PLANT-SPECIFIC FACTORS AFFECTING SHORT-RANGE ATTRACTION AND OVIPOSITION OF EUROPEAN GRAPEVINE MOTHS

by

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Abstract

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The spread of pests and pathogens is increasingly intensified by climate change and globalization. Two of the most serious insect pests threating European viticulture are the European grape berry moth, *Eupoecilia ambiguella* (Hübner) and the European grapevine moth *Lobesia botrana* (Denis & Schiffermüller). Larvae feed on fructiferous organs of grapevine *Vitis vinifera*, resulting in high yield and quality losses. Under the aspects of integrated pest management, insecticide measures are only reasonable when other control strategies become ineffective. In order to support the development of novel decision support system for the application of insecticides, the aim of this thesis was to decipher plant-specific factors, which affect the short-range attraction and oviposition of *L. botrana* and *E. ambiguella*.

The focus was set on the visual, volatile, tactile and gustatory stimuli provided by their host plant after settlement. The use of artificial surfaces as model plant showed that oviposition of both species is affected by the color, the shape and the texture of the oviposition site. To explain a susceptibility of certain grapevine cultivars and phenological stages of the berries to egg infestations, we analysed and compared the chemical composition of the epicuticular waxes of the berry surface as well as the volatile organic compounds emitted by the berries. Thereby it turned out that the attractiveness to wax extracts decreased during ripening of the berries, highlighting a preference of earlier phenological stages of the berries for oviposition. In addition, grapevine cultivars exhibited variations in their volatile composition. The principle components perceived by female's antennae could not explain the differentiation between cultivars, suggesting volatiles do not trigger orientation to certain cultivars. Furthermore, a method was developed to measure real-time behavioural response of female moths to volatiles. The setup allowed to quantify the orientation to a volatile source as well as movements of the antennae and ovipositor. They could be linked to the olfactory and gustatory perception of volatiles during the evaluation of suitable host plants for oviposition. In addition, the risk of potential alternative host plants in the vicinity of the vineyard was investigated. This confirmed that *L. botrana* in particular prefers the stimuli provided by some plants to those of grapevine. Overall, the results suggest that during oviposition, volatiles emitted by the plants and the composition of the plant surface are the most important factors for host plant differentiation.

Zusammenfassung

Die Verbreitung von Schädlingen und Krankheitserregern wird durch den Klimawandel und die Globalisierung zunehmend verstärkt. Der Einbindige Traubenwickler *Eupoecilia ambiguella* (Hübner) und der Bekreuzte Traubenwickler *Lobesia botrana* (Denis & Schiffermüller) zählen in Europa zu den wichtigsten tierischen Schaderregern im Weinbau. Die Larven ernähren sich von den Gescheinen und Beeren der Weinrebe *Vitis vinifera,* wodurch hohe Ertrags- und Qualitätsverluste zu verzeichnen sind. Im Sinne des integrierten Pflanzenschutzes ist der Einsatz von Insektiziden nur dann sinnvoll, wenn andere Bekämpfungsstrategien unwirksam sind. Um die Entwicklung eines neuartigen Entscheidungshilfewerkzeuges für den Einsatz von Insektiziden zu unterstützen, war es Ziel dieser Arbeit, pflanzenspezifische Faktoren zu entschlüsseln, die die Nahlockwirkung und Eiablage beider Traubenwicklerarten begünstigen.

Der Schwerpunkt lag auf den flüchtigen, taktilen, visuellen und gustatorischen Reizen, die von ihren Wirtspflanzen ausgehen. Der Einsatz von künstlichen Oberflächen als Modellpflanze zeigte, dass die Eiablage beider Arten von der Farbe, der Form und der Struktur des Eiablagesubstrates beeinflusst wird. Um die Anfälligkeit bestimmter Rebsorten und phänologischen Stadien der Beeren bezüglich der Eiablage zu erklären, wurden die epikutikulären Wachse der Beerenoberfläche sowie die von den Beeren abgegebenen flüchtigen Pflanzenduftstoffe analysiert und verglichen. Dabei stellte sich heraus, dass Oleanolsäure eine der Schlüsselverbindungen ist, die für die Bevorzugung früher phänologischer Stadien der Beeren zur Eiablage verantwortlich ist. Die Rebsorten wiesen Unterschiede in ihrer Duftstoffzusammensetzung auf. Die Komponenten, die über die Antennen der Weibchen wahrgenommen werden, konnten die Unterscheidung der Sorten jedoch nicht erklärt werden. Ferner wurde eine Methode entwickelt, um die Verhaltensreaktion weiblicher Falter auf Duftstoffe in Echtzeit zu messen. Dies ermöglichte die Orientierung zur Duftstoffquelle sowie die Bewegungen der Antennen und des Ovipositors zu quantifizieren. Diese wurden mit der olfaktorischen und gustatorischen Wahrnehmung der flüchtigen Stoffe in Verbindung gebracht werden. Darüber hinaus wurde das Risiko potenziell alternativer Wirtspflanzen in der Nähe des Weinbergs untersucht. Dabei bestätigte sich, dass insbesondere *L. botrana* die Reize, die von einigen Pflanzen ausgehen, denen der Weinrebe vorzieht. Insgesamt deuten die Ergebnisse darauf hin, dass während der Eiablage die Pflanzenduftstoffe und die Zusammensetzung der Pflanzenoberfläche die wichtigsten Faktoren für die Differenzierung der Wirtspflanzen sind.

Chapter I

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General Introduction

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Viticulture faces the challenge of increasing its productivity while reducing its impact on the environment (Kamilaris & Prenafeta-Boldú 2018; Zangina et al. 2021). Nonetheless, viticultural systems are affected by different problems, such as environmental conditions and pest infestations (Costa et al. 2023). Winegrowers are expected to combat an increase in pest pressure due to climate change and globalization and therefore in phytosanitary measures (Ioriatti et al. 2023; Caffarra et al. 2012; Gutierrez et al. 2012; Klein & Kerr 1995).

The grapevine moth, *Lobesia botrana* (Denis & Schiffermüller) 1975 (Lepidoptera: Tortricidae), and the grape berry moth, *Eupoecilia ambiguella* (Hübner) (Lepidoptera: Tortricidae), are two of the most important insect pests of grapevines, responsible for high productivity losses in vineyards worldwide (Gutierrez et al. 2012; Lucchi et al. 2018; Sammaritano et al. 2018; Rank et al. 2020; Ioriatti et al. 2023). The two species are distributed over America, Asia and Europe, *L. botrana* additionally occurs in Africa. Furthermore, they are categorized as quarantine pest in North America (Canada, Mexico and USA) and Asia (*E. ambiguella* in Israel; *L. botrana* in China) (EPPO 2023a, 2023b). Up to date, the highest impact of these insect pests is reported for the Western European viticulture (Ioriatti et al. 2023).

Both species pass a facultative diapause in pupal stage, generally resulting in developing two to three generations per year (Ioriatti et al. 2023; Thiéry & Moreau, 2005). Under climatic favourable conditions, e.g. in Mediterranean regions, *L. botrana* is able to initiate a fourth generation (Katerinopoulos et al. 2005). The cycle length is essentially controlled by the photoperiod and temperature (Thiéry & Moreau 2005; Almeida et al. 2022), while larvae develop in 5-6 stages (Pavan et al. 2013). Larvae of the first generation are anthophagous (flower-feeding), while the following generations are carpophagous (fruit-feeding), both on unripe and ripe berries. In general, larval damage is variety-dependent, while grapevine varieties with dense berry clusters are more severely affected than those with loose growth, because they promote larval establishment (Moreau et al. 2006; Moreau et al. 2008; Sharon et al. 2009; Pavan et al. 2018) and infections of the gray mould fungus *Botrytis cinerea* (Fermaud & Lemenn 1989; Fermaud 1998). In severely damaged vineyards, up to 30 larvae were counted per cluster (Thiéry et al. 2018). The damage caused by feeding on the inflorescences can usually be compensated by reducing the shedding of flowers (Moschos 2006) and increasing the weight of the berries (Roehrich 1978). Oviposition on leaves of vines is rarely observed (Marshall 1912; Stellwaag 1928). Mating plays a major role in initiating the oviposition process, while unmated females were capable to retain their eggs until they copulate (Maercks, 1935) and delays in copulation reduce fecundity and fertility of the females (Torres-Vila et al., 2002).

Although grapevine (*Vitis vinifera* L.) is commonly mentioned as major host plant of the two pests (Maher 2002; Benelli et al. 2023a; Ioriatti et al. 2023), *L. botrana* seems to be polyphagous and has been reported from more than 40 plant species, as recently reviewed by Benelli et al. (2023a), while flax-leaved daphne (*Daphne gnidium*) is supposed as wild host of origin (Maher & Thiéry 2006). The two moth species can co-occur on grapevine though *L. botrana* is generally considered more harmful than *E. ambiguella* (Voglino 1914). This may be explained by the variable humidity requirement of the species. *Lobesia botrana* prefers dry environmental conditions whereas *E. ambiguella* is associated with rather humid areas (Bovey 1966; Stellwaag 1928; Blümel et al. 2020). Female moths are able to lay eggs almost their entire life, which is approximately a period of three weeks (Maercks, 1935). Thereby, a peak in reproductive activity occurs early, when females reach an age of 3-7 days (Maercks, 1935).

The selection of a suitable host plant by herbivorous insects is crucial in order to reproduce and survive and in general divided into three consecutive steps: host orientation (long-range and short-range), host contact and host assessment (Renwick 1989). Lepidopterans, such as *L. botrana* and *E. ambiguella*, exhibit several sensory receptors, which are located at the labial palps, tarsi, antennae, proboscis and ovipositor (Amat et al. 2022; Maher & Thiéry 2004; Renwick and Chew 1994). In case of flying insects, host orientation from the distance is associated with the visual appearance of the plants, e.g. plant height, color and shape (Roessingh & Stadler 1990), as well as volatile organic compounds (VOCs) released by the plant (Tasin et al. 2006; Masante-Roca et al. 2007; Schmidt-Büsser et al. 2009; Tasin et al. 2010; von Arx et al. 2011). The latter are usually perceived by the non-porous sensilla and terminal pore sensilla located at the tip of the female's antennae (Maher & Thiéry 2004; Pérez-Aparicio, Torres-Vila & Gemeno 2019). Upon landing, plant contact allows to perceive tactile and/or gustatory plant stimuli, which finally decides on acceptance for oviposition (Schmiederwenzel & Schruft 1990; Maher & Thiéry 2004; Knolhoff & Heckel 2014). Therefore, females of *L. botrana* use probably contact-chemo-mechanoreceptors, which are distributed on the extremities of the legs, the distal part of the proboscis and on the ventral surface of the ovipositor, thus allow the detection of non-volatile substances (Maher & Thiéry 2004). In general, oviposition rates may vary in terms of moth's mating success, fertility and food quality (Stellwaag 1928; Muller et al. 2016; Thiéry, Monreau & Moreau 2014). The level of egg infestation determines the possible need to apply insecticides.

But the use of pesticides is not always reasonable and pesticide resistance has been termed a "dilemma" (Gould, Brown & Kuzma 2018). Integrated pest management (IPM) is a holistic and sustainable approach to control grapevine moths, and any other pest, using a combination

of preventive and curative measures, thereby becoming less dependent on synthetic pesticides (Pretty & Bharucha 2015; Pertot et al. 2017; Benelli et al. 2023b). Within the EU, it is explicitly prescribed by Directive 2009/128/EC (EC 2009), that agricultural systems within the union must agree to the principles of IPM. This also implies an application of synthetic pesticides only when there is an urgent need. Thereby, the goal is not to eradicate pests, but to manage them at low levels below economically harmful thresholds (Pretty & Bharucha 2015).

With this in mind, measures to control grapevine moths may imply, as recently reviewed by Benelli et al. (2023b): preventive strategies, e.g. monitoring and modelling, sterile insect technique, mating disruption and natural enemies. Up to date, the sex pheromone-based mating disruption technique is predominantly applied (Thiéry et al. 2018), whereby in Germany almost 60% of the vineyards are treated with pheromones (Jehle et al. 2014). In order to judge their efficiency, winegrowers are advised to monitor egg infestations in vineyards (BASF 2023), while at least 50 inflorescences should be checked. If a threshold of 5% is exceed, insecticide measures targeting larvae in their most vulnerable stage (black-head stage, immediately after egg-hatch) are advised (BASF 2023). However, accompanying monitoring is time-consuming and requires a high level of practical experience (Benelli et al. 2023b), since eggs are small (about 1mm in size) and not easy to detect on fructiferous organs due to their transparency (Lucchi et al. 2018; Stellwaag 1928). Thus, pheromone traps are often used for monitoring of female oviposition even though they are inappropriate because there is no correlation between male flight and female oviposition activity (Hoffmann and Doye 2017). One approach to tackle this issue is to develop a novel Decision Support System (DSS), an artificial "moth oviposition card", that simplifies the visual detection of eggs and thus supports winegrowers to decide on the necessity of insecticide applications (Benelli et al. 2023b). Therefore, the aim of the present thesis was to decode essential plant-specific factors, which affect attraction and egg deposition of both, *E. ambiguella* and *L. botrana* females, and can be used for the development of a DSS. The principle mechanism of female attraction to suitable host plants for oviposition is summarized in **figure 1** (left), while the plant specific factors investigated in the present work are highlighted in **figure 1** (right).

The initial step in developing the DSS involves investigating on attractive surfaces for the oviposition of *E. ambiguella* and *L. botrana*. Therefore, **chapter II** of this thesis addresses the use of artificial surfaces as model plants in order to evaluate visual and tactile stimuli that may influence oviposition of these two species after settlement. The use of synthetic materials is a deliberate choice to exclude volatile and gustatory stimuli emitted by a host plant, while surfaces were manipulated concerning physical factors such as color, shape, and texture.
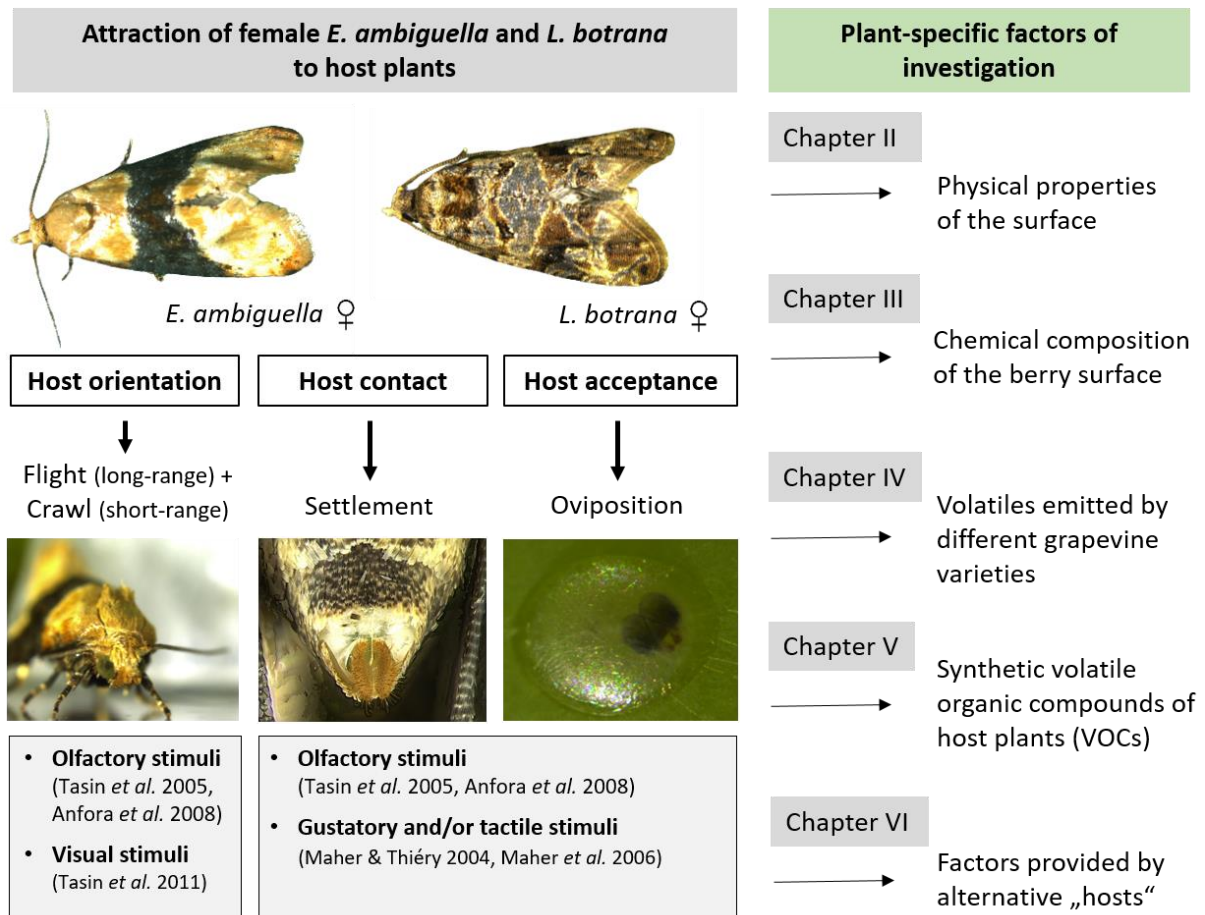
Figure 1: Scheme of (left) the attraction mechanism of the *Eupoecilia ambiguella* and *Lobesia botrana* to host plants for oviposition (A: Adult female of *E. ambiguella* in frontal view; B: Ovipositor of *L. botrana* with sensilla in ventral view; C: Egg of *L. botrana* hosting a larva in blackhead-stage) and (right) plant-specific factors investigated in Chapters II - VII in this thesis.

