 f	6 a
Diptera	Syrphidae	Syrphidae	—				—	—	—	—	—	1	—	—	—	—	—	—	—	—	—
Hemiptera	Auchenrorrchyncha (suborder)	Auchenrorrchyncha	—				—	—	—	—	—	—	—	—	—	1	—	—	—	—	—
Lepidoptera	Geometridae	*Ematurga atomaria*	—				—	—	2	1	—	—	1	—	—	—	1	—	3	—	—
Lepidoptera	Noctuidae	*Acronicta psi*	—				—	—	—	—	—	—	—	—	—	—	—	—	1	—	—
Lepidoptera	Noctuidae	*Agrotis exclamationis*	P	0.168	6.7	0.168	—	—	7 a	3 a	—	1 a	4 a	—	2 a	—	—	—	3 a	2 a	—
Lepidoptera	Noctuidae	*Dypterygia scabriuscula*	P	0.220	24.4	0.22	2 a	1 a	7 a	4 a	—	1 a	11 a	—	5 a	4 a	—	1 a	3 a	1 a	—
Lepidoptera	Noctuidae	*Dysgonia algira*	—				—	—	3	1	—	—	—	—	—	—	—	1	—	—	—
Lepidoptera	Noctuidae	*Lacanobia oleracea*	P	0.242	9.1	0.242	—	—	—	1 a	—	1 a	7 a	—	3 a	1 a	—	—	2 a	—	—
Lepidoptera	Noctuidae	*Mythimna albipuncta*	P	0.830	8.0	0.83	—	—	7 a	8 a	1 a	—	8 a	—	—	5 a	—	—	6 a	5 a	—
Lepidoptera	Noctuidae	Noctuidae	P	0.621	2.2	0.621	1 a	1 a	2 a	3 a	—	—	1 a	—	2 a	1 a	—	—	—	—	—
Lepidoptera	Noctuidae	*Trachea atriplicis*	—				—	—	—	—	—	—	1	—	1	—	—	—	—	1	—
Lepidoptera	Nymphalidae	*Apatura irisis*	—				—	—	—	—	—	—	1	—	—	1	—	—	—	—	—
Lepidoptera	Pyralidae	*Hypsopygia costalis*	P	0.862	27.6	0.862	—	—	3 a	18 b	—	—	10 ab	—	3 a	1 a	—	—	5 ab	—	—
Lepidoptera	Pyralidae	Pyralidae	—				—	—	1	1	—	—	1	—	2	—	—	—	1	2	—
Lepidoptera	Pyralidae	*Pyralis farinalis*	—				—	—	—	1	—	—	3	—	—	—	—	—	1	—	—
Lepidoptera	Sphingidae	*Deilephila porcellus*	—				—	—	1	—	—	—	—	—	—	—	—	—	—	—	—
Lepidoptera	Thyatiridae	*Habrosyne pyriotides*	—				—	—	—	3	—	—	—	—	—	—	—	—	—	—	—
Lepidoptera	Thyatiridae	*Tethea ocularis*	—				—	—	1	—	—	—	1	—	—	—	—	—	—	—	—
Lepidoptera	Thyatiridae	*Thyatira batis*	—				—	—	—	1	—	—	—	—	—	—	—	—	—	1	—
Lepidoptera	Thyatiridae	Thyatiridae	—				—	—	2	—	—	1	—	—	—	—	—	—	—	—	—
Lepidoptera	Tortricidae	*Hedya pruniana*	P	0.775	53.2	0.775	2 a	3 a	20 a	9 a	2 a	2 a	10 a	2 a	2 a	3 a	3 a	—	2 a	—	1 a
Lepidoptera	Tortricidae	*Olethreutes arcuella*	—				—	—	—	2	—	—	—	—	—	—	—	—	—	—	—
Lepidoptera	Tortricidae	*Ptycholoma lecheana*	—				1	—	—	1	1	—	—	2	—	—	1	—	—	—	—
Lepidoptera	Tortricidae	*Tortrix viridana*	—				—	—	1	—	1	—	—	1	—	—	—	—	—	—	—
Neuroptera	Chrysopidae	*Chrysoperla spp*	—				1	—	—	—	—	—	—	1	—	—	—	—	—	—	—

Table 1. Target and non-target insect species caught in traps during the first flight (May 3–17, 2018). Tested blends: A1-A15. Stat: Poisson (P) or negative binomial (NB) distribution. P-val: probability value for overdispersion with poisson distribution. Σ2: chi-square value for factor treatment, PΣ: probability for the differences between treatments.

Compound		Chemical class					B1	B2	B3	B4	B5	B6	B7
Vial for AA and 2-PET (D = different, S = Same)							D	S	S	S	S	S	—
acetic acid (AA)		acid					500	500	500	500	500	500	—
2-phenylethanol (2-PET)		benzene and subs. der.					50	50	50	50	50	—	
ethanol		alcohol					—	—	100	100	—	—	—
3-methyl-1-butanol		alcohol					—	—	100	100	—	—	—
ethyl acetate		ester					—	—	100	100	—	—	—
isoamyl acetate		ester					—	—	100	—	—	—	
isobutyl acetate		ester					—	—	100	—	—	—	—
methyl acetate		ester					—	—	100	—	—	—	—
isobutanol		alcohol					—	—	100	—	100	—	—
3-methyl-3-buten-1-ol		alcohol					—	—	100	—	100	—	—
acetaldehyde		aldehyde					—	—	100	—	—	—	—
benzaldehyde		aldehyde					—	—	100	—	—	—	—
acetoin		acyloins					—	—	100	—	—	100	—
Order	**Family**	**Species**	**Stat**	**P-val**	χ^2	**P** χ	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**
Lepidoptera	Tortricidae	*Lobesia botrana* (female)	NB	0.001	13.9	0.016	40 a	18 a	43 a	26 a	17 a	19 a	—
Lepidoptera	Tortricidae	*Lobesia botrana* (male)	P	0.895	37.6	0.000	25 b	11 ab	24 b	9 ab	11 ab	17 ab	1 a
Lepidoptera	Tortricidae	*Lobesia botrana* (total)	NB	0.000	71.2	0.000	65 b	29 b	67 b	35 b	28 b	36 b	1 a
Coleoptera	Coccinellidae	*Harmonia axyridis*	—				1	—	—	—	—	—	—
Diptera	Culicidae	Culicidae	—				—	—	—	—	—	—	1
Diptera	Muscidae	*Musca* spp.	NB	0.000	85.1	0.000	—	2 a	36 bc	11 ab	108 c	4 a	1 a
Hemiptera	Auchenrorrchyncha (suborder)	Auchenrorrchyncha	P	0.918	3.9	0.562	3 a	5 a	—	2 a	1 a	2 a	4 a
Hymenoptera	Apoidea (old family)	Apoidea	—				—	—	—	1	—	—	—
Hymenoptera	Vespidae	Vespidae	—				1	—	—	—	—	1	—
Lepidoptera	Noctuidae	*Autographa gamma*	—				1	—	—	—	—	—	—
Lepidoptera	Papilionidae	Papilionidae	—				—	—	1	—	3	—	—
Lepidoptera	Pyralidae	Pyralidae	—				1	—	—	—	—	—	—
Lepidoptera	Tortricidae	Tortricidae	P	0.120	18.7	0.002	5 a	2 a	13 a	1 a	—	2 a	3 a
Neuroptera	Chrysopidae	*Chrysoperla* spp.	NB	0.026	9.6	0.142	10 a	12 a	6 a	5 a	4 a	4 a	1 a

Table 2. Target and non-target insect species caught during the second flight (June 14 - July 5 2018). Tested blends: B1-B7. Stat: Poisson (P) or negative binomial (NB) distribution. P-val: probability value for overdispersion with poisson distribution. Σ2: chi-square value for factor treatment, PΣ: probability for the differences between treatments.

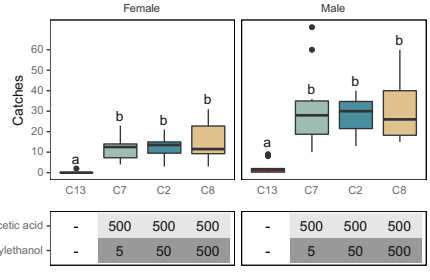

Figure 1. Comparison of capture rates of *L. botrana* males and females with a 2-component lure with an increasing load (mg) of 2-PET. Experiments were carried out in 2018 (August 2–22). Bars with different letters differ significantly. A total of 394 females and 929 males were caught.

captures did not differ when AA and 2-PET were loaded within the same or in two separate vials (Fig. 3). Male captures in sex-pheromone traps (275 males/trap) exceeded those of AA:2-PET treatments. Because sex-pheromone traps were placed in a field nearby the one where microbial volatiles were tested, the number of caught males cannot directly be correlated to the catches of the microbial lures. However, it represents an estimation of the population level (Fig. 5).

Compound		Chemical class					C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
Vial for AA and 2-PET (D = different, S = Same)							D	S	S	S	S	S	S	S	S	S	S	—
acetic acid (AA)		acid					500	500	500	500	500	500	500	500	50	5	50	—
2-phenylethanol (2-PET)		benzene and subs. der.					50	50	50	50	50	50	5	500	50	500	500	—
ethanol		alcohol					—	—	100	100	—	—	—	—	—	—	—	—
3-methyl-1-butanol		alcohol					—	—	100	100	—	—	—	—	—	—	—	—
ethyl acetate		ester					—	—	100	100	—	—	—	—	—	—	—	—
isoamyl acetate		ester					—	—	100	—	100	—	—	—	—	—	—	—
isobutyl acetate		ester					—	—	100	—	100	—	—	—	—	—	—	—
methyl acetate		ester					—	—	100	—	100	—	—	—	—	—	—	—
isobutanol		alcohol					—	—	100	—	—	—	—	—	—	—	—	—
3-methyl-3-buten-1-ol		alcohol					—	—	100	—	—	—	—	—	—	—	—	—
acetaldehyde		aldehyde					—	—	100	—	—	100	—	—	—	—	—	—
benzaldehyde		aldehyde					—	—	100	—	—	100	—	—	—	—	—	—
acetoin		acyloins					—	—	100	—	—	—	—	—	—	—	—	—
Order	**Family**	**Species**	**Stat**	**p-val**	**χ2**	**P χ**	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**
Lepidoptera	Tortricidae	*Lobesia botrana* (female)	NB	0.000	142.6	0.000	129 c	121 c	160 c	81 bc	96 c	104 c	117 c	154 c	86 c	33 b	75 bc	2 a
Lepidoptera	Tortricidae	*Lobesia botrana* (male)	NB	0.000	136	0.000	290 cd	285 cd	383 d	179 bc	271 cd	216 bd	321 cd	298 cd	211 bd	112 b	172 bc	25 a
Lepidoptera	Tortricidae	*Lobesia botrana* (total)	NB	0.000	170.5	0.000	419 cd	406 cd	543 d	260 bc	367 cd	320 cd	438 cd	452 cd	297 bd	145 b	247 bc	27 a
Coleoptera	Coccinellidae	Coccinellidae	—				1	—	1	1	2	1	1	—	1	—	1	1
Coleoptera	Coccinellidae	*Harmonia axyridis*	—				—	—	—	—	—	—	—	—	—	1	—	—
Diptera	Drosophilidae	*Drosophila* spp.	P	0.053	18.3	0.000	—	—	68 b	27 a	—	—	—	—	—	—	—	—
Diptera	Muscidae	*Musca* spp.	P	0.060	124.9	0.000	2 a	4 a	27 b	39 b	3 a	3 a	3 a	—	—	2 a	1 a	—
Hemiptera	Flatidae	Flatidae	P	0.184	7	0.800	7 a	5 a	7 a	5 a	2 a	6 a	8 a	3 a	6 a	7 a	7 a	5 a
Hymenoptera	Vespidae	Vespidae	—				2	—	1	1	—	—	—	1	—	1	—	1
Lepidoptera	Drepanidae	*Habrosyne pyriotides*	—				—	—	—	1	—	—	—	—	—	—	—	—
Lepidoptera	Erebidae	*Grammodes geometrica*	—				—	—	—	—	1	—	—	—	—	—	—	—
Lepidoptera	Noctuidae	*Agrotis exclamationis*	—				—	—	1	2	—	—	—	1	—	1	—	—
Lepidoptera	Noctuidae	*Autographa gamma*	—				—	—	—	—	—	—	—	1	—	1	—	—
Lepidoptera	Noctuidae	*Dypterygia scabriuscula*	P	0.998	3.8	0.875	2 a	5 a	1 a	2 a	3 a	—	2 a	3 a	3 a	—	2 a	—
Lepidoptera	Noctuidae	*Mythimna albipuncta*	—				—	—	1	1	1	—	—	—	—	—	—	—
Lepidoptera	Noctuidae	*Trachea atriplicis*	—				—	—	1	1	—	—	—	—	—	—	—	—
Lepidoptera	Pyralidae	*Hypsopygia costalis*	P	0.058	126.2	0.000	—	1 a	48 b	55 b	—	—	—	—	1 a	—	—	—
Lepidoptera	Pyralidae	*Pyralis farinalis*	—				—	—	1	—	—	—	—	—	—	—	—	—
Lepidoptera	Tortricidae	*Pandemis* spp.	—				—	1	—	—	1	2	—	—	—	—	—	—
Lepidoptera	Tortricidae	Tortricidae	—				—	—	1	3	—	—	—	—	—	1	—	—
Neuroptera	Chrysopidae	*Chrysoperla* spp.	P	0.123	80.7	0.000	4 ab	2 b	7 ab	—	2 b	3 b	—	29 c	10 bc	16 bc	21 ac	1 ab

Table 3. Target and non-target insect species caught in traps during the third flight (August 3–22, 2018). Tested blends: C1-C12. Stat: Poisson (P) or negative binomial (NB) distribution. P-val: probability value for overdispersion with poisson distribution. Σ2: chi-square value for factor treatment, PΣ: probability for the differences between treatments.

Addition of major microbial compounds. The addition of major fermentation compounds released from inoculated grapes (see materials and methods and[16]) including ethanol, 3-methyl-1-butanol and ethyl acetate at 100 mg each did not improve female attraction to the two-component blend of 500 mg AA and 50 mg 2-PET (Fig. 4). Esters (isoamyl acetate, isobutyl acetate, methyl acetate), aldehydes (acetaldehyde, benzaldehyde) or acetoin added to the two-component blend of AA and 2-PET did not improve catches of either sex compared to the two-component blend (Fig. 4). A lower number of males was captured by the 5-component in comparison with the 13-component blend (Fig. 4).

Bycatches. The composition of the lures strongly affected the specificity of the catch. Depending on the lure, considerable numbers of Diptera (particularly Muscidae and Tephritidae) and Lepidoptera were caught. We analyzed the specificity of the lures by expressing it as a percentage of the *L. botrana* catches, which demonstrates that specificity as a function of target species decreases with the increasing number of components in the blend (Figs. 5 and 6). The specificity of the lures was also affected by the sampling period. During the first flight period, the complex blend had a very low specificity (2-4% only). This was largely due to a combination of low *L. botrana* populations and relatively high captures of other taxa. Conversely, during the third flight period, the high population of *L. botrana* increased the specificity of all lures. Over the entire season, the 2-component blend was more specific (53-58-96%) than the major compounds (2-64-65%) or the complex blend (4-54-77%), with minor differences detected during the second flight (Fig. 5).

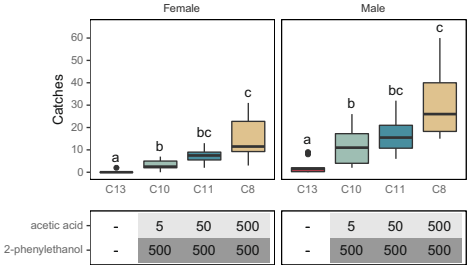

Figure 2. Comparison of capture rates of *L. botrana* males and females with a 2-component lure with an increasing load (mg) of AA. Experiments were carried out in 2018 (August 2–22). Bars with different letters differ significantly. A total of 264 females and 607 males were caught.

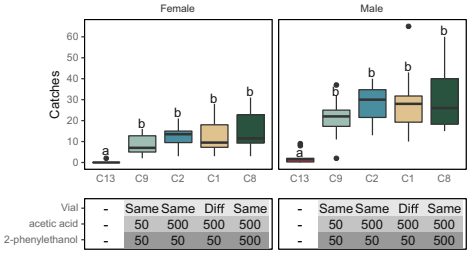

Figure 3. Boxplot of capture rates of *L. botrana* male and female in traps baited with AA and 2-PET (mg) loaded in the same (Same) or in two different (Diff) vials. Experiments were carried out in 2018 (August 2–22). Bars with different letters differ significantly. A total of 492 females and 1109 males were caught.

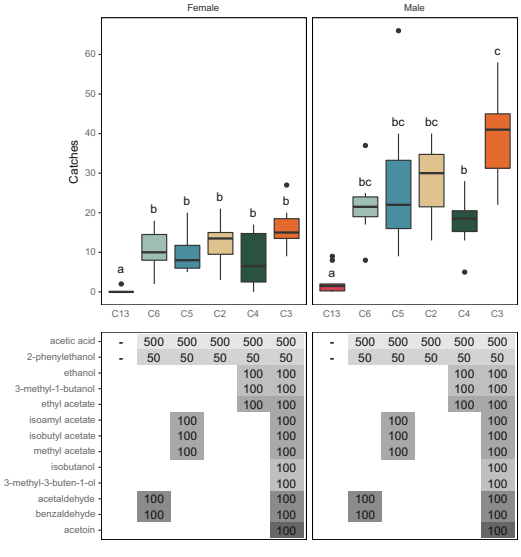

Figure 4. Boxplot of capture rates of *L. botrana* males and females in traps baited with different blends of microbial volatiles (mg). Experiments were carried out in 2018 (August 2–22). Bars with different letters differ significantly. A total of 564 females and 1359 males were caught.

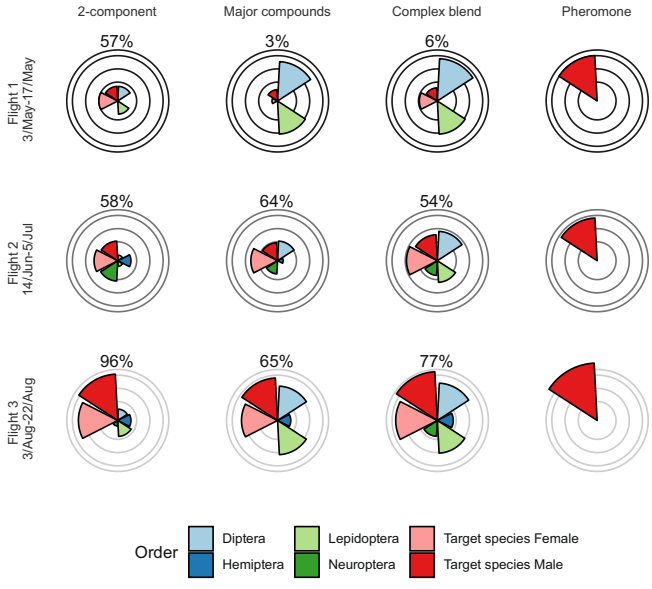

Figure 5. Total captures (square root transformed) of *Lobesia botrana* (target species) and other insect orders during three flight periods within the same fields. The flight period is presented on the y-axis. Concentric lines indicate 10, 50, 250 and 500 insects caught. The percentage of target species caught is indicated at the top of each radial plot. The composition of each of the three blends can be found in Fig. 4 (last three bars) and Table 3. The 2-component lure consisted of 500 mg AA and 50 mg 2-PET loaded in the same vial. The radial plots furthest to the right represents the number of *L. botrana* males caught in sex-pheromone traps during the three flight periods. The comparison of male catches between sex-pheromone and kairomone traps should be done with caution, because the two types of traps were placed within neighbouring plots to avoid interference with each other.

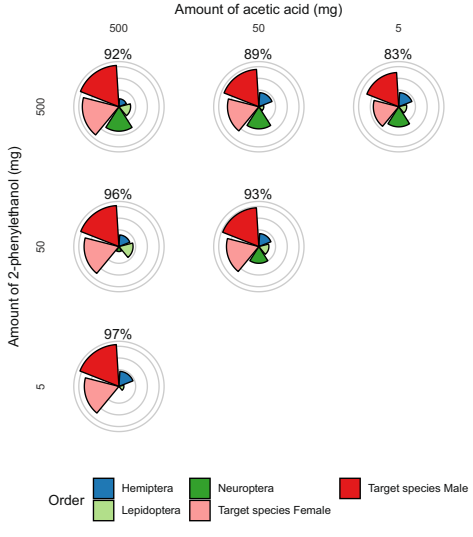

Figure 6. Total captures (square root transformed) of *Lobesia botrana* (target species) and other species during the third flight in traps baited with a different load of AA and 2-PET. Concentric lines indicate 10, 50, 250 and 500 insects caught. The percentage of target species caught is indicated at the top of each radial plot.

We further assessed specificity as a function of the ratio of the AA/2-PET blend in the third flight. Increasing the ratio of acetic acid led to an increase in the capture rate of *L. botrana* (Fig. 6). Conversely, bycatches, particularly the capture rate of lacewings, increased as a function of 2-PET load in the same blend.

Discussion

Whereas it is generally agreed that modern agriculture needs a sustainability overhaul, the best trajectory to sustainable production is less clear and progress in sustainable innovation is slow. Today, control of pests and diseases still relies heavily on cover sprays. Innovations are sorely needed that selectively target pests and reduce or eliminate cover sprays, minimising the impact on an already dwindling insect community[2,24]. Odor-based methods offer this perspective through selectively attracting or confusing target insects. Lures laced with attractants, phagostimulants and small amounts of insecticides can selectively target pest species, while their specificity avoids bycatches from the food web. Unfortunately, bycatches are not consistently reported, which makes evaluation of lures in terms of sustainable control of pests difficult. Although lures have been reported for numerous pest insects, they may be broadly attractive and similarly to insecticides, impact non-target species.

In our study, we empirically evaluated the effect of ratio, release rates and composition of a lure[16] on capture of *L. botrana* and analysed the concurrent effect on specificity. The lures attracted both male and female *L. botrana*, and could be used to support pheromone-based intervention methods. Aiming to further increase the effectiveness of the lure, we found that such increases may come at the expense of specificity of the lure. Increased release rates and blend complexity strongly decreased specificity, while not always increasing attractiveness to *L. botrana*. There thus appears to be a tradeoff between attractiveness and specificity, and the 'most attractive' in terms of total catch may not necessarily be the 'most attractive' in terms of specificity and thus sustainability.

A two-component blend alone can selectively attract *L. botrana*. Based on previous work with AA and 2-PET, where considerable numbers of *L. botrana* were caught[16], we assessed whether the lure's attractiveness could be further enhanced by changing the release rate and ratio of AA and 2-PET. Both 2-PET and AA appeared to be necessary for capturing *L. botrana*. The two compounds synergize with each other, reminiscent of components in a pheromone blend, where frequently small amounts are necessary and sufficient to increase attraction[25]. However, in another study in Hungary, 2-PET did not synergize with AA[25], although the authors used a much higher dose of AA (3000 mg instead of 500 mg) and another dispensing technique, making the results hard to compare with our study. It is rather surprising that a lure consisting of so few and such generic fermentation volatiles can be so selective. Acetic acid is a common fermentation volatile and indeed a constituent of lures for diverse insect taxa, including flies, moths, lacewings and wasps[26–31]. 2-PET is another rather general microbial volatile. It indicates the breakdown of phenylalanine and thus a protein source, with similar or derived compounds attracting various insect taxa[25,29,32,33]. That a combination of these two can be selective, may indicate that even though insects commonly rely on fermentation volatiles for adult feeding, the olfactory circuitry of different species key into different components in orientation. This is supported by recent work on tephritid fruit flies[34], where an ecological niche-driven divergence in the detection of fruit volatiles was measured, in spite of these sources generically being attractive to all species tested.

Besides, differential tuning to fermentation volatiles, the high selectivity of the 2-component blend to *L. botrana* (Fig. 6) may also result from its dominant presence in the vineyard, whereas selectively would be much lower in situations where this is not the case. The fact that in early season catches (1st and 2nd flight) selectivity was dramatically lower, underlines this. Claims about a lure's selectivity thus need verification throughout the flight season and possibly in different geographical areas.

A higher load of AA increased capture rates of *L. botrana* while higher 2-PET loads increased capture rates of lacewings. This underlines that research should not solely focus on increasing capture rates of the target insect species, but carefully balance ratio, load and composition to reduce bycatches.

Other fermentation volatiles lacked synergy, and decreased specificity of AA and 2-PET. As a blend consisting of only AA and 2-PET is far removed from a fermentation volatile mimic, we reasoned that addition of other fermenting volatiles could perhaps synergize the 2-component blend. Numerous reports have shown fermentation-based blends with quite different constituents, though often containing AA, ethyl acetate and primary alcohols, as being attractive to other insect taxa[26,35]. Among moth species, leafrollers have received considerable attention with studies on *Archips, Cydia, Pandemis*, *Spilonota*, *Epiphyas* and *Choristoneura* spp.[36,37]. Attractants comprised both constitutive plant compounds such as pear ester and induced volatiles released upon feeding damage by leafroller larvae, such as 2-PET, benzyl alcohol and benzyl cyanide. However, plant volatiles only significantly attracted when combined with AA[36,38].

Surprisingly, however, in our study none of the fermentation volatiles (as identified in[16]) increased catches of *L. botrana*. A number of reasons could underlie this. As we only tested a single load and ratio, we cannot exclude that other doses and ratios would have induced increased capture rates. Furthermore, the release rates and strong synergistic effect of 2-PET on AA may have obscured additive effects of the additional fermentation volatiles. Finally, the release rates of the compounds from the vials may have differed considerably, something that was not verified in this study. Future work could expand on the current by evaluating these factors.

Although the additional fermentation volatiles did not increase *L. botrana* captures, they did significantly increase attraction of other insect taxa, among which other pests: adding alcohols attracts *Musca* spp.; adding major fermentation compounds attracted Tephritidae in the first flight and *Drosophila* spp. in the last. The highest catches for all species were observed with a complex blend of 13 compounds. We also confirm a synergy between AA and 3-methyl-1-butanol for *Hypsopygia costalis* (Lepidoptera: Pyralidae[28,39];). Apparently, lures can be designed from generic fermentation volatiles, that, depending on their composition and release rate, can be

selective for certain insect taxa. These results only further underline the significance of the synergy between AA and 2-PET for *L. botrana* specifically.

We suggest that future studies such as this one should always carefully analyze blend ratio, composition and release rates to optimize not only attractiveness for the target pest, but also selectivity to avoid non-target species.

Conclusions

This study demonstrates that in spite of our expectations, lures consisting of only two components were sufficient to capture *L. botrana* males and females. Addition of other microbial volatiles did not enhance attraction. The fact that a very limited number of volatiles can selectively attract certain insect species, offers a perspective that selective lures can be developed, not only based on host-plant specific odors, but can even be derived from generically attractive substrates such as fermentation sources. Such selective lures will greatly support monitoring, by reducing or eliminating the need for identification of catches. In addition, the large numbers caught offer the perspective of the use of such lures in sustainable control, by targeting the damaging sex directly (in e.g. attract & kill), rather than indirectly (such as e.g. mating disruption). Further optimization of attractiveness, specificity, as well as the dispensing technology of the volatile components are needed, while on-going research on volatiles from (induced) hosts and microbial breakdown may provide additional volatile candidates for this.

With moth pheromones as hallmark of attractiveness, chemical ecologists readily focus on finding 'highly attractive' lures with a similar potency. However, orientation to feeding and oviposition substrates occurs continually over a moth's lifetime, in contrast to mate orientation. Accordingly, the probability of contacting moderately attractive food or oviposition lure is arguably much higher, as long as they are well dispersed throughout a crop[40,41]. Selective lures with reasonable attractiveness, such as the one described, may thus be 'good enough' from a control perspective, while simultaneously highly desirable from a sustainability perspective.

Finally, in our study, we took great care to analyze not only catches of *L. botrana*, but also bycatches. These show that specificity can readily be compromised by changes in release rates, ratios and composition of lures. Unfortunately, there exists as of yet no strong tradition for optimizing for specificity, or for reporting bycatches. There are neither standardized procedures (how/frequency to sample, season, geographic locations) or statistics (to what taxonomic level, what diversity indexes, etc.) with which to express these bycatches. This hampers comparing results across studies and lures, and obstructs the evaluation of results in terms of sustainability. Yet, reporting on bycatches can accelerate the development of selective lures for other pest species, such as reported here for dipteran and lepidopteran. We suggest that future studies always report on bycatches to accelerate sustainable innovations in pest control.

Data availability

All data analysed during this study are included in this published article (and its Supplementary Information files).

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Author contributions

P.R. led the field experiments with the help of G.K., M.O.Sz, S.L.H. and B.P.M. P.R. led the work of identifying the caught species with the help of G.K. and B.P.M. S.L.H. analyzed the data. M.T., T.D. and S.L.H. wrote the paper. B.P.M., T.D. and M.T. procured funding. All authors contributed to idea development, experimental design, improved the manuscript and approved the submitted version.

Competing interests

The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to S.L.H.

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III

Paper III - Translating olfactomes into attractants: shared volatiles provide attractive bridges for polyphagy in fruit flies

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LETTER

Translating olfactomes into attractants: shared volatiles provide attractive bridges for polyphagy in fruit flies

Tibebe Dejene Biasazin,[1,2] Sebastian Larsson Herrera,[1] Fikira Kimbokota,[1,3] and Teun Dekker[1]*

[1]*Chemical Ecology Unit Department of Plant Protection Biology Swedish University of Agricultural Sciences Box 102, SE-230 53, Alnarp, Sweden*
[2]*Department of Zoological Sciences Addis Ababa University P.O. Box 1176, Addis Ababa, Ethiopia*
[3]*Mkwawa University College of Education (MUCE) P.O. Box 2513, Iringa, Tanzania*

**Correspondence: E-mail: teun.dekker@slu.se*

Abstract

Tephritid flies are serious fruit pests. Despite clear niche differences, many species show considerable overlap in fruit preferences, of which we here analysed the olfactory correlate. Using the volatiles of four unrelated fruit species, antennal responses were quantified to construct a fruit-odour response database for four tephritid species. Although responses were distinct with a significant niche-correlated bias, the analyses show that the probability of detection of a volatile strongly increased with its sharedness across fruits. This also held for the unrelated fruit fly *Drosophila melanogaster* (DoOR repository-based analyses). We conjectured that shared volatiles signify 'host' to the fly 'nose' and induce attraction. Indeed, blends of volatiles shared by fruit and detected by all four species were very attractive for tephritid species, more than fruits. Quantitative whole antennal recordings en lieu of, or complementing bottom-up molecular neurogenetic approaches, enables comparative olfactomics in non-model species, and facilitate interpretation of olfaction in evolutionary, ecological, and applied contexts.

Keywords

Attractants, behaviour, drosophila, electrophysiology, olfactome, polyphagy, tephritidae, volatilome.