Chapter III focuses on the chemical composition of the berry surface of grapevine and its effect on the oviposition decision of the two moth species. An experimental setup was developed to demonstrate possible oviposition preferences for certain grape cultivars and developmental stages based on epicuticular wax extracts. Epicuticular wax patterns of four different cultivars (Regent, Pinot noir, Müller-Thurgau and Riesling) were chemically analysed and compared in order to decipher non-volatile compounds promoting oviposition.

In addition to this chapter, **chapter IV** examines the possible susceptibility of different grapevine cultivars and phenological stages of grapevine to attract *E. ambiguella* and *L. botrana* for oviposition, based on volatile organic compounds (VOCs) released by plants. The VOCs emitted by the berries of three different cultivars (Regent, Pinot noir and Müller-Thurgau) were analysed and compared and checked for perceptibility by female's antennae.

Supportive to this, **chapter V** investigates on developing a method to measure the real-time behavioural response of female moths to VOCs using a four-chamber olfactometer coupled

with a video tracking system. The system should be evaluated by offering pheromones to males and the scent of grapevine plants to females. Single synthetic VOCs examined in this study were previously reported as components of the scent of the host plants *V. vinifera* and/or *D. gnidium*: Their competence to promote the egg laying process was investigated.

Chapter VI focuses on the potential of alternative plant species in the surrounding vegetation of vineyards to provide *L. botrana* and *E. ambiguella* specific stimuli distinguishable from those of their main host, grapevine *Vitis vinifera*. The effect of volatile, gustatory/ tactile stimuli on short-range attraction and oviposition were evaluated in relation to their contribution to the spread of these insect pests within the vineyard.

Chapter VII discusses and concludes the outcomes of the presented work in the context of a contribution to IPM strategies and their implementation in the development of novel DSS.

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Physical factors influencing the oviposition behaviour of
European grapevine moths *Lobesia botrana* and
Eupoecilia ambiguella

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Abstract

Uncovering the entire process of oviposition could enable the development of innovative pest control strategies. The European grapevine moth *Lobesia botrana* as well as the European grape berry moth *Eupoecilia ambiguella* are serious insect pests, which still threaten viticulture in many parts of the world. By creating an artificial semiochemical emitting surface, in form of a card, we tried to develop an egg-monitoring tool for the oviposition of these two species in vineyards. The idea was to create a decision support system that facilitates timing and necessity of insecticide treatments. This article deals exclusively with the initial step towards the development of this tool: the influence of physical factors (colour, shape or texture) of a surface on the oviposition behaviour of *L. botrana* and *E. ambiguella* under laboratory conditions. The acceptance of different artificial substrates as oviposition site was analysed exclusive of volatile organic compounds emitted by a host plant. We investigated particular colours of the surface, which could be a visual stimulus to trigger the oviposition of both species. Apparently, the colour green, the texture of the material and the light conditions turned out to be highly important stimuli for oviposition.

1. Introduction

25.6 million tons of grapes were produced in Europe in 2015, and the vast majority (92%) was destined for vinification (Eurostat 2017). Its quality and to some extend also the amount of the vintage are threatened by both the European grapevine moth *Lobesia botrana* [Denis and Schiffermüller] and the European grape berry moth *Eupoecilia ambiguella* [Hübner] (Lepidoptera: Tortricidae). They are serious vineyard pests in many parts of the Palaearctic Region (Bovey, 1966; Roehrich & Boller, 1991). In particular, *L. botrana* is widespread in the Middle East and Europe (Louis & Schirra, 2001) and has recently invaded Chile, Argentina (Gonzalez, 2010) and North America (Varela, Smith, Cooper, & Hoenisch, 2010).

Economic losses are primarily caused in vineyards, where the two species can occur together on grapevine (*Vitis vinifera*) (Thiéry & Moreau, 2005). Female moths lay single eggs on inflorescences and berry-like fruits so that the hatching larvae can feed on the fructiferous organs. Beside the direct damage, the injury on the berries promotes the establishment of bacteria and fungi such as grey mould *Botrytis cinerea* (Savopoulou-Soultani & Tzanakakis, 1988). The occurrence of one or both of those species is thus strongly associated with yield and quality losses (Marchesini & Dalla Montà, 2004). In Europe, both species are able to develop 2–3 generations in 1 year (Thiéry & Moreau, 2005), whereas *L. botrana* completes 3–4 generations in Mediterranean regions (Gordon et al. 2005). Due to their polyphagous lifestyle, both species are able to spread and reproduce on several host plants belonging to 27 different plant families (Lucchi, 2015). Its host range might differ significantly from country to country according to the local climate conditions. *Daphne gnidium* (flax-leaved Daphne) is supposed to be the native host plant of *L. botrana* (Maher & Thiéry, 2006; Torres-Vila & Rodriguez-Molina, 2013), whereas *E. ambiguella* is known as a polyphagous species with transpalearctic distribution that occurred also outside winegrowing regions up to southern Scandinavia (Razowski, 2001; Stellwaag, 1928).

Even though *Artemisia vulgaris* was sometimes mentioned as one possible native host plant for *E. ambiguella*, Ioriatti, Lucchi and Varela (2012) conclude that grapevine is now accepted as its original host. The first generation of *L. botrana* in southern Europe is known to develop on flowering olive trees (*Olea europaea* L.) (Savopoulou-Soultani, Stavridis, & Tzanakakis, 1990). Other reported agricultural crops, where at least *L. botrana* was spotted, include black currant (*Ribes nigrum*), cherry (*Prunus avium*), gooseberry (*Ribes uva-crispa*), kiwi (*Actinidia chinensis*), nectarine (*Prunus persica*), plum (*Prunus domestica*) (Bradley, Tremewan, & Smith, 1979; Maher, Toulouse, Jolivet, & Thiéry, 2000; Moleas, 1988; Zhang, 1994),

pomegranate (*Punica granatum*), persimmon (*Diospyros* spp.) (Gorden et al. 2005) and highbush blueberries (González-Domínguez, Caffi, Ciliberti & Rossi, 2015). The selection of a suitable plant for oviposition by females is critical to the survival of their progeny.

Locating the host plant for oviposition by herbivores is generally divided into three parts: (i) host orientation, (ii) host contact and (iii) host assessment (Renwick, 1989), which are controlled by the chemical and physical characteristics of the plant. The physical characteristics are strongly associated with the visual appearance and the contact cues of the plant after landing in case of flying insects (Harris & Rose, 1990; Roessingh & Städler, 1990). Visual stimuli that attract females over longer distances may include plant height, orientation, colour or shape (Knolhoff & Heckel, 2014). In contrast, contact cues allow the evaluation of tactile and chemical plant stimuli that are non-volatile or have a low volatility (Knolhoff & Heckel, 2014). For this purpose, the females possibly use non-porous sensilla and terminal pore sensilla located at the antennal tip, the extremities of the legs, the distal part of the proboscis and on the ventral surface of the ovipositor (Maher & Thiéry, 2004a). They are assumed to be involved in evaluating the roughness and shape of the host plant, whereas the contact-chemoreceptors could allow the detection of plants attractants and/or deterrents (Calas, Thiéry, & Marion-Poll, 2006; Thiéry & Gabel, 1993). In this regard, *E. ambiguella* was less studied and it is still unclear whether this species, which shares the host plant grapevine, behaves in a similar way as *L. botrana*. In recent studies, evidences were given that also plant volatiles play a role in the location of the host plant and the post-landing process of *L. botrana* (Tasin et al., 2005). In other moth species, it might be possible that physical factors have a greater influence on the selection of an oviposition site than close-range chemical cues (Foster et al., 1997). The role of thigmotactic stimuli on oviposition has already been suggested for the tortricids *Zeiraphera diniana* (Bos & Baltensweiler, 1977) and *Cydia pomonella* (Lombarkia & Derridj, 2002).

The aim of this study was to investigate the physical characteristics that provide sensory or visual cues for oviposition of *L. botrana* and/ or *E. ambiguella*. We examined the influence of colour, shape and the texture of a surface on the oviposition behaviour of stock culture insects. Understanding the physical factors influencing the oviposition process could contribute, together with research results on the impact of volatiles and gustatory substances for oviposition, the development of an innovative decision support system for insecticide control of these pests. This tool should facilitate the timing and necessity of pest control. Based on a quantifiable egg deposition, which is visible on the card, the winegrower should be able to draw conclusions for the infestation on the adjacent grapes. Insecticide applications, which are often

only efficient during the egg stage, can thus be carried out precisely, so that plant protection products can be reduced.

2. Materials and Methods

2.1. Insect rearing

Adult females of *L. botrana* and *E. ambiguella* used in the following bioassays were taken from a laboratory culture (Figure S1) established at the Julius Kühn-Institut in Siebeldingen that each year is genetically refreshed with moths, which developed from larvae collected in surrounding vineyards. Larvae were mass-reared in 500 ml polypropylene (PP) cups (Huthamaki, Alf, Germany) on a semi-synthetic diet (1.5 L water, 60 g agar, 187 g wheat germs, 88 g casein, 38 g yeast, 74 g sugar, 4 g benzoic acid, 2.5 g cholesterol, 2.5 g nipagin, 40 g ascorbic acid, 1 ml formaldehyde (3.7%), 5 ml sunflower oil, 25 g Wesson's salt mixture, 15 g Vanderzant vitamin mixture and 1 g chloramphenicol) following Mani, Riggenbach, and Mendik (1978). The cups were lined with corrugated paper (Friedr. Schulte & Söhne, Weilerswist, Germany) to enable the immigration of the larvae for pupation and covered by a perforated lid to allow air circulation. The pupae were removed from the corrugated paper and transferred into paper bags to enable the hatching and mating of the adults.

A Plexiglas cage (Ø = 15 mm, H = 15 mm), lined with a plastic bag (2L, Toppits, Cofresco Frischhalteprodukte GmbH & Co. KG, Minden, Germany) as oviposition surface, was attached to the top of the paper bag and a cellulose plug was installed on the upper shell of the cylinder as a water supply for the moths. The freezer bag on which the females deposited their eggs was removed from the cylinder twice a week and attached to the inside of a new PP cup in which the hatched larvae can feed from the artificial diet. An illustration of the insect rearing can be found in the appendix. The insect rearing and the bioassays were performed separately from each other under controlled climatic conditions of 23:19 ± 2°C (day: night setback), 70 ± 10% relative humidity and a L14:D8 photoperiod +1 hr each of dusk and dawn. The two species were kept in separate plant growth rooms "Fitotron type SGR233" (Weiss Technik UK Ltd, Loughborough, UK).

2.2. Manipulation of the physical factors of the oviposition surface

Surface layers of different synthetic composition were chosen to find a suitable oviposition material on which the physical characteristics (like colour, shape and texture) could be examined excluding the influence of volatile organic compounds usually emitted by a plant.

The oviposition material should enable an easy detection of the eggs, to ensure that winegrowers can determine the egg infestation within their vineyard. As the monitoring tool should be used in the field, the surface has to be stable to all kinds of weather conditions, as far as possible. Preliminary studies with 27 different kinds of oviposition materials have shown that beside glass, which cannot easily be modified in its physical characteristics, only plastic films were reasonable for our scope of work (data not shown). Consequently, we used the following synthetic materials (4 × 6 cm) to check oviposition preferences, which were characterized by different contact characteristics and visual appearances (see also Table S1): polypropylene (PP) films (Renolit SE, Worms, Germany), polyethylene therephthalate (PET) films (Folienwerk Wolfen GmbH, Bitterfeld-Wolfen, Germany) and low-density polyethylene (LDPE)-coated crepe paper (CP) (W. Bosch & Co. KG, Wipperfürth, Germany), whereas the LDPE-coated CP was tested on both sides, and the other materials were checked on one side only (embossing outwards).

The materials were compared under smooth and textured surface conditions. The texture type of the oviposition surface was analysed using PP films of different embossing. PP films "Stationery POKF Type 04647" (Renolit SE, Worms) of different grain were chosen and subsequent referred to the types A, B, C, D, E, F, G and H. Figure 1 provides an illustration of the evaluated textures.
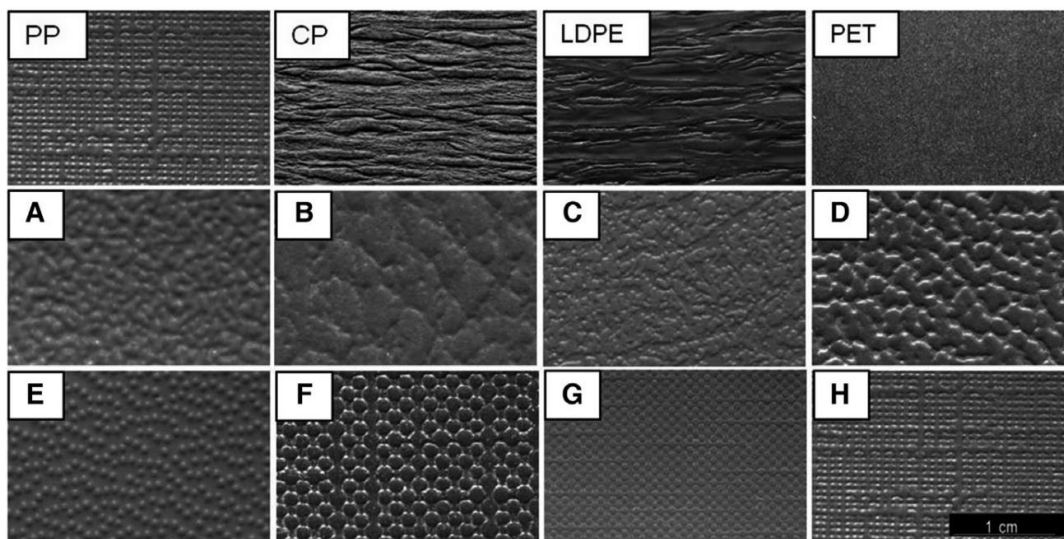
Figure 1. Detailed view of the textured materials: PP=polypropylene, CP=crepe paper, LDPE=low-density polyethylene, PET=polyethylene therephthalate and A-H (texture types of PP films). Image acquisition: Nikon D7200, 24MP (adjustment: aperture priority, exposure: +0.7), flashlight: Nikon Speedlight SB910 (adjustment: TTL-BL, indirect exposure: −1.3), objective lens: Sigma EX 105 mm/2.8 DG OS Macro HSM (aperture: 8, exposure time: 1/60 s

The PP films "Stationery POKF Type 04647" (Renolit SE, Worms) diverse in colour but of same texture (Figure 1H) were used to evaluate the visual influence of the colour on females oviposition. Six colours were checked in dual-choice experiments. Colours that occur in vineyards like brown (bark/wood), green (leaves/unripe berries), blue (ripened berries), yellow, violet and turquoise (flowers in the green cover) might influence the oviposition behaviour of *L. botrana* and *E. ambiguella* females. We also compared light grey with dark grey to determine the impact of shade within the same colour.

In addition, two different shapes of the oviposition surface were assessed (convex and plane) to understand whether the convexity of grape berries is an essential cue for oviposition. Blue PP films (Figure 1H) of identical surface area (3×5 cm) were provided with black synthetic felt underneath to form the surface (convex and plane) and prohibit transparency.

2.3. Oviposition bioassay

The oviposition behaviour of *L. botrana* and *E. ambiguella* on synthetic materials was checked in comparison with a transparent PP film (Huthamaki, Alf, Germany) as reference material, which is usually used as oviposition substrate during insect rearing. Oviposition preferences were evaluated in an arena-based bioassay. The arenas consisted of a cylindrical Plexiglas-cage ($\emptyset = 15$ cm, H = 15 cm), lined with black cardboard and covered by a stainless-steel grid (mesh size: 2 mm). An additional grid was put underneath the cage, which facilitated a constant exposure to light and air exchange. The examined synthetic oviposition substrates (4×6 cm) were fixed at the inner wall of the Plexiglas-cage by a paper clip. In each dual-choice experiment, two oviposition substrates (different in material, texture or colour) were offered on the opposite side. The remaining inner wall of the area was covered by a synthetic black felt, a material deterrent for oviposition (Hoffmann, 2008).

The substrate that performed better in the dual-choice test was chosen as reference material for the following experiments to find the most attractive of the evaluated oviposition surfaces. This process was continued until one material was left over. The materials and textures were offered to females for oviposition under dark conditions in a way that they were not able to perceive visual stimuli because not all structures were available in an identical colour.

To test the impact of convexity versus planarity, both kinds of shapes were offered in a dual-choice experiment first at the top side and in a second turn at the bottom side of the cylinder (Figure 5a). For this purpose, the materials were fixed on the stainless-steel grid with an aluminium wire (0.2 mm). In a third version, the shapes, which were fixed on the lid, were

additionally covered by a black cardboard to prevent an influence of variable light exposure, triggered by the materials. In preparation of all experiments, pupae were separated by gender according to Stellwaag (1928) about 1 week before hatching. Males and females could be distinguished by the number of body segments (females: eight; males: nine). To ensure adequate egg counts, eight pupae of both gender were transferred into cylindrical plastic vessels (Ø = 50 mm, H = 10 mm) and closed with a water-moisturized cellulose-plug. No food was provided. Moths (age<24 hr) were allowed to copulate for 48 hr before the bioassay was carried out. Each eight adult females (age <72 hr, with visible oviposition during copulation period) were separated from the males and introduced into the arena 4 hr before dusk (20–30 replicates per test). Every second arena was rotated 180° to avoid an influence of the plastic film position on the egg laying behaviour. Fluorescent lamps (Philips Master TL-D 36 W/840 Reflex, Hamburg, Germany) 110 cm above the arenas provided 14 hr of daylight (~3.500 lux) that was followed by 1 hr of dusk (at the beginning and at the end of the photophase) and 8 hr of darkness. The bioassay was ended after a total of 72 hr by removing the females from the arena. Then, the number of eggs laid on both materials was counted. Within a shorter period of time, females may not choose between different materials and the number of eggs laid on the surfaces may be reduced (Anfora, Tasin, De Cristofaro, Ioriatti, & Lucchi, 2009).

2.4. Statistical analysis

Relative attractiveness of the oviposition surfaces was calculated using the oviposition discrimination index (ODI) mentioned in Maher & Thiéry (2004b), where ODI = ([number of eggs on substrate B—number of eggs on substrate A]/total number of eggs) × 100%. The value ranges from −100 to +100%. Negative ODI values imply a favouritism of substrate A (reference material), whereas positive ODI values represent a preference of substrate B as oviposition surface. Data were analysed and illustrated using the statistical software "R"—version 3.0.3 (R Core Team 2016) and "R studio"—version 0.99.491 (RStudio Team 2015). Statistical analysis was carried out by applying nonparametric Wilcoxon signed rank test (α=0.05) for paired data sets.

1. Results

1.1.Synthetic material and texture

In the first approach, we tested the type of synthetic materials which suits best for oviposition acceptance of the two moth species and if an additional texture on the material could enhance

oviposition (Figure 2). Over all oviposition experiments, we calculated a mean per arena of 136.2 ± 3.8 eggs for *L. botrana* (n = 510) and 89.5 ± 1.3 eggs for *E. ambiguella* (n = 510). Both moth species preferred textured PP film (approximately 60% more eggs for *L. botrana* and 72% for *E. ambiguella*) over smooth PP films. *L. botrana* deposited significantly fewer eggs (p<.05) on smooth (26% less egg counts) and textured (36% less egg counts) crepe paper than on the reference material (smooth PP film). In contrast, these two CPs were suitable oviposition materials for *E. ambiguella* on which 51% (smooth: p<.05) and 75% (textured: p<.01) more eggs were found than on PP film. PET films were not more attractive for oviposition than PP films, neither for *L. botrana* nor for *E. ambiguella*. *E. ambiguella* deposited 40% (smooth: p<.05) and 64% (textured: p<.01) fewer eggs on PET films, whereas *L. botrana* did not distinguish between the materials as oviposition site (p>.05). A more suitable material for *L. botrana* oviposition was LDPE (textured LDPE: 39% (p<.01) and smooth LDPE: 20% (p<.05) more eggs than on PP), irrespective of its texture. *E. ambiguella* preferred LDPE solely against PP for oviposition when it was textured (56% more eggs; p<.05).
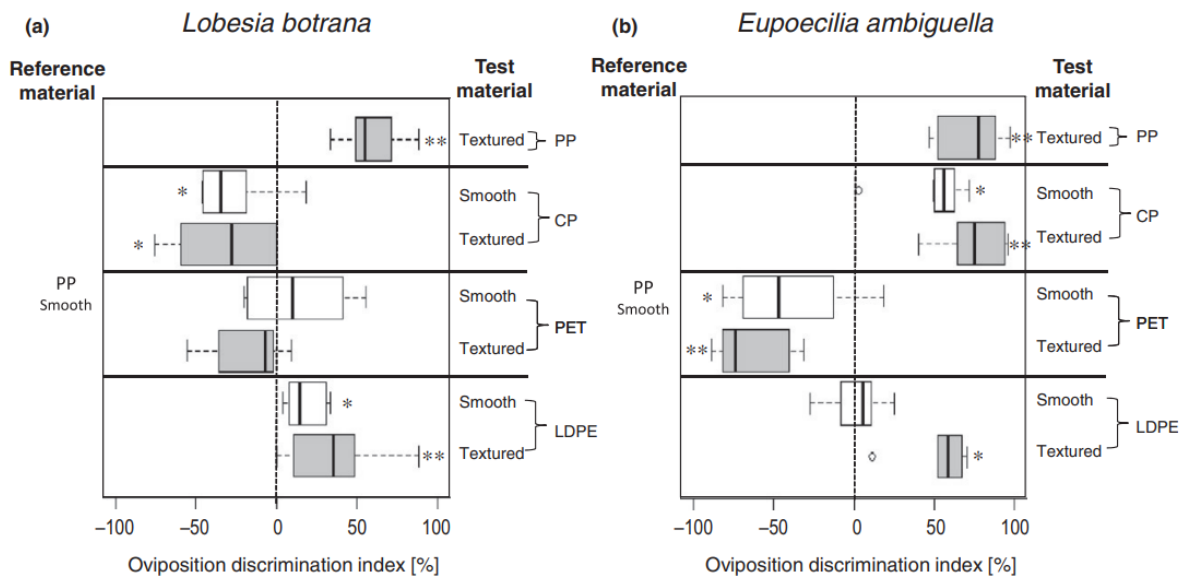
Figure 2. Oviposition preferences of (a) *L. botrana* and (b) *E. ambiguella* between the reference material (PP smooth; left) and different oviposition materials (PP=polypropylene, CP=crepe paper, PET=polyethylene terephthalate, and LDPE=low-density polyethylene) of smooth (white) or textured (grey) surface condition expressed by ODI=oviposition discrimination index varying from −100 to +100%. Asterisks indicate significant differences (*p < .05; **p < .01) between egg numbers on two different surfaces according to Wilcoxon signed rank test (N = 20 replicates per test)

As structured PP films turned out to be preferred oviposition sites by the two moth species compared to smooth ones (Figure 2), the type of the texture of the oviposition sites made of the same material (PP film) was checked for oviposition preferences. Eight different textures were

available to be checked (Figure 1), and both species were able to discriminate between them (Figure 3). The experiment was divided into three steps for evaluating the most attractive texture for oviposition.
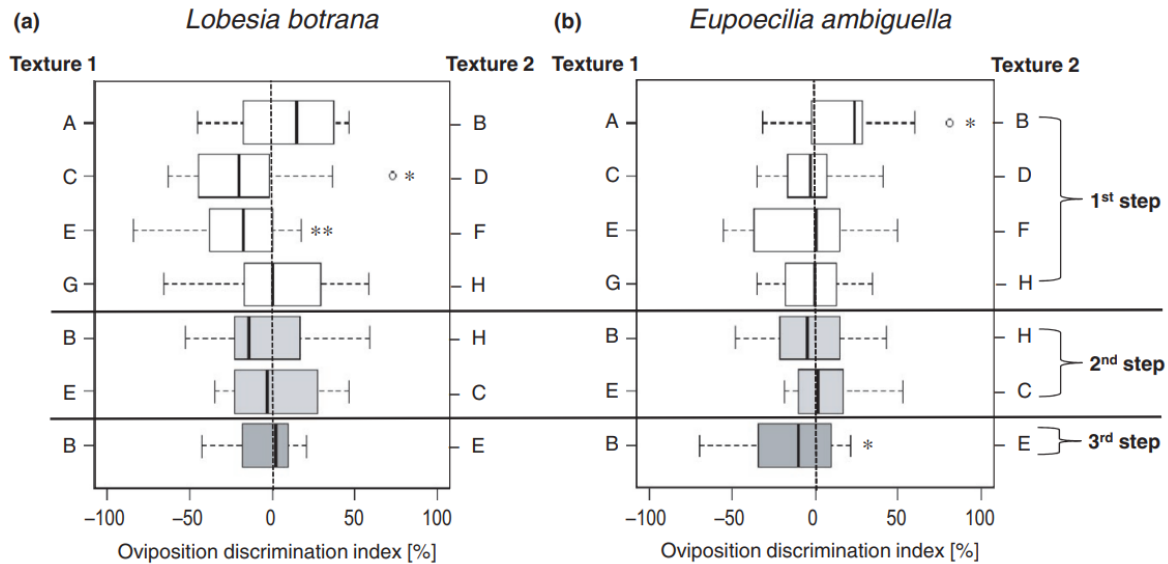
Figure 3. Oviposition preferences of (a) *L. botrana* and (b) *E. ambiguella* for polypropylene films of different texture types (code for texture types see Figure 1) expressed by ODI=oviposition discrimination index varying from −100 to +100%. Asterisks indicate significant differences (*p < .05 and **p < .01) between egg numbers on surfaces according to Wilcoxon signed rank test (N = 20 replicates per test). Stepwise comparison of favoured textures in dual-choice experiments divided into first, second and third step.

In the first step, *L. botrana* favoured texture C against texture D (p<.05) and texture E against F (p<.01). The second step demonstrated that texture C and texture E were similar (p>.05) regarding oviposition site acceptance by *L. botrana* as well as texture B and texture H (p>.05). In step two, texture B and texture E have been by trend (p>.05) the best oviposition materials and used for the third step of the oviposition assay. These two presented materials are comparable, because the ODI reached a value near 0 (−4%; p>.05). In comparison, *E. ambiguella* preferred texture B against A (p<.05). This texture B also resulted in higher oviposition rates when compared to texture C (p<.05) in the third step of experiments. No statistically significant differences could be detected between the other comparisons regarding the oviposition preference (p>.05) for *E. ambiguella*.

3.2. Colour

In a first dual-choice test, *L. botrana* and *E. ambiguella* did not demonstrate statistically significant oviposition preferences between turquoise and blue films (Figure 4). In addition,

both species did not discriminate light grey and dark grey or violet and yellow with respect to oviposition. Only *L. botrana* showed a statistically significant higher number of deposited eggs (on average 27%) on green than on brown PP films (p<.01; Figure 4a; 1st step).
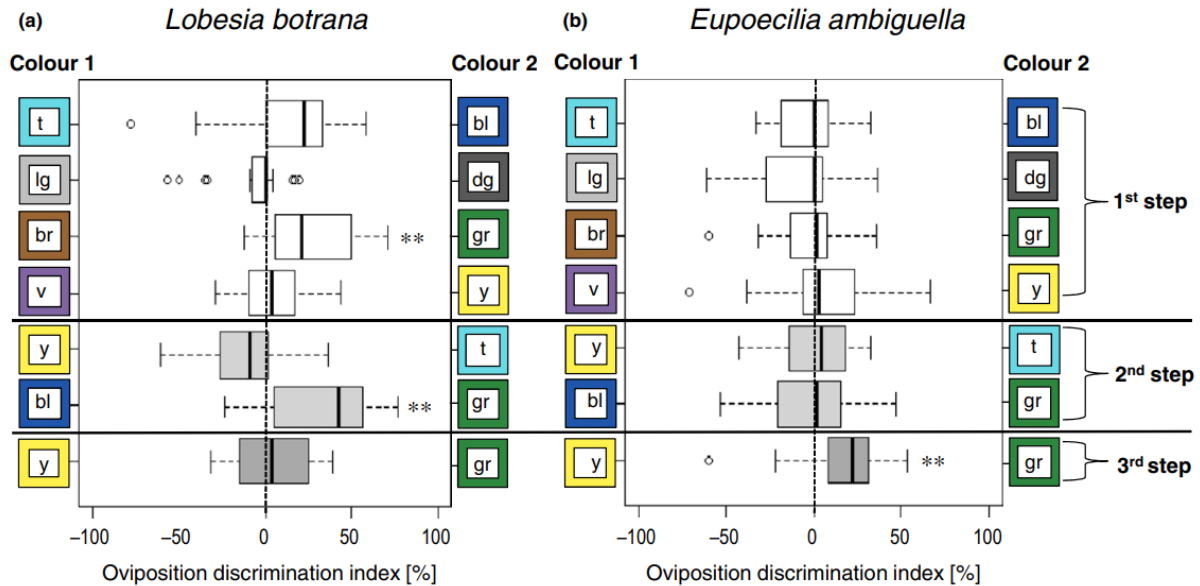
Figure 4. Oviposition preferences of (a) *L. botrana* and (b) *E. ambiguella* between polypropylene films of different colours (bl=blue, br=brown, dg=dark grey, gr=green, lg=light grey, t=turquoise, v=violet, y=yellow) expressed by ODI=oviposition discrimination index varying from −100 to +100%. Asterisks indicate significant differences (*p < .05 and **p < .01) between egg numbers on surfaces according to Wilcoxon signed rank test (N = 20 replicates per test). Stepwise comparison of favoured colours in dual-choice-experiments divided into first, second and third step.

In the second step of the experiment, the colours with the most oviposition shown in first step were chosen and compared again. In both insect species, yellow and turquoise films were not discriminated as oviposition sites (p>.05). By comparing the colours blue and green, green was significantly preferred (p<.01) for oviposition by *L. botrana*, whereas *E. ambiguella* showed no statistically significant oviposition preference (p>.05) in the second step of the experiment. Green (twice preferred by *L. botrana*) and yellow were chosen in the third step of the dual-choice oviposition assay. *L. botrana* did not prefer any of the two colours, whereas *E. ambiguella* deposited significantly more eggs (18%; p<.01) on green than on yellow.