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INTRODUCTION

The ecological breadth of a species is intimately linked to its ability to detect and respond to features characteristic for its niche. Odour cues are key in this, particularly for short-lived arthropods, as the multidimensionality of olfaction provides for a solid means to hardwire 'niche'. Chemical ecology aims to harness this to identify habitat cues used by a certain species and use this in developing lures for monitoring and control of pests. To what extent the olfactory circuit delimits niche breadth, or instead promote niche shifts, is not well-understood. Comparative olfactory response databases (olfactomes) can provide a means to address this question, and at the same time offer a much needed tool to accelerate the rational design of lures for monitoring and control of insect pests. Though, such databases seem to be slowed by the apparent need for molecular neurogenetics, which is constrained for most species by the costs and the rate it returns data, although steady progress is being made (De Fouchier *et al*. 2017). This has limited the number of such databases, with *Drosophila melanogaster*'s (Meigen) being by far the most comprehensive olfactome (Münch & Galizia 2016). Here, we tested the possibility to build olfactory response databases in non-model organisms by creating quantitative antennal response databases, by aligning these with the olfactome of a model species, and by extracting attractants.

We focussed on true fruit fly (Tephritidae) pests that are threatening horticultural production in many parts of the world. Recent invasions in new territories, including Africa, seriously affect income of smallholder farmers and export through quarantine regulation. *Bactrocera dorsalis* (Hendel) and its close relative *Bactrocera zonata* (Saunders) are highly invasive polyphagous species with a substantial overlap in niche and a preference for mango (De Meyer & Ekesi 2016). *Zeugodacus cucurbitae* (Coquillett), has a strong preference for cucurbitaceous vines, ovipositing in its fruits as well as vegetable parts. *Z. cucurbitae* also infests numerous other crops, including fruit trees (Vayssières *et al*. 2007; De Meyer *et al*. 2015; see also Virgilio *et al*. 2015 for a robust phylogenetic analysis of tephritid species). In Africa, these three invasive species aggravated an existing problem caused by native Tephritidae species, such as *Ceratitis capitata* (Wiedemann), which is also polyphagous and highly invasive outside Africa (Carey 1991). Although polyphagous tephritid species may attack a variety of fruit species, many species manifest a preference for a certain few hosts (Aluja & Mangan 2008; Rwomushana *et al*. 2008). Cutting across ecology, the above four species also include different degrees of relatedness, from within subgenus (Bd–Bz), between subgenera (Bd/Bz–Zc) and tribes (Bd/Bz/Zc–Cc). This permits asking whether olfactory tuning is shaped more by ecology or evolution.

Much of the leading edge research in insect olfaction revolves around the fruit fly *Drosophila melanogaster*, a distantly related species through the infraorder Muscomorpha that evolved a fruit-odour preference independently of Tephritidae. In contrast, our knowledge of tephritid olfaction is rather fragmentary (Fombong *et al*. 2016), in spite of their global economic significance. In semi-field trials, Cunningham *et al*. (2016) found a combination of only three short-chain aliphatic esters derived from guava that was attractive to the Queensland fruit fly, *Bactrocera tryoni* (Froggatt). While this does provide a horizon for using synthetic blends for monitoring or control of tephritids, the use of three highly related compounds leaves the vast majority of volatiles and their

cognate olfactory channels 'untapped' (Siderhurst & Jang 2006, 2010; Jayanthi *et al*. 2012; Biasazin *et al*. 2014).

A technique called gas chromatography-coupled electroantennographic detection (GC-EAD), is commonly used to screen for olfactory responses to volatiles of potential behavioural significance. Using the insect antenna as detector, it measures the sum potential differential in antenna in response to volatiles that sequentially elute of the GC column. While often used on single species, whole mount antennal recordings have occasionally been used to identify differences in sensitivities between closely related fly species and correlate these to their ecological niches (Linn *et al*. 2003). Differences in antennal responses between species may reflect, for instance, over-representation of certain classes of sensory neurons and concomitantly enlarged glomeruli, or differential tuning of these to niche-relevant odours, as observed in several Drosophilidae (Dekker *et al*. 2006; Ibba *et al*. 2010; Date *et al*. 2013; Linz *et al*. 2013; Goldman-Huertas *et al*. 2015; Jacob *et al*. 2017). However, GC-EAD studies are overwhelmingly qualitative rather than quantitative or comparative.

We explored how comparative and quantitative GC-EAD analyses can be used to create comprehensive fruit-odour response databases for non-model fruit fly species for evaluation in evolutionary ecological and behavioural contexts. We quantified olfactory sensitivities to fruits across fruit fly species, linked this to an outgroup species, *D. melanogaster*, extracted fruit-odour blends for testing in behavioural contexts, and evaluated how this could be used in evolutionary ecological studies as well as in the identification of attractants for use in pest control.

MATERIALS AND METHODS

Insects

Tephritid species originated from the International Atomic Energy Agency (IAEA) division of nuclear techniques in food and agriculture, Austria, Vienna. Adults were kept in polyester netting bugdorm cages ($325 \times 325 \times 325$ mm^3) at 26–29 °C, 60–65% RH and 12 : 12 light : dark cycle, with access to food (three-parts sugar, one-part yeast) and water (wet cotton). Mature flies were provided with an oviposition medium (Ekesi *et al*. 2007).

Experimental fruits and volatile collections

Four fruit species known to be attractive to *B. dorsalis* and other tephritid flies (Rwomushana *et al*. 2008) and which were readily available in Arbaminch (Ethiopia), were selected. Volatiles were sampled from freshly picked guava, *Psidium guajava* (L.) cv. 'locale', orange, *Citrus sinensis* (L.) cv. 'Valencia', mango, *Mangifera indica* (L.) cv. 'Kent' and banana *Musa acuminata* (Colla.) cv. 'Grand Nain' in polyethylene bags (Toppits Scandinavia AB, Helsingborg, Sweden). Charcoal-purified air entered the bags from the air pushing section of a pump (12 V, KNF-Neuberger, Freiburg, Germany). Teflon columns (*c*. 6 cm, ID 3 mm) filled with super Q adsorbents (35 mg mesh 80/100) were attached to a teflon tube at the sucking section of the pump. Aerations were run for 5 h at 1.0 L min^{-1}. Samples were eluted with n-hexane into 1.5 mL glass vials (Genetec AB, Sweden) and extracts stored at −20 °C.

Electrophysiology and compound identification

GC-EAD recordings from the tip of the antenna of 10–15 days old females were performed using protocols described in Biasazin *et al*. (2014). Gas chromatography coupled mass spectrometry (GC-MS) (Agilent 6890 GC and 5975 MS, Agilent Technologies Inc., Santa Clara, CA, USA) was used for identification. The GC-MS used a HP-5MS Ultra Inert capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA), with helium as carrier gas. GC-EAD-active peaks were identified using the Kovats' retention indices (KI) from GC-EAD, GC-MS and published records, and mass spectra were compared to three reference libraries: 'Alnarp 11', 'Wiley275' and the NIST 14. Synthetic compounds were used to confirm electrophysiological activity for 26 compounds and stereoisomers (Table S1). Remaining compounds were tentatively assigned to chemical classes using prominent and typical ion fragments and reference library suggestions.

Synthetic blends

For behavioural assays, we composed synthetic blends in paraffin oil from compounds detected by all species and shared by all fruits (first six compounds, 6-blend), or at least three fruits including mango (11-blend): (1) 2-methylpropyl-acetate > 97%, (2) ethyl butanoate > 97%, (3) 3-methylbutyl-acetate > 98%, (4) 2-methylpropyl-butanoate > 98%, (5) 3-methylbutyl-3-methylbutanoate > 98%, (6) 3-methylbutyl butanoate > 98%, (7) ethyl-hexanoate > 98%, (8) ethyl (*E*)-but-2-enoate > 98%, (9) (-)-beta-pinene > 99%, (10) ethyl-octanoate > 98%, (11) (R)-(+)-limonene > 93% (all purchased from Sigma Aldrich). Release rates from filter paper (Whatman Grade 1) were adjusted following solid-phase microextraction (SPME, DVB/CAR/PDMS, 50/30 µm; Supelco, Bellefonte, PA, USA) to closely match mango headspace (1 : 57 : 9 : 2 : 1 : 3 : 45 : 1 : 3 : 45 : 9).

Multi-choice olfactometer experiment

A glass cage ($420 \times 420 \times 420$ mm^3) was used for behavioural experiments (Fig. S1). A group of thirty 10–15 day-old females were starved for 12 h with access to water and kept in tubes (25×95 mm^2) and released into the arena. Flies could enter any of the six circularly arranged chambers (traps) on the top glass plate (50 mm diam.). The chambers were fitted with a metal cup (45 mm diam., 23 mm height) with three entry holes (6 mm), whereas the top of the chambers consisted of a disposable plastic wine cups (15 cl, Clas Ohlson, Sweden) with an airflow entering through the cut stem (see Fig. S1). A pump (Elite 802, Hagen Ltd, UK) and two glass wash bottles containing activated charcoal and distilled water provided purified and humidified air. A 0.5 L min^{-1} airflow reached each of six treatments contained in separate airtight polypropylene boxes (1.8 L) and entered

the arena via the holes in the metal cups. Teflon tubing was used throughout. Light (daylight lamp, Photo studio CFL 45 W, 5000K) was diffracted using an opaque white plexiglass panel. In each replicate preferences all treatments were tested against each other, including intact ripe orange and ripe banana, 10 μL paraffin oil containing the 11-blend in mango ratio (2-methylpropyl acetate at 5 ng μL^{-1} and remaining compounds at ratios indicated above), the 6-blend, γ-octalactone (100 ng μL^{-1}, an oviposition attractant, Jayanthi *et al.* 2014a,b) and paraffin oil (see Fig. S1). Treatments, including cups and connecting tubing, were rotated between experiments. Flies within each of the six chambers were counted after 5, 20, 30 and 60 min. Flies could exit the chambers, although this was infrequently observed. Statistical comparisons were therefore restricted to each time point.

Analysis

To make EAD responses across traces independent of absolute antennal sensitivities, we normalised each individual response through dividing it by the weighted average 'responsiveness' of a trace. This weighted average response was calculated as the back-transformed average of all log-transformed EAD responses within a trace. Absolute EAD responses to individual compounds were then averaged across traces. EAD values thus reflect a response relative to the average (being 1). For tile plotting we used ggplot2 (Wickham 2009). The heatmap of *D. melanogaster* was generated by multiplying the response to a compound of each antennal olfactory receptor (from the consensus response matrix of the DoOR database, Münch & Galizia 2016), by the number of sensory neurons expressing that receptor (Grabe *et al.* 2016), and summed across antennal receptors. Responses below 10% of the maximum response were excluded. For behavioural results a general linear model fitted with a poisson distribution (GLM) was used, followed by a pair-wise comparisons using multcomp (Hothorn *et al.* 2008). Analysis was performed in R (version 3.4.4) (R Core Team 2018). PCAs were performed using the pcomp function, while NMDS' were calculated using package vegan in R (Oksanen *et al.* 2018). For phylogenetic analysis of relationship between olfactory responses a presence/absence standardised Jaccard dissimilarity index was calculated using R package vegan (Oksanen *et al.* 2018) and plotted in ggdendro (De Vries & Ripley 2016). Traditionally used by community ecologists, we replaced species composition by olfactory responses to compounds in the headspace.

RESULTS

Headspace analysis

Autointegration with a threshold value of zero in the post-run chemstation analysis, and subtraction of a hexane blank, gave a total of 349 unique volatiles, with large qualitative and quantitative differences between fruits (Fig. S2, Table S1). Where possible, compounds were putatively assigned to functional classes using characteristic ions. Esters quantitatively dominated the headspace of all fruits (on average 57%), as well as qualitatively, with 43% of 133 compounds in mango being an ester, 30% of 125 compounds in guava, 30% of 93 compounds in orange and 28% of 155 compounds in banana (Fig. S2). Alkanes were the second most common in guava, orange and banana (20–30%), but low in mango (11%). The headspace of all fruits contained ~ 20% terpenoids (except for banana, 8%), 3.2%–8.3% alcohols and 4.3%–7.5% ketones. The putative ID of the remaining compounds were grouped under diverse classes: aldehydes, alkenes, amines, anhydrides, carboxylic acids, ethers, phenylpropanoids, 'other' (e.g. alkyl thiols, alkynes, anhydrides, benzenes and steroids), and unassigned compounds (Fig. S2, Table S1, see also Fig. 1 and Fig. S5). The headspace composition of the four fruit species were clearly distinct, with orange and guava being closer, and mango and banana furthest apart (Fig. S3a, see also Fig. S10).

Fruit-odour responses for four tephritid species

Using highly repeatable GC-EAD recordings, we subjected the antenna to volatiles in amounts as they are occurring in the headspace of fruit (white overlaid trace in the heatmap of Fig. 4). The resulting traces, heatmaps and analyses thereof reflect sensitivities to fruit volatiles in relative ratios in the fruit headspace, instead of absolute sensitivities. Antennal responses (Fig. 2, Fig. S4) show that the antennae of the four tephritid species detected a total of 111 unique compounds in the headspace extracts of the four fruits (Fig. 4, and Table S1 for a list of compounds, retention and Kovats indices). The antennal responses separated the fruit species in a similar fashion as the headspace, with orange and guava grouping closely together and mango and banana being more divergent (Fig. S3b). Responses were dominated by esters (58.6% of the 111 compounds, Fig. 1, see also Figs S5 and S6), followed by terpenoids (21.4%, with much variation between fruit species) and alcohols (8.25%). Although alkane species were abundant in the headspace, they comprised only 3.8% of antennal responses. The remaining 7.95% of responses to headspace volatiles was represented by other chemical classes (Fig. 1 and Figs S5 and S6).

Of the 111 detected compound, 56.8% elicited antennal response in all fruit fly species, 17.5% in three, and 7.4% in two species. The rest of the compounds (18.3%), were uniquely detected by single species (Fig. S2). Whereas the fly species thus shared the detection of many compounds, they differed in relative response strength to these (see heatmap Fig. 4 and Fig. S9) to the extent that the recordings of the four tephritid species separated well in a NMDS plot, with little within species variation (Fig. 2, Fig. S4). A Jaccard-Bray Curtis dissimilarity analysis shows that *Z. cucurbitae* was an outlier compared to the other three species (Fig. S10), owing to the strength of the responses to the various compounds, and the unique presence and absence of compounds in the olfacome of *Z. cucurbitae* ($n = 33$, $\chi^2 = 13.3$, $P = 0.008$ and $n = 14$, $\chi^2 = 9.7$, $P = 0.001$, resp.). *Z. cucurbitae* unilaterally lost the detection of eight fruity esters (61%, of $n = 13$ lost by *Z. cucurbitae*), but gained the detection of only one (18%, uniquely gained in Zc, of a total of six uniquely gained by *Z. cucurbitae*), indicating that the olfactory sensitivity of *Z. cucurbitae* is qualitatively diverging.