3.3. Shape

In terms of oviposition, both moth species responded in a similar way to convex and plane surfaces (Figure 5). If the surfaces were placed on the bottom side of the arena and the females had to oviposit on the top, convex shapes were favoured (in mean 52% (*L. botrana*) and 36%

(*E. ambiguella*) more eggs) in comparison with plane shapes (p < .001: *L. botrana*, p < .05: *E. ambiguella*).
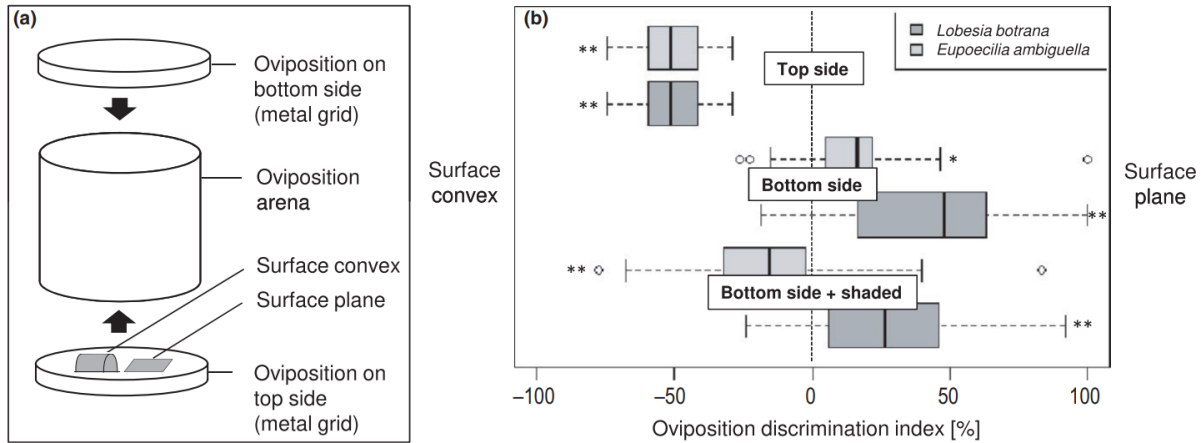
Figure 5. (a) Bioassay set-up for evaluating the oviposition behaviour of grapevine moths on plane and convex shaped surfaces (b) Oviposition preferences of (light grey) *L. botrana* and (dark grey) *E. ambiguella* between convex and plane shaped PP films expressed by oviposition discrimination indices varying from −100% (convex surface preferred) to +100% (plane surface preferred). PP films were placed alternative at the bottom and at the top side of the arena. Asterisks indicate significant differences (*p < .05 and **p < .01) between egg numbers on surfaces according to Wilcoxon signed rank test (N = 30 replicates per test).

In contrast, if the oviposition sites were placed on the top side of the arena and the females are forced to lay their eggs upside down, they reacted inversely and deposited significantly more eggs (p<.01) on plane than on convex shapes. In this case, *E. ambiguella* deposited approximately 20% and *L. botrana* 42% more eggs on plane models. It could not be excluded that changed light exposure during oviposition on the bottom side influences the oviposition behaviour. Therefore, the lid was additionally shaded with a black cardboard in a further experiment. *L. botrana* still preferred plane shapes for oviposition (26%, p<.01), whereas *E. ambiguella* reacts inversely and preferred convex (16%, p<.01) in comparison with plane shaped surfaces.

4. Discussion

To prevent economic losses, which can be caused by the European grapevine moth *L. botrana* and European grape berry moth *E. ambiguella*, it is necessary to detect threshold levels at insect's early life stage. For this purpose, a visual egg monitoring on berries is necessary, but not practicable for winegrowers. Pheromone traps equipped with female sex pheromones to catch males are often used for monitoring of female oviposition even though they are

inappropriate because there is no correlation between male flight activity and female egg-laying behaviour (Hoffmann & Doye, 2017). These traps are only suitable for monitoring the search activity of males for females. Oviposition and consequently infestation rates are regulated by mating success and climatic conditions. Most notably, in areas, where mating disruption as control strategy against grape berry moths is not possible (small vineyards, highly structured landscape), insecticides have to be applied. For these vineyards, an appropriate egg-monitoring tool is needed to determine integrated pest management thresholds and the timing of insecticide application (Gross & Gündermann, 2016). Thus, the intention of this article was to find a synthetic oviposition surface, which could be used subsequently as surface of a more complex egg-monitoring tool for evaluating moth infestation rates on grapevine.

A dual-choice oviposition assay was developed to analyse the oviposition acceptance of different artificial substrates. The bioassay was used to determine physical key factors for the oviposition of both grapevine moth species. Coscolla (1997) reviewed the number of eggs in studies of different authors. They ranged between 30 eggs/female in the field and 160 eggs/female in the laboratory (for *L. botrana*). In our oviposition experiments, we counted a mean of 136.2 ± 3.8 eggs laid by *L. botrana*, which fits well in this range. As each test lasted only 72 hr, females have laid only a part of their eggs they would have laid during their whole lifetime.

In case of *L. botrana*, egg laying primarily occurs on relatively smooth surfaces of berries and inflorescences (Bovey, 1966; Götz, 1941). This was confirmed by Maher & Thiéry (2004b) who demonstrated a preference of *L. botrana* females to lay eggs on artificial surfaces, which were smooth. This preference was considered in our design of the oviposition arena. We observed during insect rearing that under no-choice conditions, when the inner wall of the arena was smooth (polyethylene film), a huge number of eggs (136 ± 27 eggs, n = 10) were laid on the arena. If the arena was coated with felt, oviposition was extremely low (18 ± 6 eggs, n = 10). A similar phenomenon could also be observed in vineyards. Geisler (1959) indicated that inflorescence hairiness is supposed to be unfavourable for the oviposition of *L. botrana* females. In this context, most of the eggs are placed on the peduncle and not on the petal directly (Geisler, 1959). For this reason, it could be assumed that physical factors influence the localization of a suitable oviposition site of *L. botrana* as well as *E. ambiguella*. Marchal (1912) already demonstrated that a treatment of the berry surface with chalk powder or molasse inhibited the oviposition of *L. botrana* and supposed that primarily tactile cues must be responsible for this effect because the volatiles emitted by the berries could still be recognized by the females. But he was not aware of possible gustatory side effects of this treatment. Later on, Maher & Thiéry

(2004a) demonstrated that chemosensory perception of such gustatory substances could influence the oviposition behaviour. To avoid such a discrepancy between chemosensory and tactile cues, we used an artificial oviposition surface. We found synthetic materials on which the deposition of the eggs was quantifiable without the effect of VOCs emitted by the host plant. We used these materials to check whether the colour, the shape or the texture of the surface layer has an influence on oviposition acceptance.

A particular colour of a plant could be a visual signal to trigger certain activities, which will increase the fitness and survival rate of the insect itself and its offspring (Yurtsever, Okyar, & Guler, 2010). Oviposition is carried out at the afternoon (Götz, 1941) where the perception of visual cues in insects is still possible. In previous studies, it has been demonstrated that some nocturnal hawkmoths have colour sensitive eyes. They use three spectral types of cone photoreceptors to detect flowers even at dim starlight intensity (0.0001 cd/m^2) when bees and humans are totally colour blind (Kelber, Balkenius, & Warrant, 2002). In our experiments, *L. botrana* preferred the green compared to brown or blue colours for oviposition. The fact that both moth species did not discriminate light grey from dark grey, but different colours, implies that they probably do not use achromatic cues for oviposition. A previous experiment of Kelber (1999) indicated that the colour green is not attractive for feeding, but it is also a favourite oviposition colour for females of some butterflies, such as the Orchard Swallowtail (*Papilio aegeus*). In this case, the newly hatched larvae need food and brightly green leaves give them a sign of fresh and rich nutrition. Blue and green colours are stimuli for feeding and egg laying in the cabbage butterfly (*Pieris brassicae*) (Weiss & Papa, 2003). In particular, in spring, it could be observed that *L. botrana* females use the leaves, the peduncles and the leaf tendrils moreover for oviposition (Stellwaag, 1928). Marshall (1912) assumed leaves were preferred oviposition sites because they are available earlier in the season, more numerous, and represent a larger surface area. But as soon as fructiferous organs are present, *L. botrana* preferred buds and berries moreover (Maher, Thiéry, & Städler, 2006). Our results indicate that the green colour of this plant parts could be a basic signal for any oviposition site selection of the two moths. Tasin et al. (2011) indicated that vision had a positive synergistic effect in host–plant recognition in association with olfactory cues emitted by the plant for *L. botrana*. The strategy behind this preference might be a higher survival rate on unripe than on ripe (juicy) grapes, where the risk to drown is high for freshly emerged larvae. The grape berry moth *E. ambiguella* did not distinguish between green and blue coloured oviposition sites, but this species seems to prefer green surfaces compared to yellow ones, which could also reflect a favouritism of younger/unripe berries for oviposition.

In addition to the foliage colour, the leaf shape might be an important cue used to select sites for oviposition. Maher and Thiéry (2004b) documented that females preferentially lay eggs on spherical glass surfaces rather than on plane ones, in assumption that the convexity of the berry surface might play a role in the acceptance of oviposition sites. We could only observe a preference of spherical shapes in the dual-choice test when females lay eggs on the top of a surface. When the females were forced to oviposit overhead, most of the eggs were laid on plane surfaces. The effect could be observed in both insect species. During the experimental set-up, the influence of modified light exposure, triggered by the oviposition shapes fixed at the lid, could not be excluded. It could be assumed that plane shapes were more shaded by themselves than convex shapes. An interaction between exposure to light and oviposition was already discussed by Vogel (1907) who observed higher egg numbers on grape clusters shaded with paper rolls. This phenomenon was also noticed by Zahavi, Harpaz, Ben Meir, and Ben David (2017) who showed that oviposition on grapevine clusters in the field was triggered by light exposure. However, an interaction with the wind shielded situation and the temperature in these experimental set-ups could not be excluded. Our results indicate that *L. botrana* females obviously prefer to lay their eggs overhead and if this is not possible, they prefer to lay eggs with as much verticality position as possible. *E. ambiguella* on the contrary seems to prefer convex shapes in any kind of placing position, whereas this preference is triggered by light conditions.

Little is known about the physical surface texture of the berries or leaves promoting the oviposition of the moths. Maher & Thiéry (2004b) tested the oviposition behaviour of *L. botrana* on sandpaper and filter paper compared to glass plates and concluded that smooth surfaces were favoured against rough textures. Although cardboard might have interfered oviposition in past studies (Gabel & Thiéry, 1996), we used crepe paper in our oviposition experiments to check if chemical additives of the chosen oviposition materials may also have a negative effect on the oviposition of *E. ambiguella*. For this purpose, we used smooth and alternative textured LDPE-coated crepe papers to check whether the material or the texture induced a deterrent effect. As well as Maher & Thiéry (2004b), we consider crepe paper as an inappropriate oviposition material for *L. botrana*. Interestingly, *E. ambiguella* responded differently and preferred crepe paper (both smooth and textured) for oviposition compared to smooth PP films. That phenomenon suggests that *E. ambiguella* is less averse from slightly fibrous surfaces or chemical additives in the crepe paper. Because females distinguished between the materials (smooth PP, CP, LDPE and PET) as oviposition site, it could be assumed that they noticed chemical additives in the oviposition material. Moreover, as our aim was to

mimic the structure of oviposition site, we chose different textured PP films for our assay, which were as far as possible similar to the natural oviposition surfaces (berry or leaf). Foster et al. (1997) mimicked the structure of a leaf by coating glass plates with a wax layer and showed for the light brown apple moth (*Epiphyas postvittana*) that model plant regions with ridges had greater oviposition rates than regions without ridges. The use of technical embossing of the PP films in our experiments should ensure a uniform and imitable surface condition. Already our first experiments with different materials showed that structured PP films were preferred surface layers compared to non-structured ones. Maher & Thiéry (2004a) assumed that among others the sensilla on the ventral surface of the ovipositor could be responsible for a tactile evaluation of the oviposition site. Such behaviour could also be observed during our choice experiments seen as a point-by-point exploration of the egg-laying site with the ovipositor. Both moth species show a similar behaviour in regard to their preference for a particular texture.

During our studies, we could not exclude that moth polymorphism caused a change in the host acceptance for oviposition. It could be possible that the preferences of the females originating from the laboratory rearing differed from those of the wild population. That is why the laboratory rearing was refreshed with wild population from the field. A combination of the texture B, green colour and a spherical surface (plane on the bottom side and convex on the top side) provides the basic concept for the creation of an artificial egg-laying site.

In order to enable a competitiveness of the artificial surface to grapes, it is also important to analyse the impact of volatiles emitted by the host plant, which attract females in long and short range (Anfora et al., 2009; Schmitt-Büsser, von Arx, Connetable, & Guerin, 2011; Tasin et al., 2005, 2011), and the role of wax layers of the grape surface, which may also impact the oviposition decision of female berry moths (Maher & Thiéry, 2004b; Maher et al., 2006). These results should be also considered in the development of an artificial oviposition monitoring tool, which actually reflects the pest infestation. Thus, further research is necessary to reveal and mimic the entire process of oviposition, which is essential for the development of a more sophisticated egg-monitoring tool, which can actually compete against grapes.

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Supplementary Material

Figure S1: Schematic life cyle of grapevine moth *Lobesia botrana* and grape berry moth *Eupoecilia ambiguella* in the insect culture (**I**. Pupae placed in paper bag for hatch, while hatching moths copulate and lay eggs; **II**: Egg film placed in a cup with artificial diet; **III**. Larvae feeding from the diet; **IV**. Larvae enter corrugated paper to pupate; **V**. Pupae removed from the corrugated paper).
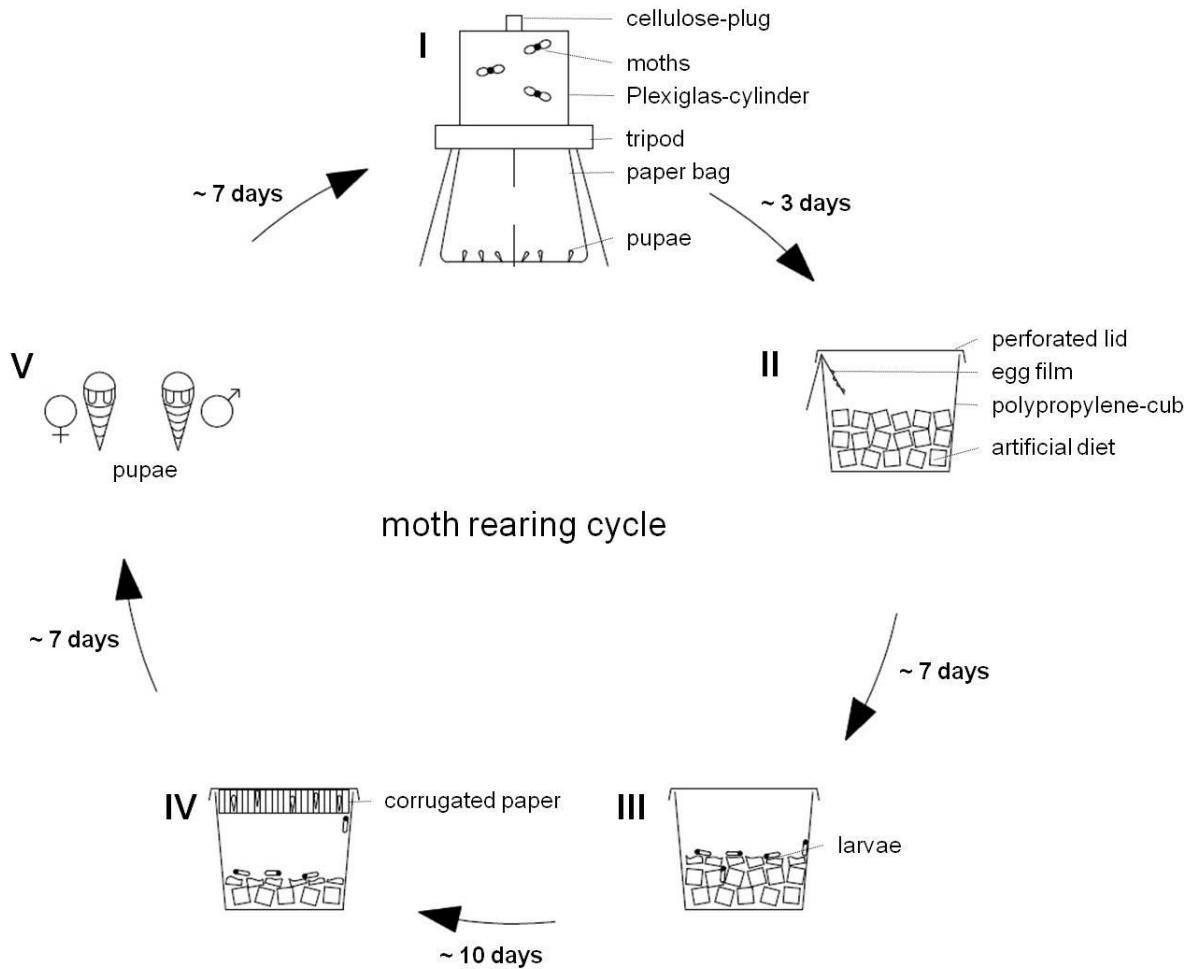
Table S1: Detailed description of the synthetic oviposition materials investigated in the studies.

physical cue	internal surface code	color	specifications of the producer			
			labelling	thickness [µm]	grain-code	color-code
material	PETs	transparent	PET-GAG film	250	smooth	-
	PETt	milky-white	PET-G film	200	rough	-
	CP-LDPEs	beige	crepe + LDPE	-	smooth	-
	CP-LDPEt	blue	crepe + LDPE	-	texture	-
color	lg	light grey	Stationery POKF	100	01679	70104
	t	turquoise	Stationery POKF	100	01679	50635
	y	yellow	Stationery POKF	100	01679	24117
	br	brown	Stationery POKF	100	01679	83409
	bl	blue	Stationery POKF	100	01679	53022
	dg	dark grey	Stationery POKF	100	01679	78100
	v	violet	Stationery POKF	100	01679	51489
	gr	green	Stationery POKF	100	01679	63274
texture	A	beige	Ecofilm PP	120	00141	90500
	B	green	Stationery POKF	100	01233	66563
	C	green	Stationery POKF	100	01055	63125
	D	brown	Stationery POKF	100	016662	84385
	E	green	Stationery POKF	100	01067	63219
	F	green	Stationery POKF	100	01827	63265
	G	brown	Stationery POKF	100	01660	87705
	H /PPt	green	Stationery POKF	100	01679	64044

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Waxy bloom on grape berry surface is one important factor for oviposition of European grapevine moths

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Abstract

Grapevine moths are severe pest insects in European viticulture. Oviposition by grapevine moths is largely influenced by several physical and chemical cues located on the surface of their host plant's fruits. The contribution of waxy bloom layer on the berry surface for oviposition decision of two European grapevine moth species, *Eupoecilia ambiguella* and *Lobesia botrana*, was investigated. An experimental setup was developed to prove oviposition preferences of both species for certain grape varieties and developmental stages based on epicuticular wax extracts. Chemical analysis of epicuticular wax patterns of four different *Vitis vinifera* varieties revealed differences. However, oleanolic acid was the main component on berry surface waxes and its relative amount decreased between early and late phenological stages. Furthermore, oleanolic acid was responsible for the preference of earlier phenological stages for *E. ambiguella* oviposition. However, ovipositional variety preferences were triggered by minor components on the wax berry layer. While the oviposition decision of *L. botrana* was mainly triggered by oleanolic acid, additional cues like olfactory and haptic ones were also important. The ovipositional preferences were discussed in accordance with the results of the chemical analysis in order to elucidate the role of wax compounds for oviposition stimulation.

1.   Introduction

The European grapevine moth, *Lobesia botrana* (Denis & Schiffermüller), and the European grape berry moth, *Eupoecilia ambiguella* (Hübner) (Lepidoptera: Tortricidae), are polyphagous insects (Bovey 1966) distributed across the Palaearctic and severe insect pests of commercial viticulture. Depending on climatic conditions, gravid females typically lay their eggs on flowers (BBCH 53–57), green berries (BBCH 73–79) and mature grapes (BBCH 81–89) (Ioriatti et al. 2011; Marchal 1912) during the first, second and third generations of the year, respectively (Lorenz et al. 1994). The main damage on wine grapes results from the colonization of damaged berries by gray mould (*Botrytis cinerea*), causing the entire bunch to rot (Fermaud and Giboulot 1992). Thus, a control of both European grapevine moth species must be continually conducted to keep damage at an acceptable level.

In Europe, the mating disruption technique of grapevine moths is a successful model for integrated pest management (IPM), an ecosystem approach to crop production and protection that combines different management strategies and practices to grow healthy crops and minimize the use of pesticides (Gross and Gündermann 2016). This technique is applied, e.g., in Germany on more of 60% area of all vineyards. However, this technique has its limitations at high pest insect population densities, in small vineyards, or in vineyards with steep slopes. Under these circumstances, insecticide treatments, preferentially at early life stages of the moths, have to be conducted (Ioriatti et al. 2011). For the development of innovative strategies for pest control, detailed knowledge on pest–host plant interaction is of primary importance. An easy and cheap monitoring of the males' fight by traps baited with pheromones and predictive models are available, but the correlation with female oviposition is poor.

Thus, to prevent immoderate insecticide application, a better decision support system for growers is needed, tracking the eggs rather than male's fight, which enables better timing and necessity of pest control. Such a tool, called "moth oviposition card" (M-OVICARD), an artificial oviposition substrate consisting of volatile/non-volatile compounds supported by visual and tactile cues, could contribute to the monitoring and is currently under development (Markheiser et al. 2018). Based on a quantifiable egg deposition (e.g. by a smartphone app, Sprute et al. 2016) on such a card, the winegrower is able to draw conclusions for the infestation of grapes by moth eggs. Insecticide applications, which are often only efficient during the egg stage, could be carried out more precisely, resulting in lower amounts of applied plant protection products (Markheiser et al. 2018). The results of the presented study will contribute to the development of the tool in means of coating the card with wax extracts in order to improve

its attractiveness for moth oviposition. Epicuticular waxes are deposited on the outer surface of the plant cuticle and are the last barrier of aerial organs of plants. The epicuticular wax layer is typically constituted of a mixture of hydrocarbons (alkanes, aldehydes, wax esters, free fatty acids, primary and secondary alcohols) and secondary metabolites, such as triterpenoids and phenylpropanoids (Jetter et al. 2006). The waxy bloom on grape berries' surface controls cuticular transpiration (Schönherr 1982), accounts for resistance against fungi (Comménil et al. 1997) and serves as physical and chemical cues used for host recognition by insects (Müller and Riederer 2005). Epicuticular wax composition changes along maturation and among grape varieties (Casado and Heredia 2001; Comménil et al. 1996; Pensec et al. 2014). After veraison, the total amount of epicuticular waxes increased strongly (Palliotti and Cartechini 2001). For all of the previous reasons, it is obvious that developmental stages of grapevine fructiferous organs do not provide the same chemical and physical information to grapevine moth females throughout the season.

After alighting on a plant, a gravid insect uses a combination of physical and chemical stimuli at the surface to assess the plant's acceptability for oviposition (Schoonhoven et al. 2005). During plant surface examination, grapevine moth females exhibit a sweeping behavior of the ovipositor over the surface, which is the ultimate step before oviposition. In this context, *L. botrana* utilizes the ventral surface of the ovipositor, which is covered with mechanoreceptors and contact-chemoreceptors (Maher and Thiéry 2004b). This confirms the ability of the moths for sensing surface structures and chemicals on the oviposition substrate. This is probably the case for most moths (Ramaswamy 1988; Städler 2002). It is already proven that sensilla on the ovipositor of *L. botrana* are sensitive to polar compounds such as fructose and glucose (Maher and Thiéry 2004b; Maher et al. 2006). The perception ability of these sensilla for apolar compounds is still uncertain. However, behavior data show that tortricids are able to perceive waxy compounds, especially those present on eggs (C16-C18 fatty acids and esters) (Gabel and Thiéry 1996). It is possible that the waxes convey information on grape quality to grapevine moth females as shown for other Lepidoptera (Brooks et al. 1996; Juma et al. 2016; Li and Ishikawa 2006; Udayagiri and Mason 1997). Moreover, *L. botrana* show oviposition preferences for certain varieties (Sharon et al. 2009; Thiéry et al. 2014). In this context, it may be possible for certain varieties or developmental stages to be considered as particularly susceptible, since their wax layer is attractive for oviposition.

To determine the impact of the waxy bloom of grape berries on the oviposition of grapevine moths, chemical analysis of the waxes and behavioral oviposition experiments were conducted with both, *L. botrana* and *E. ambiguella* females. Patterns of epicuticular waxes of distinct

maturation stages of grape berries belonging to several varieties (*Vitis vinifera*; 'Regent,' 'Pinot Noir,' 'Müller-Thurgau' and 'Riesling') were analyzed by gas chromatography and compared. Furthermore, the key compounds that are responsible for the separation of the groups were defined. In order to elucidate the impact of phenological stages and varieties of grapevine, oviposition preference experiments with both grapevine moth species were conducted. The contribution of contact signals of several varieties for oviposition preferences was assessed by dual-choice bioassays. Furthermore, contact cues were evaluated firstly by oviposition preference bioassays after selective wax removal from grape berries, and secondly by studying the influence of a certain wax extract to the number of eggs laid. Additionally, crosswise dual-choice experiments were performed to elucidate ovipositional preferences for varieties or developmental stages within the varieties based on chemical contact cues. Finally, synthetic candidate substances revealed by the chemical analysis were tested for oviposition stimulation.

2. Materials and methods

2.1. Insects

Lobesia botrana (Denis & Schiffermüller, 1775) and *Eupoecilia ambiguella* (Hübner, 1796) moths used in behavioral experiments were reared in stock cultures originating from JKI, Siebeldingen, Germany. The culture is each year genetically refreshed with moths captured in the surroundings of JKI as described in Markheiser et al. (2018). Pupae were sent by mail to Dossenheim and kept under controlled conditions [L16:D8 photoperiod (2000 lux) with each 1 h of dusk and dawn (20% change in light intensity every 12 min), 23:19±0.5 °C and 60±10% relative humidity (r. h.)] in a climate cabinet (RUMED, Modell 3501, Rubarth Apparate GmbH, Laatzen, Germany). The moths were kept in cages (bug dorms, 30×30×30 cm) provided with a water source until further treatment. The oviposition assays were conducted with 3–4-day-old gravid females. Mated moths were obtained by pairing virgin males and females in a cage. Insects were observed, and copulating pairs were transferred to experimental cages.

2.2. Wax extract of Vitis vinifera berries

Vitis vinifera berries of 'Regent,' 'Pinot Noir' and 'Müller-Thurgau' were collected in vineyards belonging to S. & W. Stein, Großsachsen, Germany (49°30′44.5″N 8°40′19.6″E), in the year 2015, and of 'Riesling' from potted grapevine plants provided by JKI Siebeldingen. They were cultured in the greenhouse (1 L substrate pots, Fruhstorfer Erde Typ Poinsettien, Hawita Gruppe GmbH, Vechta, Germany) under 23±5 °C and 30±10% r. h. at the respective

BBCH-stages (BBCH 73=Berries groat-sized, BBCH 77=Berries beginning to touch, BBCH 85=Softening of berries). The classification of the BBCH-stages is defined according to Lorenz et al. (1994). The abbreviation BBCH derives from Biologische Bundesanstalt, Bundessortenamt and CHemical industry (Meier et al. 2009). The petiole was abscised from the berries, and the berries of 2–3 bunches were submerged in chloroform depending on berry amount (50–100 mL, Sigma-Aldrich) at room temperature for exactly 30 s to get only epicuticular waxes (adapted from Shin et al. 2010). The extract was filtered (Type 604, Schleicher & Schuell, Whatman International Ltd., GE Healthcare, UK), and the solvent evaporated on a rotary evaporator (Rotavapor RE 120, BÜCHI Labortechnik GmbH, Essen, Germany) under reduced pressure to yield dry wax samples (600 mbar, 50°C). The dry wax was weighted and dissolved in chloroform to reach a concentration of 300 mg/mL.

2.3. GC-FID analysis

The composition of the mixtures was studied by capillary gas chromatography with fame ionization detector (GC-FID) (Clarus 580, PerkinElmer, Rodgau, Germany). n-Tetracosane (Aldrich, Sigma-Aldrich, Schnelldorf, Germany; C24 alkane; 625 µg) was added to the sample as internal standard. The samples were completely evaporated under a gentle stream of nitrogen (using Reacti-Vap™, Thermo Scientific, Waltham, MA, USA) at 50 °C, dissolved in 300 µL pyridine (Sigma-Aldrich, USA) and derivatized with 300 µL bis-N,O-(trimethyl silyl) trifuoroacetamide (BSTFA; Fluka, Sigma-Aldrich) to transform all hydroxyl- and carboxyl-containing compounds into the corresponding trimethyl silyl derivates. Derivatization conditions were 2 h at 70°C and were maintained with Reacti-Therm™ (Thermo Scientifc). A total of 60 mg were used of each wax extract sample. Derivatized samples were injected onto a capillary GC-column (Rxi-5 ms, 30 m×0.25 mm ID×0.25 µm, Restek, Bad Homburg, Germany) with helium as carrier gas at 100 kPa.

The injector temperature was 320°C, and the oven temperature was set at 50°C for 2 min, raised at 40 K/ min to 200°C, held for 2 min at 200°C, raised 3 K/min to 300°C, held for 2 min, raised 3 K/min to 320°C and held for 20 min at 320°C. Authentic standard substances were introduced for the identification of some components [oleanolic acid (OA): Roth, Karlsruhe, Germany; ursolic acid (UA): Sigma-Aldrich, Schnelldorf, Germany; montanic acid: Fluka, Sigma-Aldrich]. All other substances were set as "known unknowns" defined by their retention time. Peak areas were integrated, and a compositional dataset was generated (N=40; BBCH 75–79: nRE=10, nPN =3, nMT=5, nRI=3; BBCH 85–89: nRE=10, nPN =3, nMT=5, nRI=3).

2.4.Contact/non-contact oviposition bioassay

The attractiveness of grape varieties for oviposition was quantified by means of a dual-choice experiment. A preference for 'Regent', 'Pinot Noir', 'Müller-Thurgau' and 'Riesling' at the phenological stage BBCH 83–85 was checked via a cross-comparison of the varieties. In each comparison, two varieties were tested against each other in six reciprocal crosses. The comparability of the BBCH-stage was ensured due to shifted growth of the potted grapevines. Every variety was offered for oviposition in clusters of 4 berries, which were incorporated in the lid of a 10-mL polypropylene cup (Kastelplast GmbH, Mainz-Mombach, Germany) providing water. Berries were removed from potted grapevine plants, which have been cultivated at the Julius Kühn-Institut, Siebeldingen, Germany.

During a first experimental setup (following Tasin et al. (2009)), females of *L. botrana* and *E. ambiguella* were exposed to only olfactory and visual stimuli (OV), whereas in a second setup females were exposed to olfactory, visual and additional contact stimuli (OVC). Transparent polypropylene cups (100 mL, Kastelplast GmbH) were used for an exclusion of contact stimuli in the OV-experiment. The cups were perforated using a needle (60 holes per cup, Ø=1.1 mm) allowing volatiles emitted by the berries to evaporate. The berries (with or without cups) were placed in the middle of a gauze cage (60×40×40 cm, The Caterpillar Castle, Live Monarch Foundation, Boca Raton, USA), 20 cm apart from each other. Eight couples of moths of the same species, sexed 48 h prior the beginning of the experiment, were introduced into the cage for oviposition to enable adequate egg quantities. For controlled climatic conditions, cages were kept in condition chambers 'Fitotron type SGR233′ (Weiss Technik UK Ltd, Loughborough, UK) at 23:19 °C±2 °C, a L14/D8 photoperiod with 1 h each of dusk and dawn and 70±10% r. h. The experiment was stopped after 72 h by removing the moths from the cage and counting eggs deposited on the berries or cups, respectively. Each comparison was repeated 10 times.