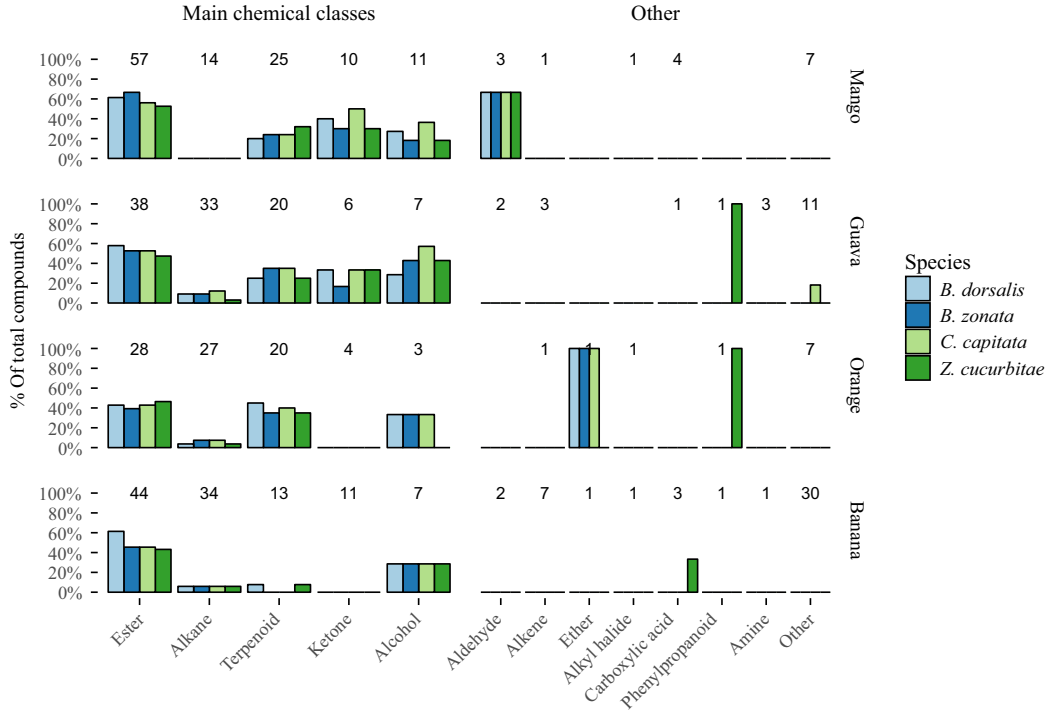

Figure 1 Likelihood of a volatile from a certain chemical class to induce an antennal response in a given fruit fly species (number in the olfactome/number in the volatilome). The number above each class of compounds represents the number of volatiles in that class. The graph demonstrates that esters not only dominate the headspace (see also Figs S1 and S3), but antenna were on average more tuned to these, as compared to volatiles from other chemical classes (see Figs S4 and S5 for additional analyses).

Esters disproportionally dominate responses and are disproportionately shared

We asked whether the dominance of esters (see above) was the result of ester species dominating the headspace of the fruits (volatilome bias), or whether tephritid olfactory circuits detect esters disproportionately over other compounds (olfactome bias). Whereas on average 32% of the volatiles from a fruit was an ester (Fig. 1, Figs S2 and S5), of those detected by the antenna, 69% was an ester (Fig. S5). Similarly, an average of 59% of the ester species released by any fruit was detected by the antenna, whereas this was 21% or less for other chemical classes (detailed per fruit in Fig. 1, Fig. S5). Furthermore, whereas the proportion of esters (either present in the headspace or detected) in each sharedness class (shared by 1, 2, 3 or 4 fruits, or fruit flies, respectively) was directly proportional to the fraction of the compounds in each of these classes (R^2 = 0,996, F = 13 671, $P < 0.0001$ and R^2 = 0.972, F = 70.0, $P < 0.01$, respectively), those compounds shared BOTH among fruits AND fruit flies (i.e., present in all, or at least in three fruits and detected by all fly species) were strongly ester biased (81% being an ester). Thus, tephritid fruit fly antennae appear to be disproportionately sensitive to shared esters qualitatively (% that induce sensory physiological responses), although not quantitatively (relative response strengths to esters were comparable to responses to other compounds, Figs S6–S8).

Volatiles shared by fruits are disproportionately detected by tephritids

Next, we analysed if there was a correlation between the number of fruits in which a volatile was found (sharedness of volatiles) and the likelihood of its detection by one or several fruit fly species (shared detection). A significant correlation (R^2 = 0.95, F = 40.29, P = 0.02) was found between the sharedness of a volatile and the probability that it induced an antennal response in any tephritid antenna. Similarly, for the 111 compounds detected by fruit flies, sharedness of detection increased with sharedness across fruits, being 100% for those volatiles shared by all fruits (R^2 = 0.99, F = 667, P = 0.001, Fig. 3), with a weak correlation between response strength and the sharedness across fruits (R^2 = 0.90, F = 17.64, P = 0.053). Tephritid antenna appear disproportionately tuned to detecting volatiles shared between fruits.

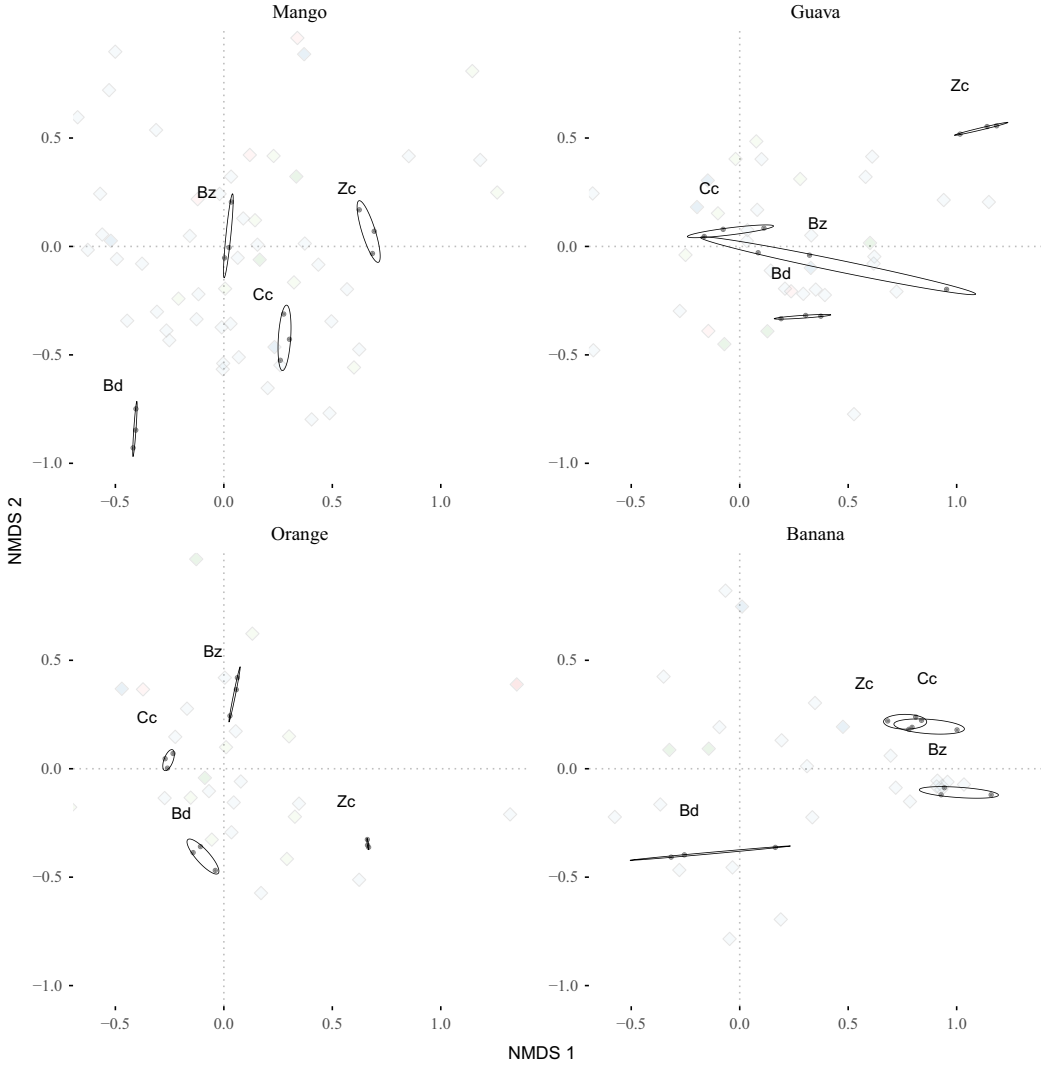

Figure 2 Nonmetric multidimensional scaling (NMDS) analysis of the compounds that induced antennal responses illustrates that the replicates of EAD runs within the same species are highly similar (with the exception of *Bactrocera zonata* in guava, possibly due to differences in recording electrode position), while distinct between species. The latter illustrates that, in spite of the large overlap in EAD responses between species (see also sample traces Fig. S4), the response profiles and olfactomes are highly distinct. Stress values of the 4 NMDS plots were between 0.10 and 0.17.

The olfactome of *D. melanogaster* is also tuned to shared fruit volatiles

To evaluate how the responses of the four tephritid species compared to the model fruit fly species *D. melanogaster*, which independently evolved a preference for fruit, we calculated provisional antennal responses of *D. melanogaster* from the online receptor response repository, DoOR (Münch & Galizia 2016). Compounds shared across fruits and across tephritid fly species (heatmaps Fig. 4 and Fig. S9, top of the heatplots) were also abundantly represented as ligands for *D. melanogaster* in the DoOR database. Conversely, fruit volatiles that were not shared across fruits were frequently either not present in the DoOR database (white), or induced no or poor responses (light blue) in *D. melanogaster* (Fig. 4, Fig. S9). Similar to tephritids, the probability of a response in *D. melanogaster* strongly correlated with the sharedness of a volatile among fruit species ($R^2 = 0.99$, $F = 199$, $P = 0.005$),

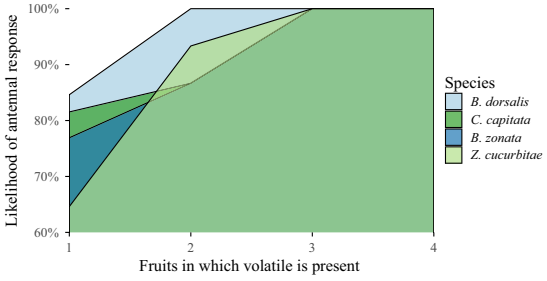

Figure 3 The likelihood of a compound to be detected by the antenna of a certain species increased with the 'sharedness' of the compound in the headspace of the four fruits. Detection was 100% for compounds found in all four fruits. All volatile compounds that were detected by at least one species (111 compounds) were included.

and was 100% for esters shared across four fruits (62% for all volatiles).

As the DoOR database was not directly comparable to the responses obtained in this study, we exposed *D. melanogaster* to a blend of the top 11 components (shared by 3 or 4 fruits, see 'Materials and Methods' and below – behaviour) in a ratio reflecting mango. Eight of 11 compounds in the ratios presented induced a response in *D. melanogaster*. A PCA analysis using the 11-component blend separated *D. melanogaster* weakly from the other species (Fig. 5 and Fig. S5).

A blend of shared compounds is more attractive than fruit odour

As sharedness of volatiles appeared to be strongly correlated with antennal responses in all fruit fly species, we conjectured that these shared compounds may constitute some sort of backbone of attraction (Fig. 6). We therefore composed synthetic blends based on compounds that were detected by all fruit fly species and shared by at least three fruits (nine esters + two terpenoids), or all four fruits (six compounds, all esters). We designed a novel multi-choice olfactometer, allowing gravid female flies to enter traps from which an airflow containing fruit odours flowed (Fig. S1). The blends were calibrated to ratios found in the headspace of mango, and tested on their attractiveness for female *B. dorsalis*, *Z. cucurbitae*, which is most distant to *B. dorsalis* regarding ecology and antennal responses (Fig. S10), and the 'unrelated' *D. melanogaster*. For *B. dorsalis* and *Z cucurbitae* these blends were more attractive than intact fruits (Fig. 6, see also Biasazin *et al.* 2014) and γ-octalactone, an oviposition attractant (Jayanthi *et al.* 2014a, see also Fig. S11). *D. melanogaster* was also attracted to the 6 and 11 blend, but preferred banana over these.

DISCUSSION

Today's olfactory research increasingly involves omics, using bottom up genetics and molecular tools to unravel the inner workings of olfaction. In insect olfaction, omics are largely led by studies on the olfactory model *par excellence*, *D. melanogaster*, providing an increasingly fine-grained understanding of the olfactome, the circuitry connectome and its translation into olfactory behaviours (Hansson & Stensmyr 2011; Mansourian & Stensmyr 2015). The resulting mechanistic understanding of olfactory coding and behaviours should ultimately lead to 'in silico' models of odour coding that support the rational design of novel attractants and repellents for use in monitoring and control, although endeavours to translate olfactomics into application are largely absent.

Progress in other model species is substantially slower, as the tools are limited, and the process time consuming and expensive, although progress is made in some model species (e.g. De Fouchier *et al.* 2017). However, comprehensive olfactomics in non-model species (i.e., the vast majority insect species, including Tephritidae) is rarely on the research agenda. We therefore explored the potential for complementary olfactomic alternatives using readily accessible tools with high throughput. While GC-EAD analyses are admittedly course, lacking the molecular detail of sensory neuron and receptor combinations (Hansson & Stensmyr 2011), and the spatial detail of calcium imaging in the antennal lobes (Sachse & Galizia 2002; Wang *et al.* 2003), they do provide a robust overview of the overall antennal sensitivities to ecologically relevant odours.

Our research concentrated on Tephritidae, a family of fruit flies of which several members are highly invasive, and threaten horticultural production and livelihoods in large parts of the tropics and subtropics. Whereas many species appear polyphagous, they generally display a stronger preference for a limited few hosts (Bush 1969; Rwomushana *et al.* 2009). How this is regulated by the sense of smell, and whether flies cue in on specific host-signifying volatiles in a complex blend, or rely on general compounds in particular combinations and ratios, is not known (Visser 1986; Bruce *et al.* 2005). Aligned olfactory responses of four species to four fruit species surfaced surprising correlates of preference, and olfactory evolution and ecology of these four fly species and permitted translation in terms of attraction.

Sharedness, phylogenetic relatedness and pre-adaptive bridges for host shifts

Whether host preference shifts cause or are caused by shifts in the olfactory circuitry is an unresolved question. It is equally obscure how an insect can get from one olfactory optimum to another in a presumably largely non-adaptive landscape (Linn *et al.* 2003; Cha *et al.* 2012). This is particularly enigmatic when optimal olfactory codes are disjunct and intermediate preferences suffer high fitness costs, such as in moth pheromone communication (Groot *et al.* 2016). Here we show that, in spite of evolutionary distance (*C. capitata* and *Bactrocera* spp.) and differences in ecology (*Z. cucurbitae*), the fruit odour responses of four tephritid species showed a large degree of overlap. The overlap may provide adaptive corridors that support a broad host breadth and facilitate host shifts along with seasonal variations in fruit availability. Such a scenario with a relaxed selection for specificity or conversely selection for broad host acceptance, could promote rapid radiation to available hosts and lead to

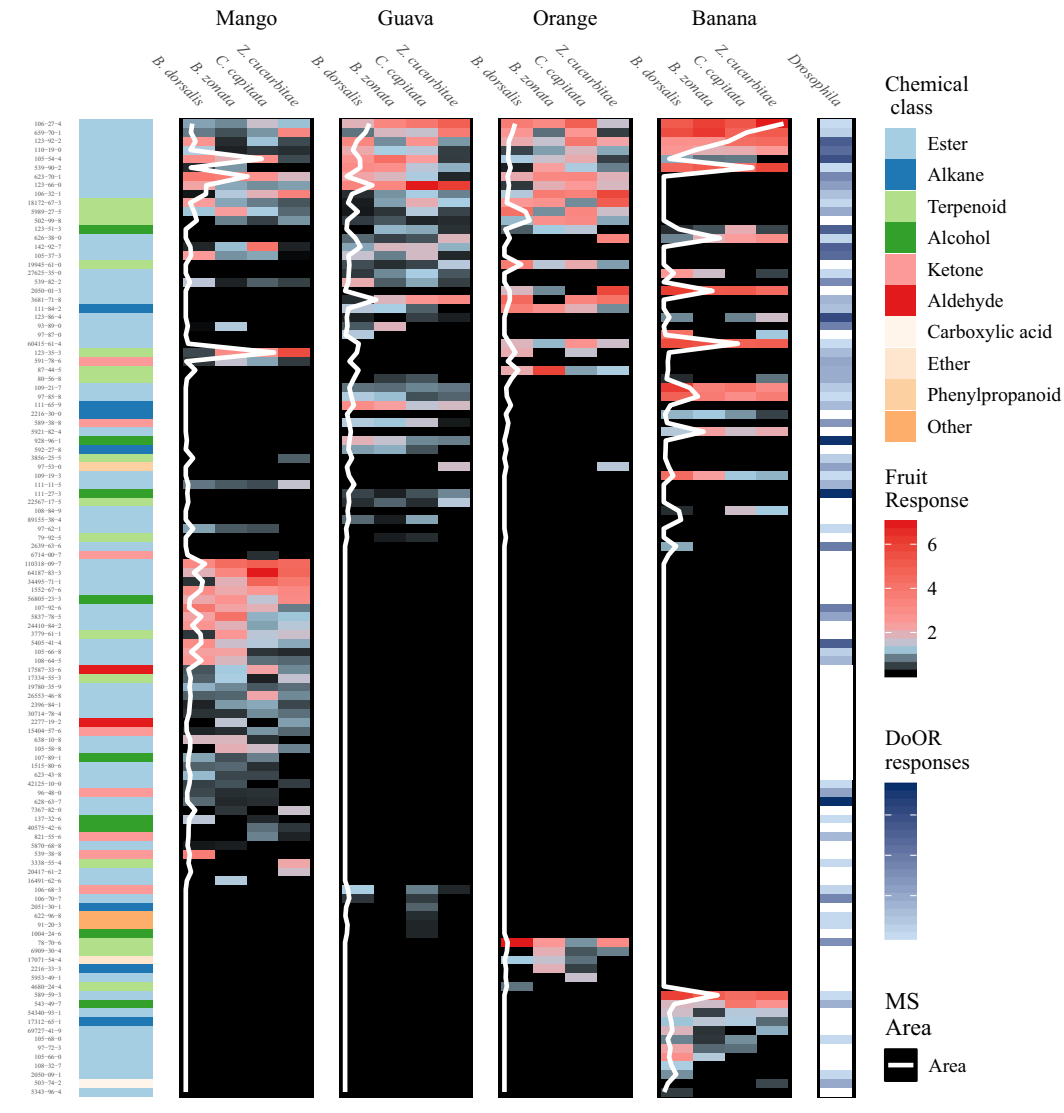

Figure 4 A heat map from left to right shows (1) CAS number to the compounds, (2) functional classes of the compounds (3) sensitivity of the four fruit fly species (*Bactrocera dorsalis*, *Bactrocera zonata*, *Ceratitis capitata* and *Zeugodacus cucurbitae*) to compounds in the four fruits (mango, guava, orange and banana) with the MS area (the sum of ions) in white, and (4) olfactory response of *Drosophila melanogaster* to the compounds. The compounds are arranged from top to bottom in order of decreasing sharedness (first across fruit and within each cluster across fruit fly species) The chemical groups include alkane (light blue), ester (dark blue), terpenoid (light green) and other (dark green). The average relative sensitivity of the fly's antenna ranges from light gray (0) to dark pink (6 × averaged EAD response, see 'Materials and Methods'). The amount of each of the compounds is overlaid as a white trace on the heatmap (axis at the bottom of the figure). The compounds are vertically arranged in decreasing order of sharedness across fruits (presence in the headspace of the fruits), and within these sharedness in antennal response (from detected by all to detected by none of the species). Note further that the strength of a response in the tephritid heatplot to a certain compound is relative to the overall response of the antenna of that species and fruit. Cross column comparisons therefore reflect relative, not absolute, differences between species in responsiveness to compounds (details of the calculations see m&m). The antennal responses of *D. melanogaster* simulated from DoOR database increases from light blue to dark blue, with a white bar indicates compounds not present in the DoOR database. Note that the DoOR response is not adjusted for amounts coming from the fruits, but extracted from DoOR-reported response strengths (which are based largely on stimulations typically at 100 µg of compound on filter paper). White bars indicates compounds not present in the DoOR database.

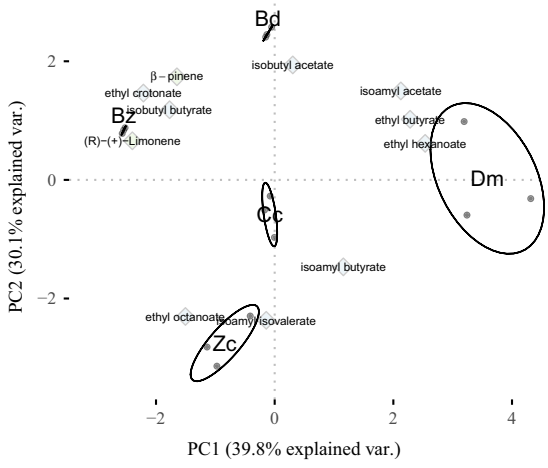

Figure 5 PCA plot of antennal recordings of four Tephritidae species and *Drosophila melanogaster* to the 11-blend of shared compounds. The plot shows that 70% of the variation was explained by PC1 and PC2. Names of the compounds illustrate which of the volatiles contributed to the separation in which direction. Recordings (black dots, $n = 3$ per species) separated out the five species in distinct clusters. Surprisingly, *D. melanogaster* did not separate out very strongly from the four distantly related tephritid species.

species with overlapping but distinct preferences, such as we see in Tephritidae (Duyck *et al.* 2004).

The concept that odours that are shared between hosts could promote hosts shifts, dates back to Dethier (1941), who postulated that host acceptance by papilionid caterpillars is facilitated by shared host volatiles that form pre-adaptive bridges, and is presumed to have been an important factor in the evolution of host-specialisation and hosts shifts in another clade of tephritid fruit flies, *Rhagoletis* (Linn *et al.* 2003; Cha *et al.* 2012; Powell *et al.* 2012) The 'ecological fitting' hypothesis has a similar tenet, although not based on chemical ecology (Agosta & Klemens 2008). Our data support the idea that 'shared volatiles' may function as bridge between hosts and promote broad host ranges: there was a remarkably strong correlation between shared volatiles and shared detection, and blends of these shared compounds were highly attractive, more so than fruits. The huge phylogenetic distances between the fruits tested here (closest via order sapindales: mango/citrus, clade rosids: guava; angiosperms: banana), strongly indicates that these shared compounds are a common volatile denominator of a broad range of fruit species, and perhaps therefore constitute a set of volatiles whose detection is selected for. The fact that *D. melanogaster*'s published olfactome (Münch & Galizia 2016) converged on a roughly similar set of volatiles, which were also attractive, underlines the significance of shared volatiles. In a study on *Helicoverpa armigera*, a similar idea was tested although without reference to olfactomics: a range of attractive flowering plant species were scrutinised on common volatiles, and a blend of these was attractive (Del Socorro *et al.* 2010).

Whether shared detection of shared volatiles is due to common ancestry and functional conservedness of ORs, or convergent selection through 'independent' lineages of receptors, needs further study. In *Drosophila* large parts of the peripheral coding for odours appears to be functionally conserved, with some lineage specific divergences, frequently fitting with

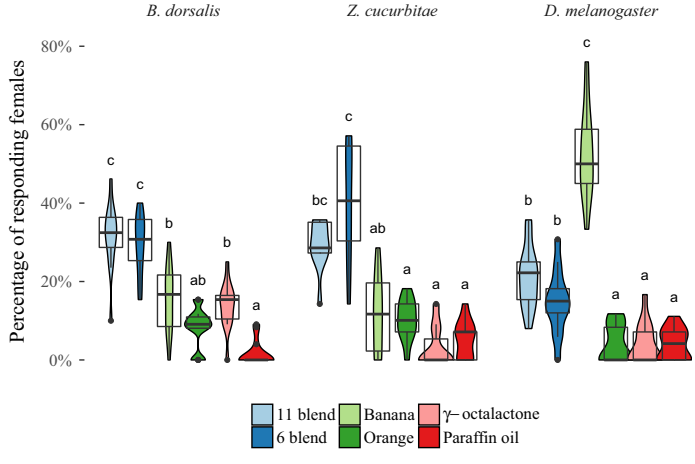

Figure 6 Capture of *Bactrocera dorsalis*, *Zeugodacus cucurbitae* and *Drosophila melanogaster* in a six-choice olfactometer assay after 30 min assay (for trap scores at other time points, see Fig. S11). Treatments in each test included the 11-component blend (shared across all flies and 3 fruits, each of the blends in mango headspace ratios), the 6-component blend (shared across all flies and fruits), orange, banana, γ-octalactone and paraffin oil. Graphs are plotted using a combination of boxplot and violin plot. The box plot indicates the interquartile ranges, whereas the violin plot indicates the distribution of the data. Black dots are outliers in the data set. Number of flies per experiment = 30; number of replications = 14, 10 and 13 for Bd, Zc and Dm respectively. At the 30 min time point the total % that had entered was 44%, 40% and 51%. For *B. dorsalis*, *Z. cucurbitae* and *D. melanogaster*, respectively. Letters above each time point indicate significance at 0.05 (glm model fitted with a poisson family).

ecological niches (e.g. Stensmyr *et al*. 2003; Dekker *et al*. 2006; De Bruyne *et al*. 2010; Linz *et al*. 2013; Goldman-Huertas *et al*. 2015). This might also hold for Tephritidae. Irrespective of the evolutionary scenario, however, ecologically the detection of shared volatiles may function in finding alternative food and oviposition sources and e.g. bridge seasonal absence of 'preferred' hosts.

Our study further demonstrates that, in spite of the significant overlap, tephritid antennal responses were also divergent. Interestingly, this did not follow relatedness (Virgilio *et al*. 2015; Yaakop *et al*. 2015), as the distantly related (tribe, supergenus) *C. capitata* did not separate well from *B. dorsalis* and *B. zonata* (Fig. S10), whereas *Z. cucurbitae*'s olfactory responses (same subgenus as *B. dorsalis* and *B. zonata*) were the most distant of the four. *Z. cucurbitae* has both significantly gained and lost the detection of volatiles, which is possibly linked to the fact that *Z. cucurbitae* is ecologically distinct and prefers herbaceous cucurbits (De Meyer *et al*. 2015; Yaakop *et al*. 2015), whereas *C. capitata* ecologically strongly overlaps with *B. dorsalis* and *B. zonata*. Ecological niche may thus override phylogenetic relatedness in olfactory tuning. Also here, whether such patterns are caused by conservedness of receptor repertoires, functional convergence of evolved receptors (between *B. dorsalis*, *B. zonata* and *C. capitata*), or for instance divergence or expansion of certain receptors and loss of others (particularly in *Z. cucurbitae*), requires comparative receptor and connectome studies (Goldman-Huertas *et al*. 2015; Jacob *et al*. 2017).

Esters are overrepresented

What furthermore emerges from our analyses is that the esters dominate the responses in all fruit flies, up and above the already disproportionate presence of esters (Fig. 1 and Fig. S2). As this was not due to increased absolute sensitivity of the antennae to esters (Fig. S6), possible explanations for the high detectability of esters include a large set of ORs tuned to esters than to other classes of compounds (as seems the case in *Drosophila*, Münch & Galizia 2016), and/or a broader tuning and therefore receptive range of underlying receptors. Our DoOR-based analysis shows that *D. melanogaster* also disproportionately detects esters, with more than half of the deorphanised adult receptor repertoire is tuned to esters (DoOR database, see also De Bruyne *et al*. 2010).

Regardless of the mechanism, since esters dominate the headspace of all fruits, the tuning of antenna to esters species may have been selected for to secure a broad detectability of fruits and overcome periods in which favoured fruits are scarce (see above). As dominantly shared volatiles, both in headspace and detection, esters could thus be the key in maintaining a broad host range in tephritids and serve as the aforementioned pre-adaptive bridges (Dethier 1941). The fact that highly attractive blends of shared compounds consisted of 80–100% esters (11 and 6-blend respectively) supports this.

Summary and further research

GC-EADs are frequently used more qualitatively and illustratively. Here, we show the potential of using this low-cost, high throughput method for building cross-species consensus olfactome databases (*sensu* DoOR, Münch & Galizia 2016) that can complement frontline, bottom up approaches, particularly for non-model species. Such databases can provide important comparative information on olfactome tuning in evolutionary and ecological contexts, and fasttrack basic olfactomics (bottom up molecular neurogenetics) more effectively into application, something that rarely happens today.

The research presented here also raises many new questions, which cannot be addressed in the context of this paper, such as the role of 90% remaining volatiles (see for diverse roles of olfactory channels, Mansourian & Stensmyr 2015). Furthermore, we are cognizant of the fact that the current database underrepresents the actual olfactory breadth. Future efforts should include different fruit varieties and species (see e.g. Jayanthi *et al*. 2012, 2014a,b), fly species (e.g. *B. tryoni*, Cunningham *et al*. 2016) and environmental cues (e.g. food and host plant odours, contextual odours), and pay particular attention to volatiles whose detection may have fallen below noise levels due to positional effects or low abundance of corresponding sensory neurons (Olsson & Hansson 2013; Biasazin *et al*. 2014, Jacob *et al*. 2017).

We hope that the current work will promote standardisation and collaboration on olfactomics in diverse species, particularly non-model, for use in fundamental evolutionary and ecological research, as well as discovery of innovative solutions for pest control. This is sorely needed in an era of increased impact of globalisation and climate-change induced pest pressure, while already grappling with mounting issues of sustainable production, global food security and safety (Paini *et al*. 2016).

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AUTHORSHIP

Conception of ideas (TDB, SLH, TD), working out experimental protocols (TDB, FK, SLH, TD), gathering primary data (TDB, FK, SLH), analysis (TDB, SLH, TD), writing first draft (TDB, TD), revisions and editing (TDB, FK, SLH, TD).