2.5.Wax/non-wax oviposition bioassay

For studying the importance of the epicuticular wax for oviposition decision, an oviposition bioassay with *L. botrana* and *E. ambiguella* females was conducted. Five couples of each species were introduced during copulation into small cages (Ø=14 cm, h=25 cm; Port-A-Bug, Insect Lore, USA) and kept therein for 72 h. The couples separated themselves and females started to oviposit. The moths had to decide between two small berries bunches (4 berries/bunch), placed within the cage. One berry bunch was offered untreated (with intact wax layer), while the other was treated with cellulose acetate (without wax layer, see below). The

berries were supplied with a water source. The number of eggs on the berries was counted and compared statistically.

The cuticular wax layer of berries (*V. vinifera* 'Regent' at BBCH 77 or 85) was removed according to the cellulose acetate stripping method described in (Baker et al. 1983; Müller and Hilker 2001). Cellulose acetate (Sigma-Aldrich, USA) was dissolved in acetone (7% w/v) by ultrasonic treatment for 2 h. This methodology ensures the removal of surface waxes without further damage of the plant tissue. Berries were brushed with the solution. After evaporation of the acetone, a white film appeared on the surface of the berries and was carefully removed with fine forceps. Accidently injured berries were not used for experiments. The surface areas of berries with and without wax layers were visualized by a digital microscope (VHX 5000 with VH-Z500R/Z500T, Keyence, Neu-Isenburg, Germany, Fig. 4a).

2.6. Variety/BBCH oviposition bioassay of berry epicuticular wax extracts

A small glass arena, composed of a concave glass block (4×4×1.4 cm; Bioform, Nürnberg, Germany) and its cover glass (4×4 cm, Bioform), was used for testing the influence of different wax extracts on oviposition behavior of *L. botrana* and *E. ambiguella* (Fig. 1). As the experimental arena a circle (Ø=3 cm) was drawn on the cover glass and separated into two semicircles, each for one of the two treatments. The females could oviposit upside down on the plane glass plate. 20 µL (of 300 mg/mL=6 mg) of chloroform-wax extract was pipetted on the cover glass and distributed within the semicircle. The chloroform evaporated completely resulting in a homogenous and visible wax layer on the glass plate.

Fig. 1 Small glass arena composed of a concave glass block and its cover glass for testing the influence of different wax extracts on oviposition behavior of *L. botrana* and *E. ambiguella*. The insects were set within the block and could oviposit upside down. The semicircles were covered with different wax extracts or synthetic compounds. Ovipositing *L. botrana* is shown

We introduced single couples of grapevine moths during copulation into each small glass arena. After the couples had separated (<8 h), the males were carefully removed to prevent disturbing

the females during oviposition. The experiment was stopped after 72 h and the eggs laid on each semicircle as well as on the non-treated base area (concave indentation from the glass block) were counted. The following comparisons were performed with both moth species: all extracts (BBCH-stages and varieties) against a solvent control (chloroform); all BBCH-stages extracts against each other within one variety; all variety extracts against each other within the same BBCH-stage, and two standard substances (oleanolic acid and ursolic acid) at a concentration of 10 mg/mL. The number of replications was at least 12.

2.7. Statistics

For investigation of the wax pattern of berries, a compositional dataset out of 59 peaks was calculated and used for statistical analysis. Permutational multivariate analysis of variance (PERMANOVA, Anderson (2001)) was used as multivariate test for discrimination of groups. Furthermore, to elucidate the dispersion of the wax patterns and to assign that the significant PERMANOVA result is based on location and not on dispersion effects, a permutational analysis of multivariate dispersions (PERMDISP, Anderson (2006)) test for homogeneity of multivariate dispersion was conducted. PERMANOVA and PERMDISP were calculated with Bray–Curtis dissimilarities (Brückner and Heethof 2017).

Random Forests (RF) are used in chemical ecology as a universal approach to assign samples to a priori defined groups and return the variable importance ('E,' mean decrease accuracy) (Breiman 2001; Brückner and Heethof 2017). The inequation $E > 0$ defines compounds that contributed to the classification, whereby an increasing value of 'E' reflects the importance of the single substance. RF was used for untransformed compositional data. RF generates confusion matrices along with an out-of-bag (OOB) estimated error rate and multi-dimensional scaling (MDS) plots based on proximity matrix obtained from RF. Kruskal–Wallis tests followed by Wilcoxon rank-sum tests were performed for the comparison of the relative amounts of oleanolic acid in dependency of BBCH-stage as well as variety. Significance level was set at $p < 0.05$. All analyses were performed using R version 3.3.1 (2016-06-21) "Bug in Your Hair" (R Core Team 2016) with packages 'lsmeans' (Lenth 2016), 'vegan' (Oksanen et al. 2017), 'randomForest' (Liaw and Wiener 2002) and 'ggplot2' (Wickham 2009).

Preferences for berries or wax extracts in two-choice experiments were assessed by Wilcoxon signed-rank test (*$p < 0.05$, **$p < 0.01$) on the proportions of eggs laid on the particular treatments due to nonparametric dataset. Graphs were visualized by an oviposition discrimination index (ODI). It was calculated from the number of eggs laid on treatment 'A'

minus the egg number on treatment 'B,' divided by the overall amount of eggs laid, according to the formula ODI (%) =[(A−B)/(A+B)] ×100.

Generalized linear models (GLMs) were used to analyse the effect of variety (levels: 'Regent', 'Pinot Noir', 'Müller-Thurgau' and 'Riesling') and BBCH-stage (levels: BBCH 73, BBCH 77 and BBCH 85) and their interaction on the proportion of eggs laid on extracts relative to the chloroform control. Quasi-binominal family was chosen in GLMs for proportions due to the observed overdispersion. The total amount of laid eggs was considered by using "cbind"-function for original data on success (number of eggs laid on extract) and failure (number of eggs laid on control). Because the interaction between 'variety' and 'BBCH-stage' and the factor 'variety' had no influence on the proportion of laid eggs for both moth species (Table S1), quasi-binominal GLMs were calculated with two more levels on 'variety': oleanolic acid and ursolic acid. Significance of terms within the models was tested using F-test and the function drop1. The models were simplified by removing non-significant interactions (p>0.05) and then non-significant factors.

Post hoc comparisons between BBCH-stages were obtained from least-square means and confidence intervals from statistical models using the function 'lsmeans'. P values were adjusted using the method of Tukey (p<0.01).

3. Results

3.1. Pattern of berry epicuticular wax

A total of 59 peaks could be detected in chromatograms of derivatized wax samples (Fig. S1). Oleanolic acid was the most abundant substance in all samples and contributed from 63% up to 83% to the overall wax amount (Fig. 2d).

Based on PERMANOVA, the interaction of BBCH-stage and variety had no influence on the results. The BBCH-groups differed statistical significantly (Table 1, Fig. 2a) based on the whole surface wax pattern of berries and this difference explained 19.77% of the overall variance (Table 1; PERMANOVA). Additionally, 10.35% of variance was explained by the varieties (Table 1, Fig. 2b, c). The variability between the patterns did not differ either between BBCH-stage or between varieties (Table 1; PERMDISP). For determining the group belonging, a Random Forest (RF) algorithm for classification was conducted and visualized by a multi-dimensional scaling (MDS) plot of the proximity matrix (Fig. 2a–c). The wax pattern differed between the BBCH-stages (Fig. 2a; N-trees: 10 000, mty=8, OOB=5%). Because of the strong influence of BBCH-stages, a RF calculation for each BBCH-group was performed. The wax

patterns within the BBCH-stage 75–79 differed between the four varieties, but a good grouping was not observable (Fig. 2b; N-trees: 10,000, mty=8, OOB=47.62%).
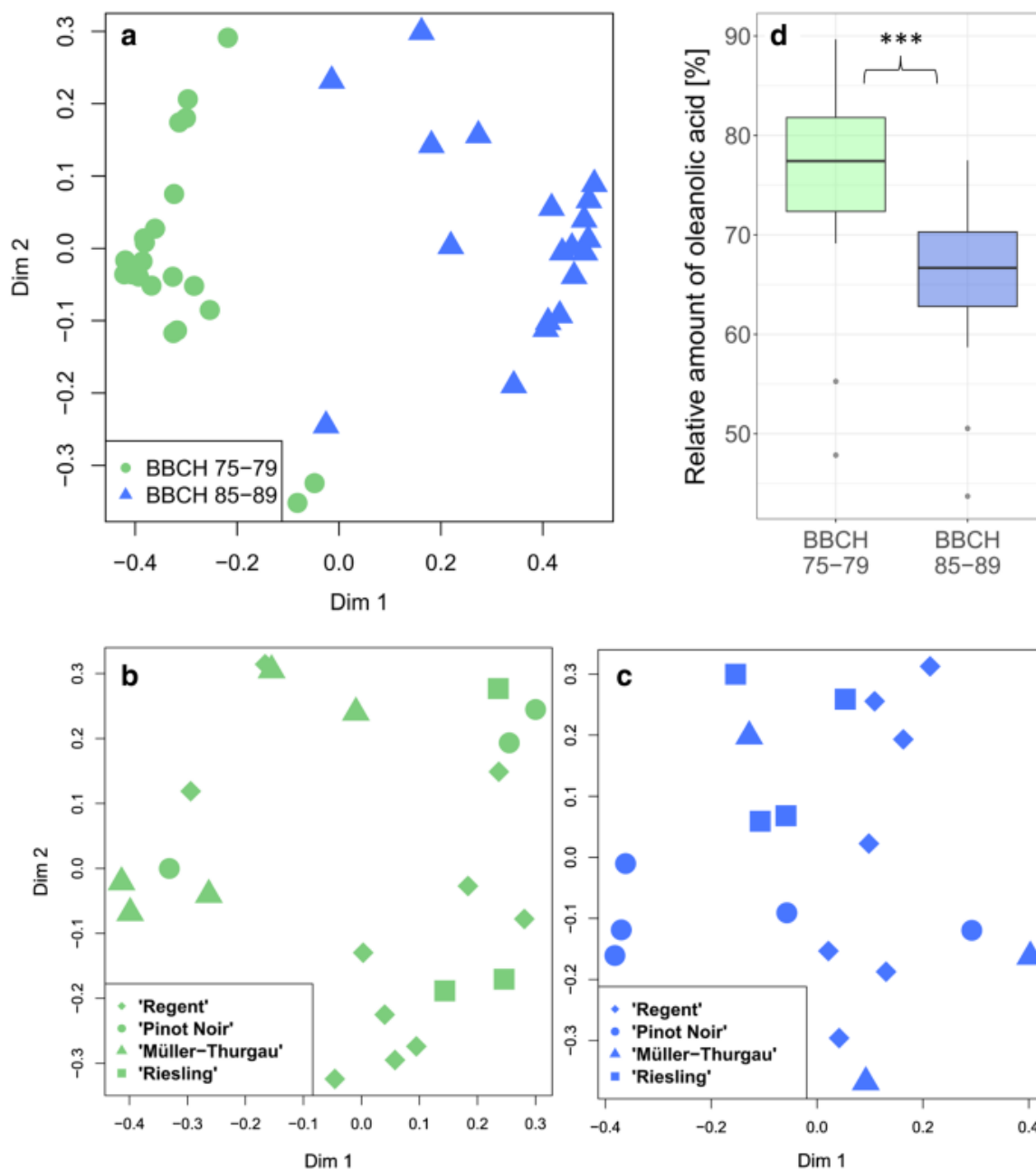
Fig. 2 Multi-dimensional scaling (MDS) plots of the proximity matrix obtained from RandomForest (RF) (N-trees=10,000, mtry=8) classifcation for whole wax pattern obtained by 59 compounds. a Grouping by BBCH-stage (OOB=5%); grouping by variety for each BBCH-stage: b BBCH 75–79 (OOB=47.62%) and c BBCH 85–89 (OOB=47.37%). d Relative amount of oleanolic acid in wax extract samples from BBCH-stage 75–7

At BBCH-stage 85–89 a better grouping behavior of the varieties could be observed, especially for the pattern of 'Regent' (Fig. 2c; N-trees: 10,000, mty=8, OOB=47.37%, class error from 'Regent': 0%). Oleanolic acid (E=70) and montanic acid (E=186) along with three unidentified

compounds contributed most to the classification into two BBCH-groups (highest value for 'E') obtained from RF. For the classifications of the varieties within each BBCH-stage were other minor unidentified substances responsible, but not oleanolic acid (BBCH 75–79: E=12.24; BBCH 85–89: E=−15).

Table 1 Results of multivariate statistical tests PERMANOVA/PERMDISP based on Bray–Curtis dissimilarities for the berry wax pattern of different BBCH-stages and varieties

PERMANOVA	Df	Pseudo-F	R^2 (%)	p value
BBCH-stage	1	9.90	19.77	9.999e−05
Variety	3	1.73	10.35	0.0481
Residuals	35		69.88	
PERMDISP	Df	F		p value
BBCH-stage	1	0.0765		0.7878
Variety	3	0.5574		0.6695

N (permutations) = 10,000

The relative amount of oleanolic acid did not vary between the varieties (Kruskal–Wallis, df = 3, $\chi2$ = 6.3, p>0.05), but the phenological stage had an influence on the amount of this triterpenoid (Kruskal–Wallis, df = 1, $\chi2$ = 12.9, p < 0.01). Oleanolic acid was higher in wax extracts obtained from green berries (BBCH 75–79) than from mature berries (BBCH 85–89) (Fig. 2d; Wilcoxon rank-sum test, p<0.01), regardless of the variety.