DATA ACCESSIBILITY STATEMENT

Data will be publicly available from www.tephri.org

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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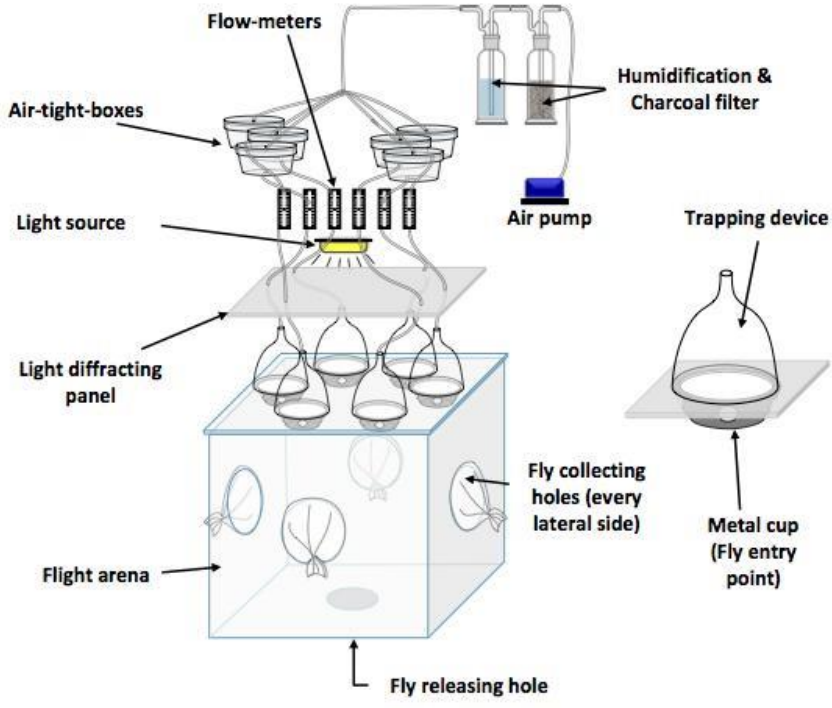
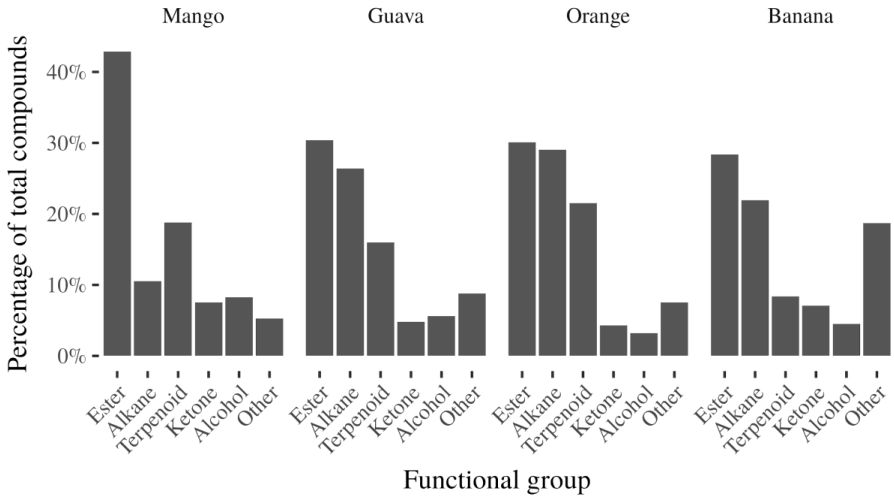
Paper III supplementary material 1 - Translating olfactomes into attractants: shared volatiles provide attractive bridges for polyphagy in fruit flies

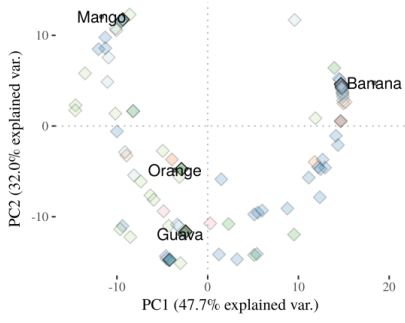


Supplemental Figure 1. Schematic drawing of the olfactometer apparatus used in multiple-choice experiment. The apparatus is made up of a 40 x 40 x 40 cm cubic glass cage with 6 circular holes on the top, and large circular holes on the lateral sides and bottom providing access for release and collecting flies. Air was active-charcoal filtered, humidified and passed airtight boxes containing each on of six treatments (11-blend, 6-blend, gamma-octalactone, orange, banana and paraffin oil). The flow was monitored and kept at 0.5 L min-1 and reaches the flight arena through modified metal cups with 3 holes of 0.5 cm diameter. The effect of light was minimized using light diffracting panel. The behavioral tests were carried out with 6 different treatments simultaneously (see Fig 6 and S11). Traps, tubing and boxes were rotated between replications.

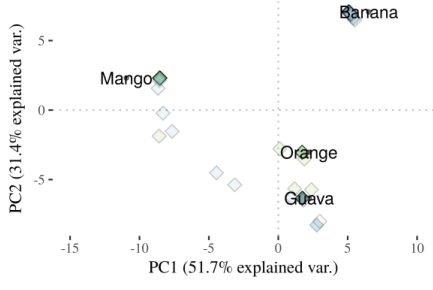


Supplemental Figure 2. Percentage of main chemical classes of volatile compounds in the headspace of fruits. For more details see Fig 2 and 3

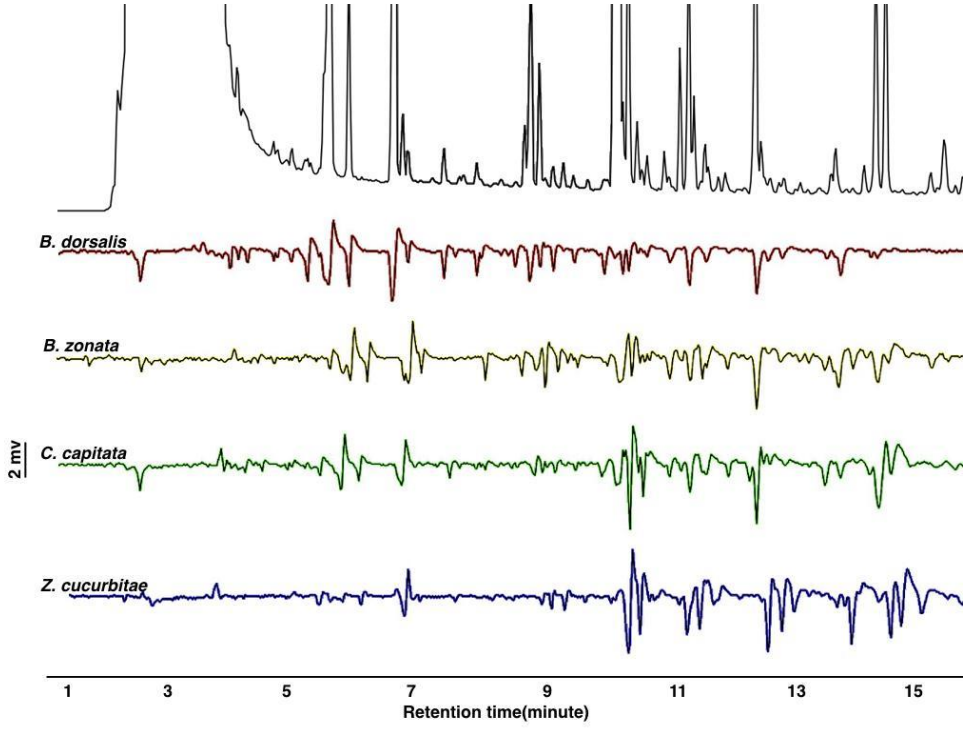
A



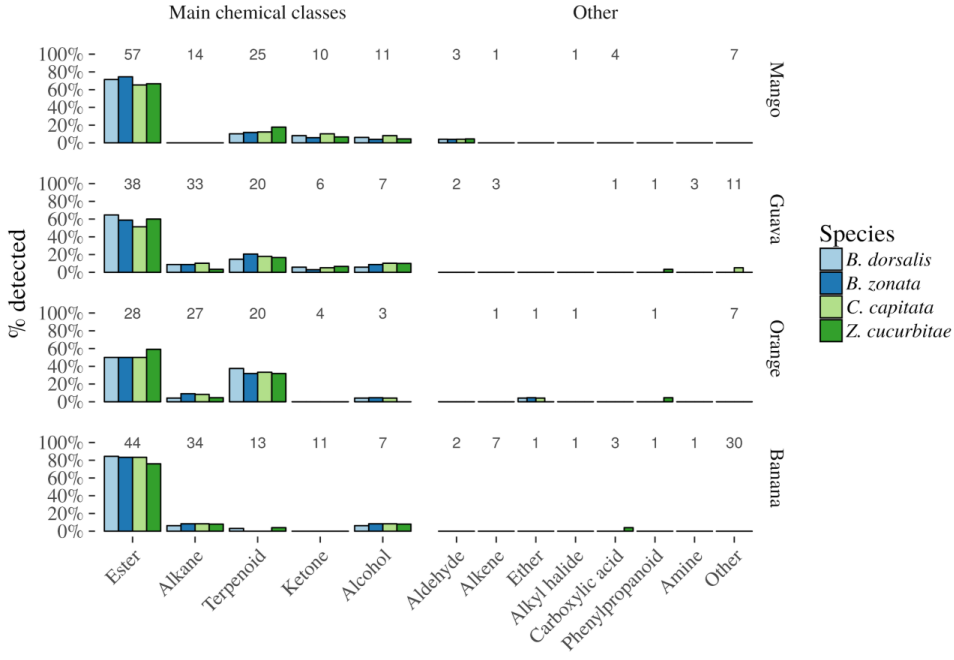
B



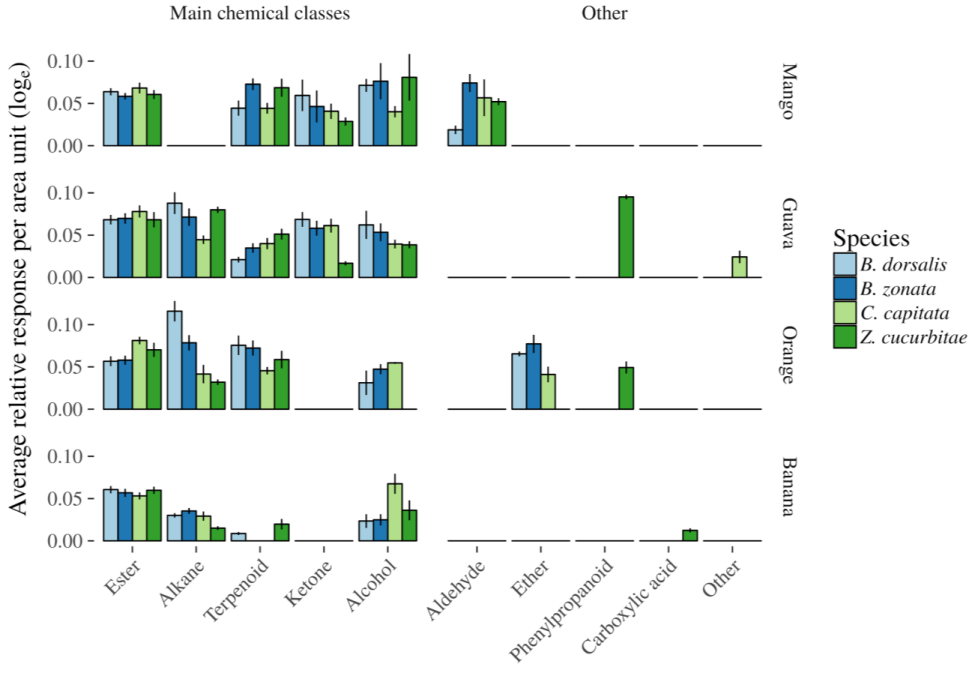
Supplemental Figure 3. Principal Component Analysis of the volatilome (A) and corresponding combined olfactory responses (B). Both PCA analyses demonstrate that across two components, explaining around 80% of the variation, orange and guava were closely linked, whereas banana and mango were furthest apart both in the volatiles from the fruits as well as the response of tephritid fruit flies to these.



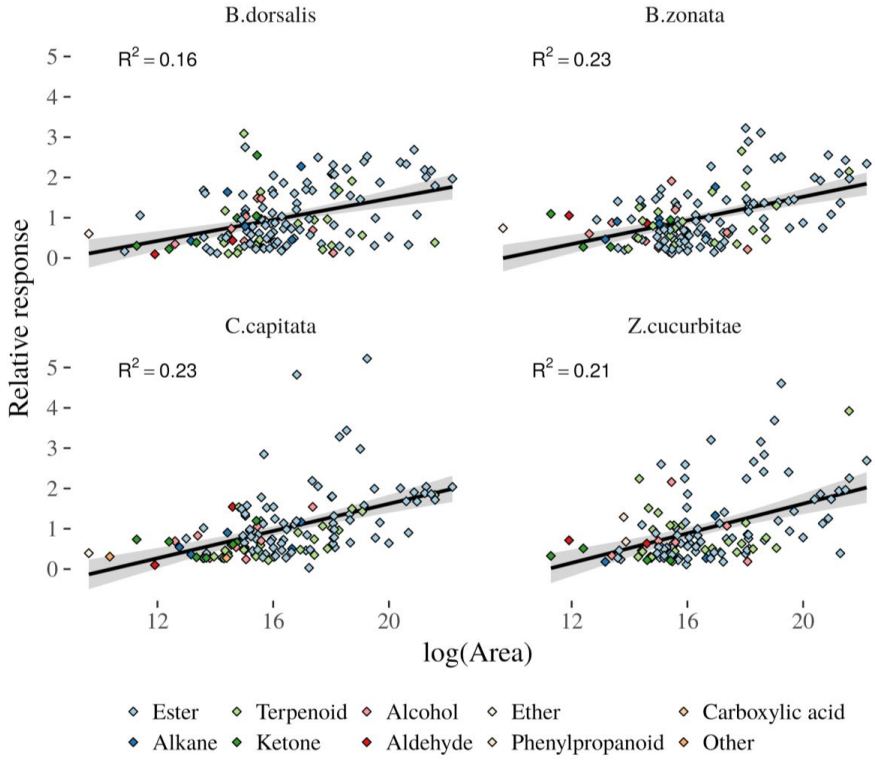
Supplemental Figure 4. Gas chromatograph- coupled electroantennogram detection responses of female *B. dorsalis*, *B. zonata*, *C. capitata* and *Z. cucurbitae* to volatiles from ripe mango. Top (black). FID trace of ripe mango, bottom 4 traces (colored) are EAD peaks for the four tephritid fly species.



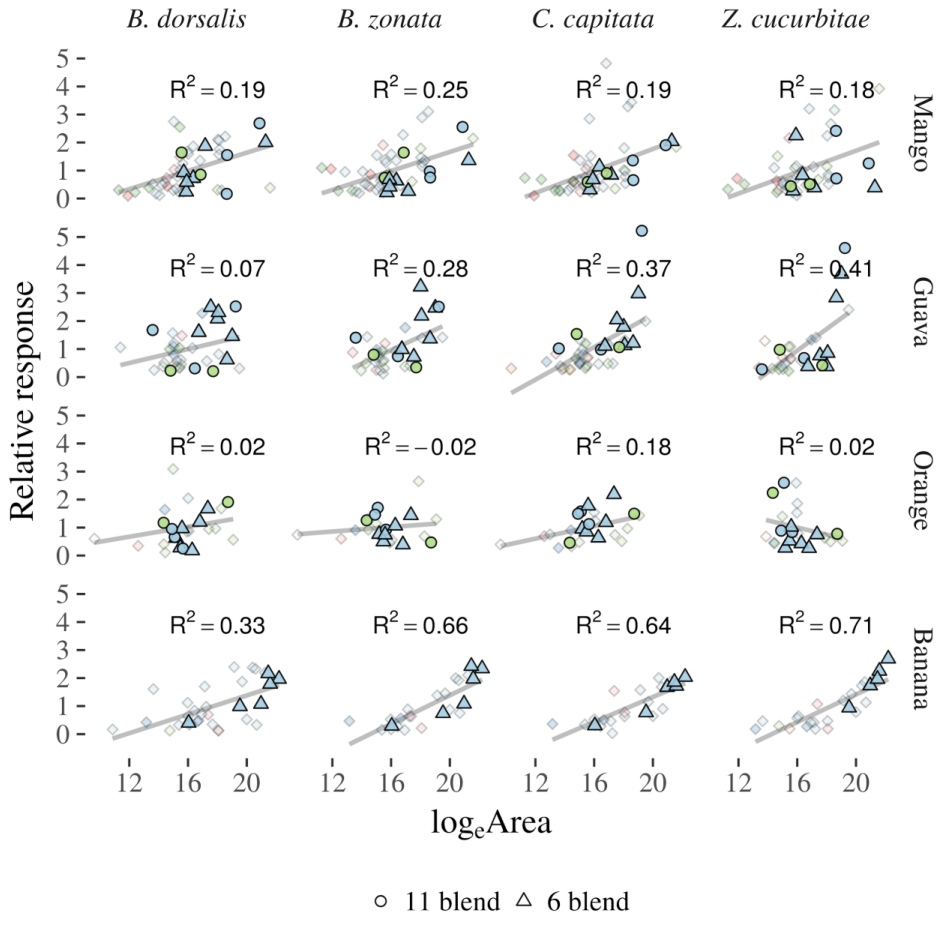
Supplemental Figure 5. Volatiles belonging to a certain chemical group expressed as a fraction of the total number of compounds that gave a response in the antenna of a all four tephritid species. The number above each class of compounds represent the number of volatiles in that class for that fruit species. The graph demonstrates that esters dominate the fruit-odor input in the fruit fly antenna.



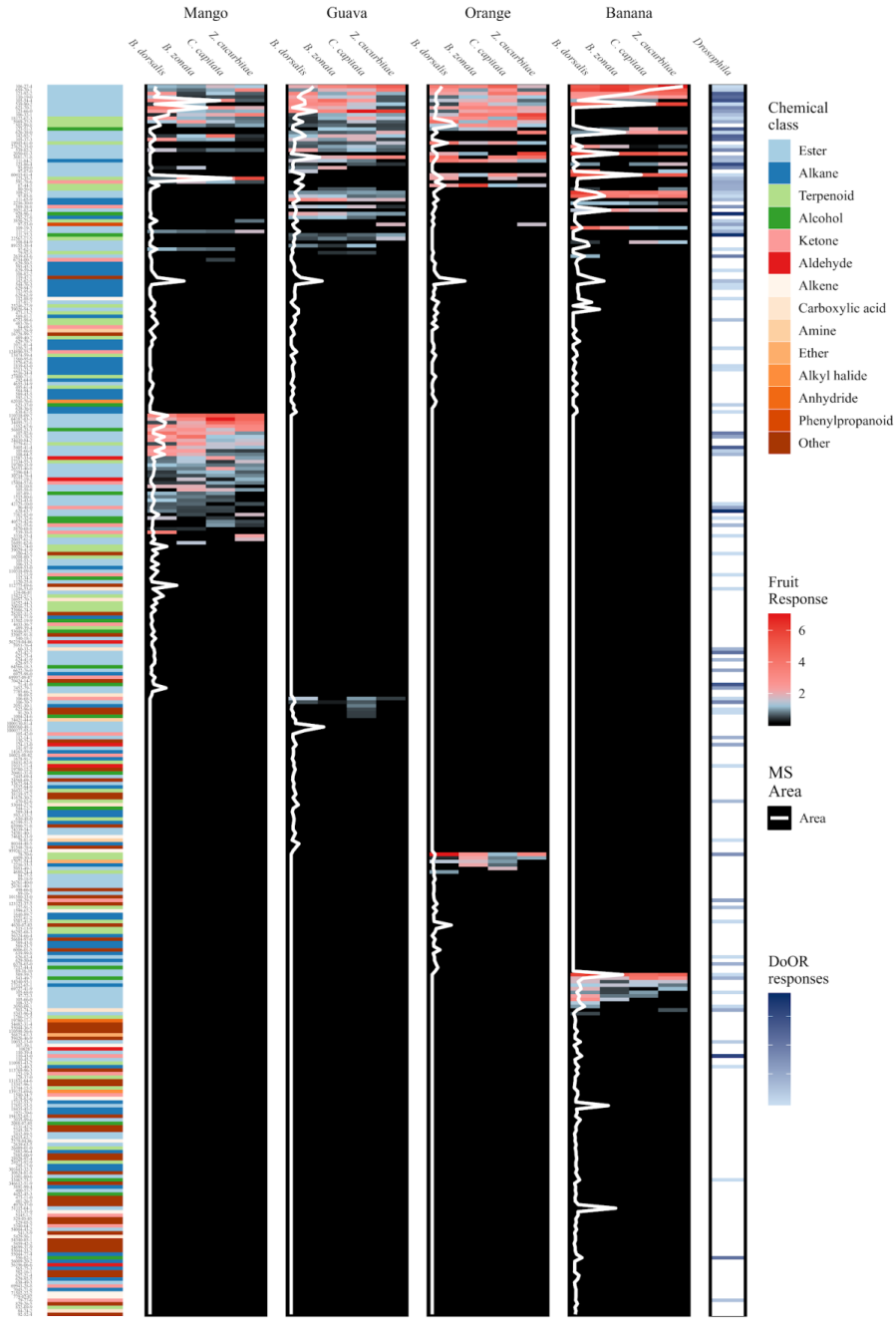
Supplemental Figure 6. The average relative antennal response (+- SE) divided by the natural log of the MS area (the sum of ions). Whereas antenna pick up a disproportionate fraction of esters compared to other chemical classes (Fig S5), they appear not to display a higher sensitivity (in relative response strength per $\log_e$(area)) to esters than to compounds of various other chemical classes. Note that the area under a peak in the MS is representative of the sum of ions and thus depends on the fractionation of compounds. Since this differs between compounds, the surface area is confounded by the number of ion of a compound. The above figure represents therefore only an indication.



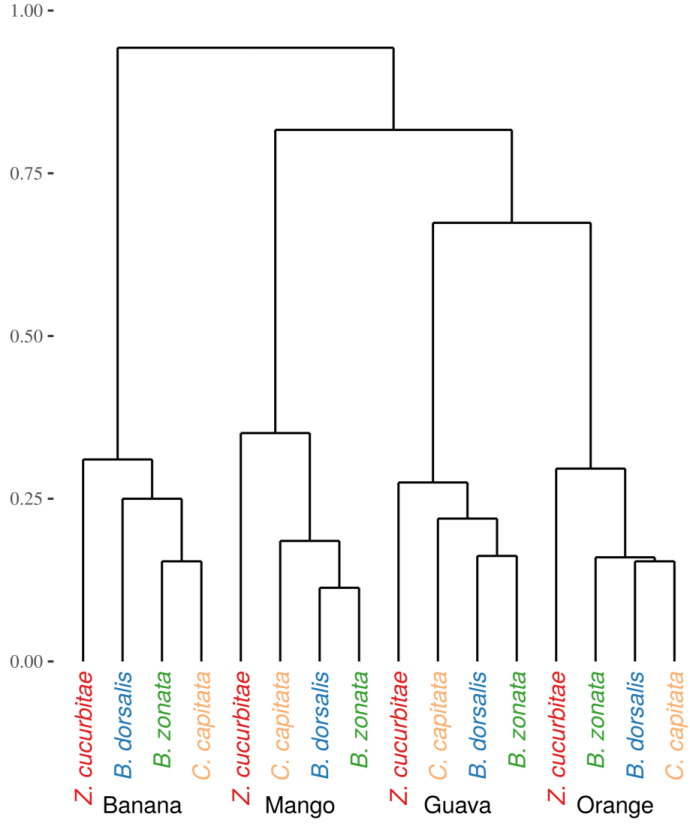
Supplemental Fig 7. The relative EAD response strength over the stimulus intensity for each tephritid species. It demonstrates the poor correlation between stimulus intensity ($\log_e$(area)) and antennal response strength. See also fig S6 and S8.



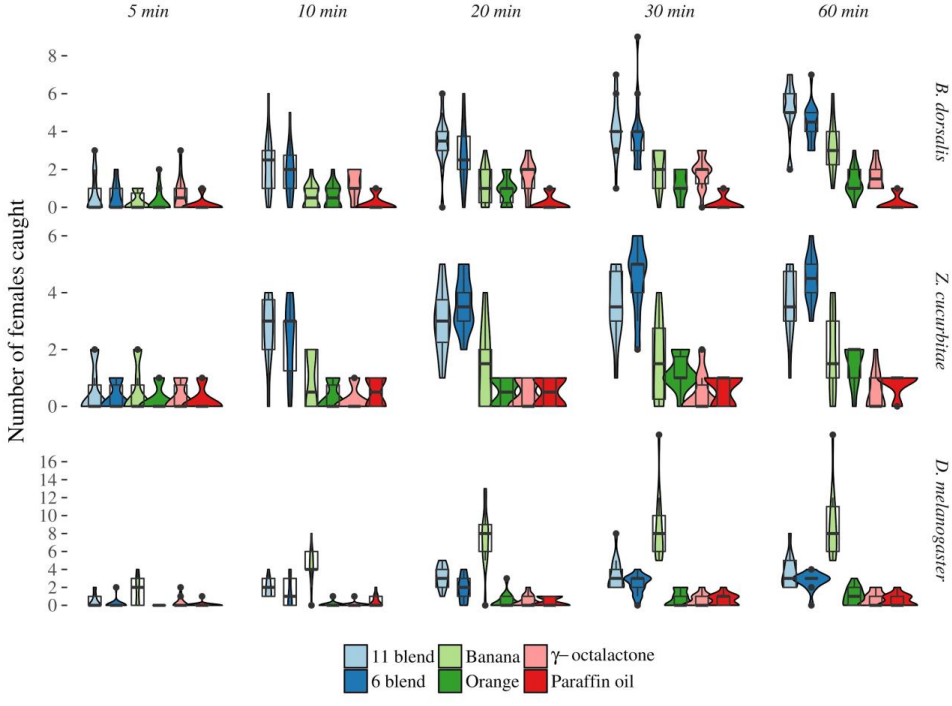
Supplemental Fig 8. The relative antennal response strength over the logged stimulus intensity split out per fruit and fruit fly species. Regressions show that there is a poor correlation between the response strengths and $\log_e$(area). Shapes that are more prominent (clear) are those present in the six-component blend (triangles) or eleven-component blend (triangles and circles). Remaining (shaded) symbols are other responses. Blue color are esters and green terpenoid. See also Fig. S6 and 7.


Mango
Guava
Orange
Banana
B. dorsalis
B. zonata
C. capitata
Z. cucurbitae
Drosophila
Chemical class
Ester
Alkane
Terpenoid
Alcohol
Ketone
Aldehyde
Alkene
Carboxylic acid
Amine
Ether
Alkyl halide
Anhydride
Phenylpropanoid
Other
Fruit Response
6
4
2
MS Area
Area
DoOR responses

Supplemental Figure 9. A heat map from left to right shows 1) CAS number to the compounds, 2) functional classes of the compounds 3) sensitivity of the for fruit fly species (*B. dorsalis*, *B. zonata*, *C. capitata* and *Z. cucurbitae*) to compounds in the four fruits (mango, guava, orange and banana) with MS area (the sum of ions) in white, and 4) olfactory response of *D. melanogaster* to the compounds. The compounds are arranged from top to bottom in order of decreasing sharedness (first across fruit and within each cluster across fruit fly species). The chemical classes include alkane (dark blue), ester (light green), terpenoid (dark green) and other (pink). Compounds that didn't fall into any of the chemical classes are presented as "?" (light blue). The average relative sensitivity of the fly's antennae ranges from light gray (0) to dark pink (>6), the number representing the strength of the antennal response relative to the weighted average (see m&m). The compounds are vertically arranged in decreasing order of sharedness across fruits (presence in the headspace of the fruits), and within these sharedness in antennal response (from detected by all to detected by none of the species). Note that the strength of a response in the heatplot to a certain compound is relative to the overall response of the antenna of that species and fruit. Cross column comparisons therefore reflect relative, not absolute, differences between species in responsiveness to compounds (see m&m for details). The antennal responses of *D. melanogaster* simulated from DoOR database increases from light blue to dark blue, with a white bar indicates compounds not present in the DoOR database. Note that the DoOR response is not adjusted for amounts coming from the fruits, but extracted from DoOR-reported response strengths (which are based largely on stimulations typically at 100 µg of compound on filter paper). White bars indicates compounds not present in the DoOR database.



Supplemental Figure 10. Dissimilarity indices is calculated using R package Vegan and function vegdist, which is traditionally used for community ecologists, instead of using species composition mean relative response for each compound is used (Oksanen *et al*.2018). The resulting dissimilarity index is used as a input for hierarchical clustering using stats::hclust and the resulting clustering is plotted using the ggdendro package (De Vries and Ripley 2016). The olfactome for each fruit fly species and fruit is calculated as individual "sites", species names are annotated correctly in post-analysis. The dissimilarity index used is "Jaccard", which is calculated from a Bray-Curtis dissimilarity index. To account for absence of responses to compounds a presence/absence standardization before calculating the dissimilarity index was used.



Supplemental Figure 11. Extension of figure 6. Counts of *B. dorsalis*, *Z. cucurbitae* & *D. melanogaster* in a six-choice olfactometer assay at regular time intervals during the assay. Treatments in each test included the 11-component blend (shared across all flies and 3 fruits, each of the blends in mango headspace ratios), the 6-component blend (shared across all flies and fruits), orange, banana, γ-octalactone and paraffin oil. Graphs are plotted using a combination of boxplot and violin plot. The box plot indicates the interquartile ranges, whereas the violin plot indicates the distribution of the data. Black dots are outliers in the data set. Number of flies per experiment = 30; number of replications = 14, 10 & 13 for Bd, Zc & Dm respectively. The percentage that entered a trap at 5, 10, 20, 30 and 60 min was as follows for each species: Zc: 7, 23, 31, 40, 43%; Bd: 9, 22, 35, 44, 53%; Dm 9, 28, 45, 51 and 55% respectively. Letters above each time point indicate significance at 0.05 (glm model fitted with a poisson family).

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Vries de A. & Ripley B.D. (2016). ggdendro: Create Dendrograms and Tree Diagrams Using 'ggplot2'. R package version 0.1-20. https://CRAN.R-project.org/package=ggdendro)

Paper III supplementary material 2 - Translating olfactomes into attractants: shared volatiles provide attractive bridges for polyphagy in fruit flies

Supplemental Table 1. Kovats retention indices of volatile compounds from; mango (*Magnifera indica* cv. 'Kent'); guava (*Psidium guajava*, local cultivar); orange (*Citrus sinensis* cv. 'Valencia') and banana (*Musa accuminata* cv. 'Grand nain') that elicit responses in four Tephritidae species: *Bactrocera dorsalis*, *Bactrocera zonata*, *Ceratitis capitata* and *Zeugodacus cucurbitae*. Bold letters indicate that the identity of the compound was doubly confirmed using GC-EAD with injection of synthetics