3.2. Contact/non-contact oviposition bioassay

Dual-choice oviposition bioassays were conducted with *E. ambiguella* and *L. botrana* in crosswise tests with berries at the BBCH-stage 83–85 of four *V. vinifera* varieties. In the first trial, the moths were allowed to choose their oviposition substrate based on olfactory and visual cues only. *Eupoecilia ambiguella* showed no preference in oviposition for one of the two offered bunches of different varieties (Fig. 3a, OV). In the second trial, where additionally a direct contact to the berries was enabled and the test insects were allowed to oviposit directly on the berries, females of *E. ambiguella* preferred 'Regent' over 'Riesling' and 'Pinot Noir' over 'Riesling' (Fig. 3a: OVC, Wilcoxon signed-rank test, p<0.05). In contrast, *L. botrana* females, guided through olfactory and visual cues but without direct contact to the berries, preferred to lay more eggs on the cup surrounding 'Müller-Thurgau' berries than on 'Regent' (Fig. 3b: OV, Wilcoxon signed-rank test, p<0.05).
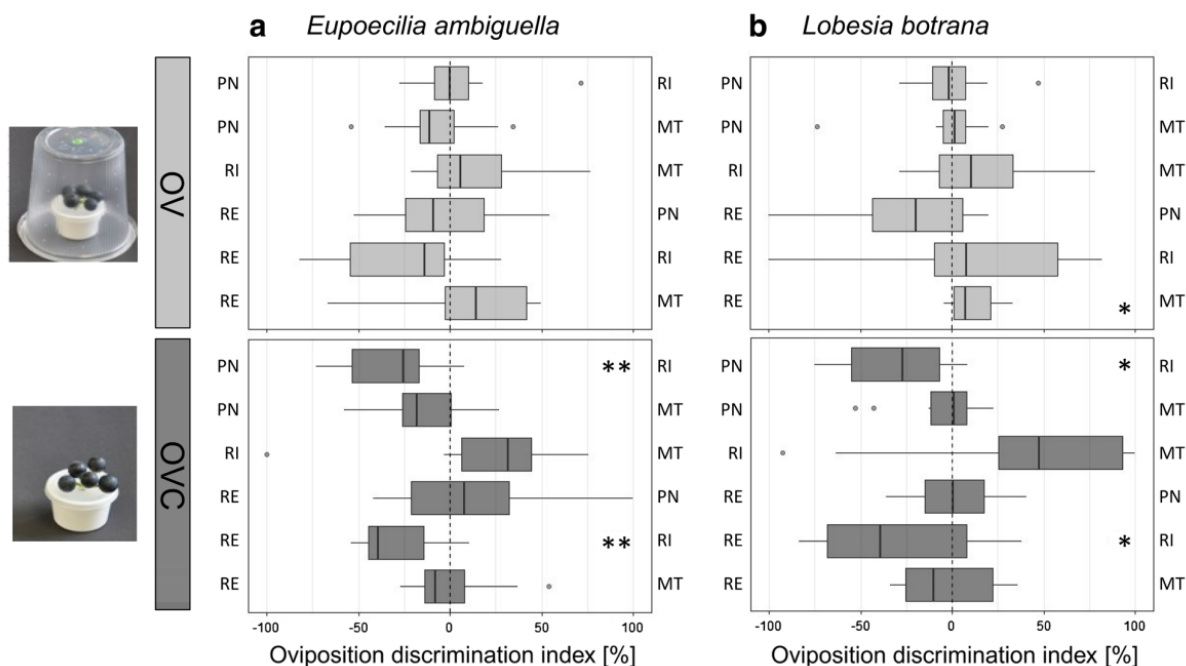
Fig. 3 Oviposition discrimination of berries of several *V. vinifera* cultivars in dual-choice contact/non-contact oviposition bioassays with moth species **a** *E. ambiguella* and **b** *L. botrana* at BBCH-stage 83–85. OV (above): moths could use visual and olfactory signals; OVC (bottom): moths could use visual and olfactory signals with additional contact to the berries. RE='Regent', PN='Pinot Noir', MT='Müller-Thurgau', RI='Riesling'. Box–whisker plots of the ODI were shown, whereby lines represent median, boxes the interquartile range (IQR), whiskers 1.5 * IQR and closed circles outliers. Statistically different results are indicated by asterisks (Wilcoxon signed-rank test; *pp<0.05, **p<0.01; n=10)

By getting direct contact to the berries, 'Müller-Thurgau' berries were not preferred for oviposition anymore. More eggs were deposited on 'Regent' than on 'Riesling' and on 'Pinot Noir' than on 'Riesling' (Fig. 3b: OVC, Wilcoxon signed-rank test, p<0.05) directly. The mean and SD of eggs laid in each experimental setup was calculated for *E. ambiguella* (OV: 50±35 (SD); OVC: 90±61) and *L. botrana* (OV: 67±50; OVC: 63±46).

3.3. Wax/non-wax oviposition bioassay

For assessing the importance of the epicuticular wax on oviposition decision of both moth species, dual-choice tests between a treated (wax layer removed=dewaxed) versus an untreated berry cluster were conducted (Fig. 4). *Eupoecilia ambiguella* females preferred the berries with wax layer over the dewaxed berries for oviposition at both tested BBCH-stages 77 and 85, green and mature berries, respectively, of 'Regent' (Fig. 4b; Wilcoxon signed-rank test, p0.05). *Lobesia botrana* showed a tendency to lay more eggs on control berries than on dewaxed berries (Fig. 4c; Wilcoxon signed-rank test, p>0.05).
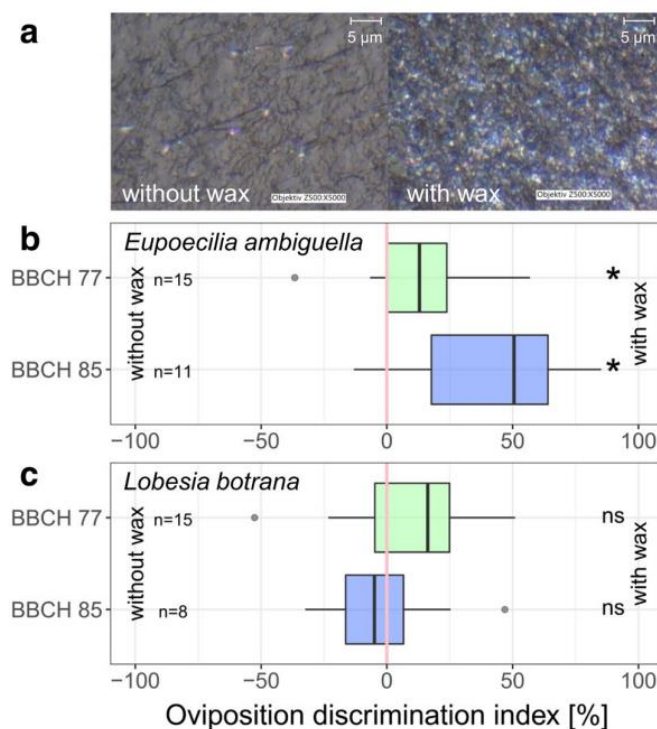
Fig. 4 a Berry surface from 'Regent' BBCH 85 without wax after removing by cellulose acetate stripping (left) and natural wax layer (right) (resolution: 5000x). Oviposition preferences of **b** *E. ambiguella* and **c** *L. botrana* for treated (without wax, left) versus untreated (with wax, right) 'Regent' berries at the BBCH-stages BBCH 77 and BBCH 85 illustrated by Box–whisker plots of the ODI (lines represent median, boxes IQR, whiskers 1.5 * IQR and closed circles as outliers. Asterisks denote statistical differences between treated and untreated berries (Wilcoxon signed-rank test; *p<0.05)

3.4. *Variety/BBCH oviposition bioassay of berry epicuticular wax extracts*

The models stressed that the interaction 'variety' and 'BBCH-stage' and the factor 'variety' had no influence on the proportion of laid eggs for both moth species (Table S1). Furthermore, the models stressed that only the BBCH-stage had an influence on the preference to lay eggs on the extracts for both moth species (Fig. 5, right; GLM of *E. ambiguella*: N=170, df=3, F value=19, p<0.01, pseudo-R^2=25.5; GLM of *L. botrana*: N=144, df=3, F value=13, p<0.01, pseudo-R^2=22.6). *Eupoecilia ambiguella* females laid statistically significant more eggs on extracts taken at BBCH 73 (Fig. 5a, pink boxplots; n=36, Median (Mdn)=0.77) and BBCH 77 (Fig. 5a, green boxplots; n=58, Mdn=0.81) than on extracts from berries at BBCH 85 (Fig. 5a, blue boxplots; n=55, Mdn=0.63; GLM, post hoc: least-squares means with Tukey adjustment method, p<0.01).
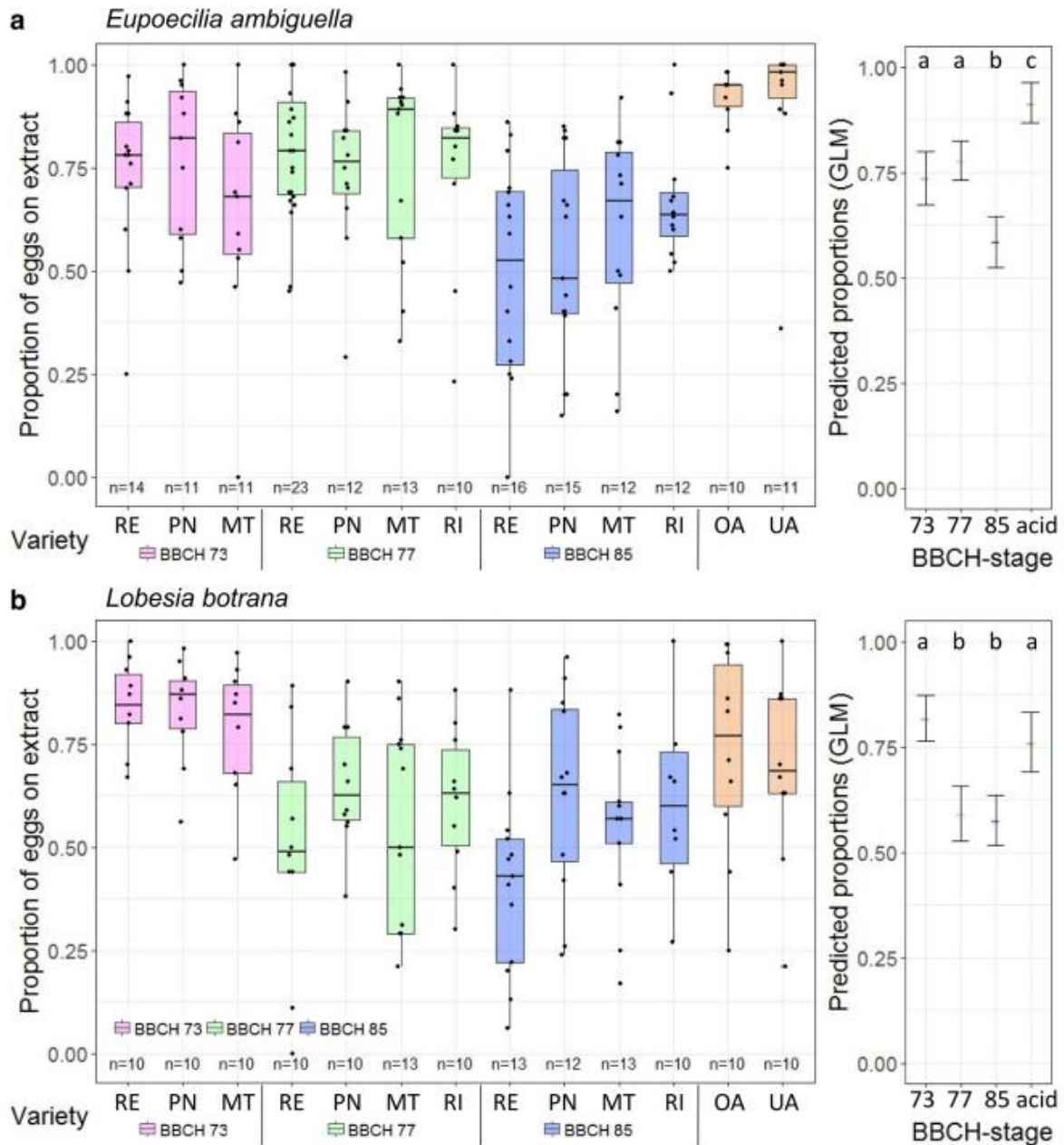
Fig. 5 Left box: proportion of eggs laid by **a** *E. ambiguella* and **b** *L. botrana* on glass substrate that was either untreated or treated with wax extracts of three BBCH-stages (73, pink; 77, green; 85, blue) or synthetic substances (OA, oleanolic acid; UA, ursolic acid; orange) of four varieties (RE, 'Regent'; PN, 'Pinot Noir'; MT, 'Müller-Thurgau'; RI, 'Riesling'). Box–whisker plots with jittered raw values as closed circles. Lines represent median, boxes the IQR and whiskers 1.5 * IQR. Right box: predicted proportion with confidence intervals obtained from quasi-binominal GLMs. Different letters denote statistical differences obtained by least-square means with Tukey adjustment method, p<0.01. More details are described in the text.

Furthermore, *E. ambiguella* females laid the most eggs on synthetic oleanolic acid (OA) and ursolic acid (UA) (Fig. 5a, orange boxplots; n=21, Mdn=0.95; GLM, post hoc: least-squares means with Tukey adjustment method, p<0.01). *Lobesia botrana* females laid statistically significant more eggs on extracts taken either at BBCH 73 (Fig. 5b, pink boxplots; n=30,

Mdn=0.86) or on synthetic substances (Fig. 5b, orange boxplots; n = 20, Mdn= 0.71) than on extracts obtained at BBCH 77 (Fig. 5b, green boxplots; n=43, Mdn=0.58) or BBCH 85 (Fig. 5b, blue boxplots; n=48, Mdn=0.56; GLM, post hoc: least-squares means with Tukey adjustment method, p<0.01). The mean number of eggs/female in this bioassay was 56±29 (SD) in case of *L. botrana*. 72% (±25%) were laid within the circle of the glass plate. *Eupoecilia ambiguella* females laid an average of 28±16 eggs, and 64% (±23%) were within the circle of the glass plate (Fig. 1).

Furthermore, to elucidate ovipositional preferences for a particular wax layer, dual-choice oviposition experiments were performed with *E. ambiguella* and *L. botrana* females. Wax extracts of all four varieties (MT, RE, RI, PN) were tested against each other at distinct phenological stages (BBCH 73, 75, 85). All statistical significant differences were ascertained by Wilcoxon signed-rank test. The number of eggs laid by *E. ambiguella* on extracts of 'Regent'/'Pinot Noir' was significantly higher than on extracts of 'Müller-Thurgau' at BBCH 73 (Fig. 6a, pink boxplots; p<0.05, p<0.01). *Lobesia botrana* exhibited no ovipositional preference for one tested wax extract of the three varieties at BBCH 73. *Eupoecilia ambiguella* did not distinguish between extracts of different varieties at the BBCH77, whereas *L. botrana* preferred 'Pinot Noir' extracts over 'Riesling' extracts (Fig. 6b, green boxplots; p<0.01). *Eupoecilia ambiguella* females never showed a preference for 'Müller-Thurgau' extracts if compared against one of the other three extracts at BBCH 85 (Fig. 6a, blue boxplots), but showed statistically significant ovipositional preferences for extracts of 'Regent'/'Pinot Noir' over extracts from 'Riesling' and preferred 'Pinot Noir' over 'Regent' (Fig. 6a, blue boxplots; p<0.01, p<0.01, p<0.05). In contrast to that, *L. botrana* preferred extracts from 'Müller-Thurgau' (BBCH 85) over extracts of 'Regent'/'Pinot Noir'/'Riesling' (Fig. 6b, blue boxplots; p<0.05, p<0.05, p<0.01) for oviposition. Additionally, more eggs of *L. botrana* were found on extracts of 'Regent'/'Pinot Noir' than on 'Riesling' (BBCH 85; Fig. 6b, blue boxplots; p<0.05, p<0.01) and they did not distinguish between extracts of 'Pinot Noir' and 'Regent'. Furthermore, *E. ambiguella* females preferred oleanolic acid over ursolic acid (Fig. 6a, orange boxplots; p<0.01), in contrast to *L. botrana*, which showed no preference for one of the triterpenoids. *Eupoecilia ambiguella* females preferred the extracts obtained from BBCH 77 over extracts from BBCH 85 in case of all varieties except 'Pinot Noir' (Fig. 6a, bottom; p<0.01, p<0.01, p<0.05). Irrespective of the cultivar, *L. botrana* females preferred none of the extracts at different BBCH-stages (Fig. 6b, bottom).
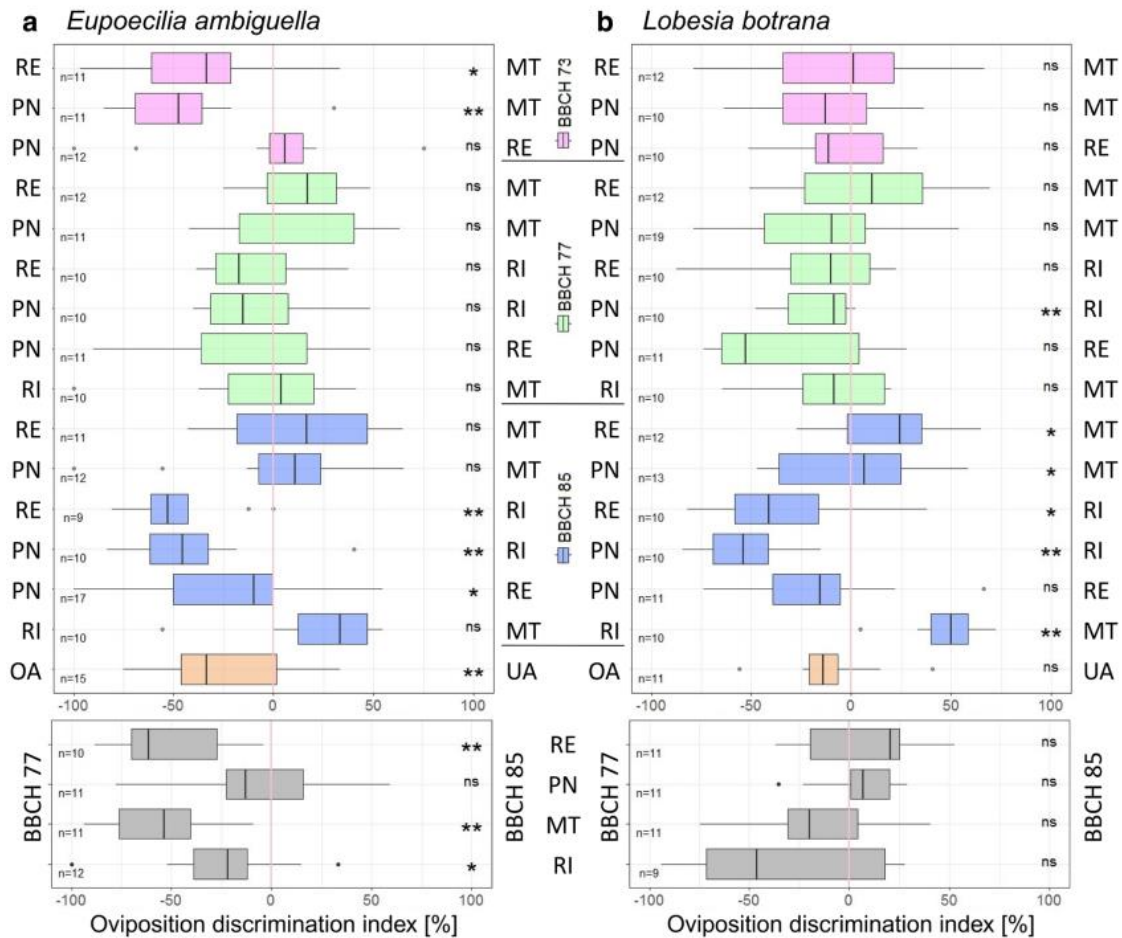
Fig. 6 Crosswise dual-choice oviposition experiments of wax extracts with females of **a** *E. ambiguella* and **b** *L. botrana*. Above: test of preferences of a certain variety (RE, 'Regent'; PN, 'Pinot Noir'; MT, 'Müller-Thurgau'; RI, 'Riesling') at three different distinct BBCH-stages (73, pink; 77, green; 85, blue) and synthetic substances (OA, oleanolic acid; UA, ursolic acid; orange). Bottom: oviposition experiments of wax extracts of two different BBCH-stages (left: BBCH 77 vs. right: BBCH 85) within each variety (RE, PN, MT, RI). Box– whisker plots of ODI, while lines represent median, boxes the IQR and whiskers 1.5 * IQR. Statistical preference for one wax extract is indicated by asterisks (Wilcoxon signed-rank test; **p<0.05)