			Kovats retention indices				
	Cas	Compound	*B. dorsalis*	*B. zonata*	*C. capitata*	*Z. cucurbitae*	Published
		Mango, *Magnifera indica* cv. 'Kent'					
1		unknown mango 1	1.17*	1.16*	1.17*	1.07*	
2	105-37-3	ethyl propanoate	712.08	715.69	713.09	712.93	705
3	137-32-6	2-methylbutan-1-ol	720.16		720.34		
4	107-89-1	3-hydroxybutanal	729.56	733.35	730.75		726
5	97-62-1	ethyl 2-methylpropanoate	756.49	760.17	756.98		755
6	623-43-8	methyl (E)-but-2-enoate	760.07	763.76	761.11		756
7	**110-19-0**	2-methylpropyl acetate	774.33	777.51	774.89	774.45	772
8	539-38-8	3-hexanone	790				784
9	105-58-8	diethyl carbonate		793.10	791.37	790.45	784
10	**105-54-4**	ethyl butanoate	806.97	809.75	808.99	807.71	803
11	591-78-6	hexan-2-one	803.52	805.32	802.72	804.40	800
12	107-92-6	butanoic acid	820.55	821.82	821.20	820.60	820
13	**623-70-1**	ethyl (E)-but-2-enoate	848.79	850.50	849.62	850.37	844
14	108-64-5	ethyl 3-methylbutanoate	859.23	860.12	859.80	859.67	859
15	**123-92-2**	3-methylbutyl acetate	883.06	883.64	883.96	882.97	883
16	628-63-7	pentyl acetate	890.52	890.57	890.67		893
17	6714-00-7	(E)-hept-5-en-2-one			901.55		866
18	539-82-2	ethyl pentanoate	908.02	908.86	908.49	907.83	902
19	**105-66-8**	propyl butanoate	905.06	905.22	905.34	905.20	898
20	42125-10-0	[(Z)-pent-2-enyl] acetate	920.63	921.15		922	909
21	96-48-0	oxolan-2-one	925.97	926.29	926.67		922
22	638-10-8	ethyl 3-methylbut-2-enoate	930.60	930.44	930.24		924
23	5405-41-4	ethyl 3-hydroxybutanoate	940.04	940.69	941.22	942.15	943
24	5837-78-5	ethyl (E)-2-methylbut-2-enoate	947.09	947.31	947.67	947.29	938
25	40575-42-6	oct-1-en-4-ol			950.42	950.76	
26	24410-84-2	ethyl (E)-pent-2-enoate	956.08	955.83	956.52	956.22	
27	**539-90-2**	2-methylpropyl butanoate	962.98	963.01			958
28	5870-68-8	ethyl 3-methylpentanoate	966.41	965.71			960
29	19780-35-9	ethyl 3-methyloxirane-2-carboxylate	969.66	969.91	970.22	969.91	
30	**18172-67-3**	(-)-beta-pinene	990.06	990.38	990.01	989.64	988
31	30714-78-4	butyl ethyl carbonate	987.61	987.21	988.19	987.22	980
32	**123-35-3**	myrcene	997.30	999.15	999.54	1000.44	995
33	**123-66-0**	ethyl hexanoate	1002.84	1002.61	1003.93	1001.97	1001
34	64187-83-3	ethyl (Z)-hex-3-enoate	1007.55	1007.40	1007.91	1007.78	993
35	26553-46-8	ethyl (E)-hex-3-enoate	1013.57	1013.34	1013.86	1014.53	1012
36	142-92-7	hexyl acetate	1017.08	1016.83	1017.46	1018.02	1017
37	1515-80-6	methyl hexa-2,4-dienoate	1020.45	1021.10	1021.33		1019
38	**5989-27-5**	(R)-(+)-limonene	1036.67	1035.78	1036.85	1035.64	1032
39	**3338-55-4**	(Z)-beta-ocimene				1040.82	1041
40	**502-99-8**	alpha-ocimene		1043.85	1044.90	1044.83	
41	1552-67-6	ethyl (E)-hex-2-enoate	1050.61	1050.27	1051.97	1049.87	1046
42	3779-61-1	(E)-beta-ocimene	1060.16	1059.20	1060.59	1059.87	1056
43	**106-27-4**	3-methylbutyl butanoate	1063.07	1063.23	1063.81	1063.31	1064
44	2396-84-1	ethyl (2E,4E)-hexa-2,4-dienoate	1078.28	1077.61	1080.05	1078.41	1093
45	2396-84-1b	ethyl (2E,4E)-hexa-2,4-dienoate			1105.33	1106.46	
46	821-55-6	nonan-2-one			1094.77	1095.19	1093
47	110318-09-7	ethyl hexa-2,4-dienoate	1099.43	1099.49	1100.62	1100.22	1093
48	**659-70-1**	3-methylbutyl 3-methylbutanoate	1108.65	1109.10	1110.61	1110.87	1104
49	111-11-5	methyl octanoate	1119.74	1118.73	1122.50	1119.89	1120
50	17587-33-6	(E,E)-3,6-Nonadien-1-ol	1155.49	1153.18	1154.84	1154.54	1153
51	2277-19-2	(Z)-non-6-enal	1160.39	1159.49	1161.62	1160.86	1153
52	56805-23-3	(E,Z)-3,6-Nonadien-1-ol	1166.32	1165.41	1167.29	1165.71	1161
53	93-89-0	ethyl benzoate	1177.27	1175.89			1175
54	15404-57-6	1,3,3,7-tetramethylnorbornan-2-one	1187.06	1186.37	1190.36	1188.21	
55	34495-71-1	ethyl (Z)-oct-4-enoate	1194.73	1195.73	1197.34	1197.30	1187
56	**106-32-1**	ethyl octanoate	1204.39	1204.47	1207.50	1205.61	1204
57	7367-82-0	ethyl (E)-oct-2-enoate		1220.81		1223.15	
58	16491-62-6	cyclohexyl but-2-enoate		1240.69			
59	20417-61-2	ethyl 2-formylcyclopropane-1-carboxylate				1289.37	
60	3856-25-5	(-)-alpha-copaene				1375.38	1376
61	17334-55-3	calarene	1433.35	1432.84	1439.15	1434.61	1434
		Guava, *Psidium guajava*, local cultivar					
1		unknown guava 1	1.17*	1.16*	1.16*	1.16*	
2	105-37-3	ethyl propanoate	701.26	704.63	707.74	2.28*	705
3	**123-51-3**	3-methylbutan-1-ol		712.76	726.14	714.73	727
4	592-27-8	2-methylheptane	746.57	757.01	752.89		759
5	**110-19-0**	2-methylpropyl acetate	764.50	774.70	771.03	761.24	770

			Kovats retention indices				
	Cas	Compound	*B. dorsalis*	*B. zonata*	*C. capitata*	*Z. cucurbitae*	Published
6	589-38-8	hexan-3-one	781.28	791.32	787.49	778.18	784
7	111-65-9	octane	798.61	804.36	802.13	794.37	800
8	**105-54-4**	ethyl butanoate	801.69	807.85	805.92	800.46	803
9	123-86-4	butyl acetate	816.14		818.47	813.81	815
10	**623-70-1**	ethyl (E)-but-2-enoate	846.04	849.88	848.47	844.82	844
11	626-38-0	pentan-2-yl acetate	852.31	855.73	854.50	852.23	843
12	928-96-1	(Z)-hex-3-en-1-ol	856.19	859.39	859.11	855.71	858
13	111-27-3	hexan-1-ol	872.81	875.05	874.50	870.79	874
14	**123-92-2**	3-methylbutyl acetate	880.33	883.20	882.28	879.17	883
15	111-84-2	nonane	903	905.20	904.49		900
16	539-82-2	ethyl pentanoate	905.73	908.13	906.54	904.50	902
17	97-85-8	2-methylpropyl 2-methylpropanoate	919.60	921.68	920.73	918.87	914
18	1004-24-6	(4-methylidenecyclohexyl)methanol			925.46		
19	106-70-7	methyl hexanoate	930.32	931.78	931.45		930
20	80-56-8	alpha-pinene		947.72	950.37		
21	79-92-5	camphene		956.74	956.36		954
22	97-87-0	butyl 2-methylpropanoate	959.24				955
23	**539-90-2**	2-methylpropyl butanoate	961.64	963.03	962.82	961.43	961
24	622-96-8	1-ethyl-4-methylbenzene			972.10		968
25	2051-30-1	2,6-dimethyloctane			976.82		
26	**18172-67-3**	(-)-beta-pinene	984.56	986.43	986.61	985.56	988
27	106-68-3	octan-3-one	993.79		993.97	993.16	990
28	**109-21-7**	butyl butanoate	1002.21	1002.24	1002.76	1001.57	998
29	**123-66-0**	ethyl hexanoate	1005.93	1006.24	1006.90	1006.30	1001
30	3681-71-8	[(Z)-hex-3-enyl] acetate	1013.82	1013.06	1014.96	1014.13	1016
31	142-92-7	hexyl acetate	1020.02	1019.48	1019.97	1019.62	1017
32	**5989-27-5**	(R)-(+)-limonene	1032.03	1032.40	1032.74	1032.37	1032
33	27400-71-7	beta-ocimene				1043.68	1041
34	**502-99-8**	alpha-ocimene	1051.46	1052.55	1053.83	1053.41	1052
35	**106-27-4**	3-methylbutyl butanoate	1063.09	1063.04	1063.73	1063.37	1064
36	89155-38-4	2-propylheptanoate	1100.80	1099.32	1100.37		1095
37	27625-35-0	3-methylbutyl 2-methylbutanoate	1106.13	1105.07	1105.86	1105.56	1103
38	**659-70-1**	3-methylbutyl 3-methylbutanoate	1112.10	1111.12	1112.62	1112.01	1110
39	19945-61-0	(3E)-4,8-dimethylnona-1,3,7-triene	1170.04	1167.26	1169.30	1168.91	
40	93-89-0	ethyl benzoate	1178.99	1176.52			1175
41	91-20-3	naphthalene			1197.81		1196
42	**106-32-1**	ethyl octanoate	1203.96	1202.54	1206.13	1205.78	1204
43	97-53-0	2-methoxy-4-prop-2-enylphenol				1375.22	1373
44		unknown guava 2			1395.32		1393
45	22567-17-5	gamma-gurjunene	1433.66	1433.23	1440.58	1437.70	1434
		Orange, *Citrus sinensis* cv. 'Valencia'					
1		unknown orange 1	1.18*		1.17*		
2		unknown orange 2			2.24*		
3	**123-51-3**	3-methylbutan-1-ol	735.62	729.80	722.77		734
4	**110-19-0**	2-methylpropyl acetate	779.50	774.18		775.72	781
5	**105-54-4**	ethyl butanoate	809.83	807.26	803.10	808.63	805
6	**623-70-1**	ethyl (E)-but-2-enoate	851.52	849.66	846.26	849.82	844
7	626-38-0	pentan-2-yl acetate				855.98	843
8	2216-33-3	3-methyloctane		859.42	856.64		
9		unknown orange 3	861.56				
10	**123-92-2**	3-methylbutyl acetate	884.20	882.79	880.97	882.70	883
11	111-84-2	nonane	905.75	904.98	903.38	904.63	900
12	5953-49-1	hexan-2-yl acetate			949.73		937
13	**539-90-2**	2-methylpropyl butanoate	963.13	963.16	962.98	962.59	961
14	**18172-67-3**	(-)-beta-pinene	986.99	986.32	986.32	985.73	988
15	**123-35-3**	myrcene	997.08		996.79		1001
16	**123-66-0**	ethyl hexanoate	1002.25	1002.86	1002.84	1001.72	1001
17	3681-71-8	[(Z)-hex-3-enyl] acetate	1006.74		1007.10	1005.85	1007
18	2050-01-3	3-methylbutyl 2-methylpropanoate	1013	1012.76		1012.56	1013
19	**60415-61-4**	pentan-2-yl butanoate	1019.72	1019.48	1020.30	1019.73	1017
20	**5989-27-5**	(R)-(+)-limonene	1032.69	1030.22	1033.56	1031.65	1032
21	**502-99-8**	alpha-ocimene	1053.28	1055.40	1056.72	1054.32	1053
22	**106-27-4**	3-methylbutyl butanoate	1062.23	1062.95	1064.22	1062.31	1064
23	78-70-6	linalool	1103.99	1105.88	1104.33	1104.40	1104
24	**659-70-1**	3-methylbutyl 3-methylbutanoate	1111.07	1111.02	1109.15	1111.58	1110
25	19945-61-0	(3E)-4,8-dimethylnona-1,3,7-triene	1122.76	1122.32	1126.46	1122.59	1117
26	4680-24-4	(+)-(E)-limonene oxide	1136.54				1138
27	6909-30-4	(+)-trans-limonene oxide	1169.75	1168.56	1173.95	1168.03	
28	17071-54-4	1-hexoxyoctane	1182.91	1183.17	1182.30		
29	**106-32-1**	ethyl octanoate	1202.99	1205.32	1210.51	1203.11	1201
30	97-53-0	2-methoxy-4-prop-2-enylphenol				1375.86	1373
31	87-44-5	beta-caryophyllene	1431.37	1434.21	1453.09	1432.99	
		Banana, *Musa accuminata* cv. 'Grand nain'					
1		unknown banana 1	1.17*	0.99*	1.23*		
2	**123-51-3**	3-methylbutan-1-ol	740.53	722.83	729.76	734.01	730
3	**110-19-0**	2-methylpropyl acetate	785.09	769.61	774.93	778.65	772

			Kovats retention indices				
	Cas	Compound	*B. dorsalis*	*B. zonata*	*C. capitata*	*Z. cucurbitae*	Published
4	**105-54-4**	ethyl butanoate	813.54	804.17	807.89		818
5	123-86-4	butyl acetate	825.56		821.43	821.86	823
6	2216-30-0	2,5-dimethylheptane	854.12	848.25	850.22	851.98	
7	**626-38-0**	pentan-2-yl acetate	861.27	856.51	857.36	857.73	843
8	5343-96-4	3-methylbutan-2-yl acetate	863.96				850
9	503-74-2	3-methylbutanoic acid				870.43	875
10	**123-92-2**	3-methylbutyl acetate	887.46	885.92	884.74	885.83	883
11	105-66-0	butanoic acid, propyl ester	907.87	907.35			909
12	543-49-7	heptan-2-ol	909.72	910.03	908.19	908.73	909
13	**97-85-8**	2-methylpropyl 2-methylpropanoate	923.97	922.80	922.48	923.23	914
14	80-56-8	alpha-pinene	947.96			947.55	960
15	108-84-9	4-methylpentan-2-yl acetate	955.46		952.61	955.41	910
16		unknown banana 2	958.97				
17	97-87-0	butyl 2-methylpropanoate	962.22				955
18	97-87-0	butyl 2-methylpropanoate				961.20	
19	**539-90-2**	2-methylpropyl butanoate	965.75	966.94	965.62	966.43	961
20	105-68-0	3-methylbutyl propanoate	978.68	978.81	977.20		972
21	97-72-3	2-methylpropanoyl 2-methylpropanoate	980.75	980.73	979.59		
22	54340-93-1	pentan-2-yl 2-methylpropanoate	988.07	987.83	988.86	988.50	
23	**109-21-7**	butyl butanoate	1003.20	1004.20	1003.32	1003.53	996
24	**589-59-3**	2-methylpropyl 3-methylbutanoate	1014.73	1016.72	1015.52	1015.49	1013
25	2050-01-3	3-methylbutyl 2-methylpropanoate	1020.77	1023.44	1022.69	1022.43	1021
26	**60415-61-4**	pentan-2-yl butanoate	1034.48	1036.42	1035.29	1035.85	1012
27	5921-82-4	heptan-2-yl acetate	1049.48	1052.37	1052.54	1051.88	1043
28	109-19-3	butyl 3-methylbutanoate	1053.50	1055.61	1056.34	1055.26	1048
29	**106-27-4**	3-methylbutyl butanoate	1064.02	1067.09	1067.07	1065.61	1064
30	108-32-7	4-methyl-1,3-dioxolan-2-one	1099.08				
31	27625-35-0	3-methylbutyl 2-methylbutanoate	1106.60	1109.72	1110.01	1107.50	1104
32	**659-70-1**	3-methylbutyl 3-methylbutanoate	1113.79	1118.01	1117.27	1113.74	1110
33	2050-09-1	3-methylbutyl pentanoate	1160.40				1134
34	2639-63-6	hexyl butanoate	1194.78				1192
35	69727-41-9	[(Z)-hex-4-enyl] butanoate	1202.06	1207.11		1204.96	1203
36	17312-65-1	3,3-dimethylundecane	1223.02	1229.10	1229.80	1223.02	

* Kovats could not be calculated

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There is an urgent need to develop novel and sustainable tools for control of pests. In this thesis a selective lure is constructed from generic microbial volatiles for the grape pest, *Lobesia botrana*. Secondly true fruit flies, Tephritidae, were used as model organisms to design a novel workflow, olfactomics, from primary research to lure design. The work shows that selective lures can be designed from generic volatiles and that this process can be strongly accelerated through comparative olfactomics.

Sebastian Larsson Herrera completed his graduate education at the Department of Plant Protection Biology, SLU, Alnarp. He received his M.Sc. in Biological Sciences from the Swedish University of Agricultural Sciences.

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