4. Discussion

After visual and/or chemical attraction to grapevines from a distance (Anfora et al. 2008; Tasin et al. 2005), the moths will come into contact with epicuticular wax layers after landing on or close to their oviposition substrate, the grape berry. The chemical and/or physical characteristics of epicuticular waxes affect the ovipositional acceptance or rejection behavior of insects (Müller 2006). Several Lepidoptera use components of the wax layer as cues in host plant selection (Brooks et al. 1996; Juma et al. 2016; Li and Ishikawa 2006; Udayagiri and Mason 1997). In addition, it was shown that ovipositing *L. botrana* females use their contact chemosensilla distributed throughout their ovipositor to probe the plant surface, enabling the insect to detect the chemicals from the berry cuticle (Maher and Thiéry 2004b). As shown in

this study, *E. ambiguella* preferred berries with waxy bloom compared to dewaxed berries at both tested BBCH-stages. Thus, the overall occurrence of the wax layer is important for oviposition decision of *E. ambiguella*. Further, it was discovered that oleanolic acid is the major compound in the epicuticular wax layer of different varieties of grape berries (Fig. S1), which is in accordance with earlier findings (Pensec et al. 2014; Radler and Horn 1965). By analyzing the whole pattern of the waxy bloom in this study, oleanolic acid, besides montanic acid, was the main compound ultimately responsible for the difference between the patterns of epicuticular waxes of green (BBCH 75–79) and mature (BBCH 85–89) grape berries (Fig. 2a). *Eupoecilia ambiguella* females could perceive this difference and preferred wax extracts of young green berries (BBCH 77) for oviposition (Fig. 6a, lower box). This may be based on the higher amount of oleanolic acid in the epicuticular wax layer of green berries (Fig. 2d). In general, the eggs are deposited rather on berries than on leaves. The wax layer of leaves contains almost no oleanolic acid (Possingham et al. 1967; Radler 1965). This supports the theory that oleanolic acid acts as chemical cue for grapevine moth's oviposition acceptance behavior. Furthermore, it was confirmed by testing oleanolic acid alone in oviposition experiments (Fig. 5a) that the occurrence of oleanolic acid in wax layers is the key factor for oviposition of *E. ambiguella*. In contrast, *L. botrana* females did not prefer one of the two wax extracts (BBCH 77 vs. BBCH 85) when offered simultaneously (Fig. 6b, lower box). Thus, the absolute amount of oleanolic acid did not seem to be as relevant for oviposition site preference as it was for *E. ambiguella*. Other still unidentified components and their relation to oleanolic acid in the epicuticular wax layer seem to be relevant for *L. botrana* with regard to oviposition preference based on contact-chemical cues. Nevertheless, oleanolic acid is also important for oviposition recognition of *L. botrana* (Fig. 5b) in general. The influence of other apolar components (e.g. montanic acid) and the optimal concentration of OA should be in the focus of further studies.

Compositional changes in cuticular components of grape berries have been suggested to influence the susceptibility to *B. cinerea* infection in grapes (Comménil et al. 1997). A decrease in the oleanolic acid content of grape berries during maturation, as shown in this study for four different varieties, may explain the raised susceptibility of riper berries to fungal infection (Comménil et al. 1997). Recently, it was shown that oleanolic acid had a regulatory effect on the germination of *B. cinerea* (Silva-Moreno et al. 2016). *Lobesia botrana* larvae act as dispersal agents for the fungus and facilitate penetration and development of *B. cinerea* (Fermaud and Le Menn 1992; Cozzi et al. 2006). Moreover, the fungus had positive effects on the biology and physiology of *L. botrana* larvae (Mondy and Corio-Costet 2000), and more eggs were found on infected grape clusters (Mondy et al. 1998). However, with progressive

infection the oviposition preference of adults and the larval performance decreased (Savopoulou-Soultani and Tzanakakis 1988; Tasin et al. 2012). In consequence, it was suggested that there is a mutualistic relation between *L. botrana* and *B. cinerea* (Mondy et al. 1998) which is positive for *L. botrana* at intermediate infections. Because oleanolic acid reduce the germination rate of *B. cinerea*, but does not prevent it completely, oleanolic acid alone, possibly together with moderate amounts of volatile organic compounds (VOCs) emitted by *B. cinerea*, could lure grapevine moths to low or intermediate infected berries. In conclusion, the occurrence of oleanolic acid allows an infection but prevents a severe one, which promises a good environment for the progeny of *L. botrana* (Mondy et al. 1998). This supports the preference performance hypothesis (Gripenberg et al. 2010; Tasin et al. 2012) often suggested for *L. botrana* (Moreau et al. 2006; Thiéry and Moreau 2005).

Pensec et al. (2014) found ursolic acid only as methyl esters in contrary to free forms in grape berry wax extracts. We could not detect it in our wax extracts. Ursolic acid is an isomer of oleanolic acid that only differs in the position of methyl groups at C29 and C30, which was for instance found in surfaces waxes of olive berries (Bianchi et al. 1992). As it was shown that *L. botrana* used at least olive flowers as oviposition sites (Savopoulou-Soultani et al. 1990), we evaluated a potential oviposition stimulating ability of ursolic acid for both moth species, which was confirmed for both species (Fig. 5). *Eupoecilia ambiguella* was more attracted to oleanolic acid, due to its preference in dual-choice experiments, compared to *L. botrana* (Fig. 6). This indicates different degrees of specialization of the two moth species in contact-chemoreception. Waxes of a certain host plant can contain different information for insects with different degrees of specialization (Müller 2006). The polyphagous species *E. ambiguella* is favoring oleanolic acid and thus may be more intensively driven by this gustatory stimulus than *L. botrana*. Further studies are necessary to elucidate if oleanolic acid is the key stimulus for oviposition in nature and if it also contributes to the waxy bloom of other appropriate host plants. For *L. botrana*, we expect that during the oviposition process additional key stimuli are also important under natural conditions.

At BBCH 77, no ovipositional variety preferences based on non-volatile chemical cues of the wax pattern have been observed, except in one case for *L. botrana* (Fig. 6). Variety preferences are based on the whole wax pattern. After veraison (BBCH 81–89), the amount of epicuticular waxes increased strongly (Palliotti and Cartechini 2001) and the wax pattern of varieties differed more than on BBCH 75–79 (Fig. 2, Table 1). A variety preference of the moths was more pronounced at BBCH 85. Cuticular waxes are a complex mixture of hydrocarbons (mixtures of alkanes, aldehydes, primary and secondary alcohols, ketones, and alkyl esters) and

contain secondary metabolites such as triterpenoids and phenylpropanoids (Jetter et al. 2006). In the presented study, 59 different compounds were detected in the waxy bloom of the four investigated varieties. We identified only the main compounds oleanolic and montanic acid responsible together with two unidentified compounds for the separation of the two BBCH-groups. However, the unidentified substances may be responsible for the differences in oviposition behavior detected in this study at BBCH 85.

Sugars can be sensed by sensilla located on the ovipositor of *L. botrana* and oviposition was stimulated by sugars (Maher et al. 2006). Additionally, Maher and Thiéry (2004a) tested two apolar extracts (obtained by chloroform and hexane) on oviposition stimulation of *L. botrana*. Their chloroform extracts were achieved from mature berries and elicited no oviposition stimulation, as shown in this study in Fig. 5b. Furthermore, extracts obtained by polar extractants (methanol and water) in the same experimental setup were preferred for oviposition (Maher and Thiéry 2004a). At this late phenological stage (BBCH 85) sugars may be more important for oviposition decision for *L. botrana* than apolar compounds. In contrast, wax extracts from berries at BBCH 73 stimulated oviposition (Fig. 5b). *Lobesia botrana* can produce up to four succeeding generations depending on weather conditions (Harari et al. 2007). Waxes seem to be an important signal for the 1st generation. But indeed, the oviposition behavior of *L. botrana* is adapted on the phenological phases of grapevines and the 2nd and 3rd generations' oviposition occurs on green (BBCH 77) and mature (BBCH 85) berries, respectively. At these phenological stages, sugars and epicuticular waxes may act synergistically in attracting *L. botrana* for oviposition. Furthermore, still unknown substances may interfere with oviposition behavior, because the substances detected in an extract are just an excerpt of the actual pattern on the berries' surface depending, e.g., on polarity of the extractant (Müller and Riederer 2005). The wax layer is also a good matrix for the adsorption of VOCs. Such molecules could equally be sensed by the moths. Furthermore, grapevine moths (*L. botrana* and *E. ambiguella*) can also distinguish between physical structures on their oviposition surface (Markheiser et al. 2018). Thus, the physical occurrence of wax crystals on grape berries' surface could also serve as signal for oviposition decision, because they change their structure in correlation to ripening (Casado and Heredia 2001). Hence, in the presented study the influence of the tertiary structure was excluded due to destruction of the wax order by producing solved extracts for oviposition tests. This aspect should be in the focus of future investigations. To our knowledge, oviposition experiments with grape berry extracts on oviposition behavior of *E. ambiguella* had never been published before. Contact-chemoreception stimuli from grape berries' surface play a role in the acceptance of the berry

for oviposition. *Eupoecilia ambiguella* did not show an ovipositional preference for any variety based on just olfactory and visual cues (Fig. 3a). After getting contact to berries' surface, the varieties. 'Regent' and 'Pinot Noir' were preferred over 'Riesling' at BBCH 85–89 (Fig. 3a). This is in agreement with the oviposition preferences of wax extracts at the same BBCH-stage for the same varieties (Fig. 6a). Hence, we conclude that the ovipositional preference for 'Regent' and 'Pinot Noir' over 'Riesling' was mediated by the wax layer. While volatile and visual cues from grapevine plants transport the information of appropriate host plants over long distances (Anfora et al. 2008), epicuticular waxes of grape berries give the crucial information for oviposition preference of *E. ambiguella*.

The attractiveness of 'Pinot Noir'/'Regent' over 'Riesling' by *L. botrana* could be observed in oviposition experiments with whole berries (Fig. 3b) and with wax extracts of the respective varieties (Fig. 6b). This result confirms that the oviposition preference of *L. botrana* is actually triggered by the epicuticular wax compounds. Furthermore, *L. botrana* preferred 'Müller-Thurgau' wax extracts over all other tested wax extracts (BBCH 85) for oviposition (Fig. 6b). In contrast, a cultivar preference could not be observed for 'Müller-Thurgau' berries, when *L. botrana* females were allowed to sense all cues, as visual, olfactory and contact cues, until oviposition acceptance. In a former study, it was hypothesized that the 'Müller-Thurgau' may even convey repellent substances (Maher and Thiéry 2003). Because the attractiveness of 'Müller-Thurgau' in the presented experiments was based on wax-chemical cues only, further attractive or repellent cues like volatile organic compounds or sugars may compensate this effect in the vineyard. For testing this and detecting some varieties as more susceptible for grapevine moths than others, this should be evaluated in a vineyard under natural conditions with a wild population, because other abiotic and biotic factors, such as the larval food, influenced oviposition as well (Moreau et al. 2016).

Recent findings on *L. botrana* showed that this species, in contrast to E. *ambiguella*, needs the synergistic interaction of visual, volatile and contact cues (Tasin et al. 2011) for oviposition preferences. Volatiles and visual characteristics of berries are important cues for the long-distance and near-distance luring (Tasin et al. 2005). We argue that, although wax extracts elicit oviposition behavior in *L. botrana*, oviposition sites were not rejected when the wax layer is missing. Other cues may compensate the "medium optimal" waxy bloom, suggesting that *L. botrana* is more variable in choosing oviposition sites than *E. ambiguella*. Additionally, *L. botrana* seems to be more dependent on haptic cues for oviposition site acceptance, as shown by Markheiser et al. (2018). However, despite sharing some cues, there is evidence that these two moth species use different cues, or with different emphasis, for recognizing the same plant.

For monitoring the oviposition behavior of the European berry moths, an egg monitoring on host plants is necessary but currently not practicable. To prevent immoderate insecticide application, a decision support system for growers is needed, which enables the timing and necessity of pest control. Such a tool, called "moth oviposition card" (M-OVICARD), is currently under development (Markheiser et al. 2018). The number of eggs, deposited on such a monitoring card, should correlate with actual pest infestation in grapevines and may help to determine the perfect spraying time, resulting in a reduced amount of applied insecticides. The waxy bloom on grape berries is one important factor for oviposition decision of grapevine moths, and oleanolic acid the main compound, which highly stimulated the oviposition behavior of both *E. ambiguella* and *L. botrana*. By coating oleanolic acid on the surface of a M-OVICARD, its suitability for egg monitoring could be definitely improved.

5. Conclusion

Unraveling the signal hierarchies which contribute to oviposition behavior of other lepidopteran pest insects could help to develop similar egg-monitoring tools of other economical important lepidopteran pest insects, such as codling moth *Cydia pomonella* on apple and pear, or pea moth *Cydia nigricana* on Fabaceae, especially in areas where no mating disruption is possible or established (Witzgall et al. 2010). The knowledge on insect oviposition will help to find a better timing for ovicide spraying, reducing the amount of insecticides and will improve future IPM strategies. Beside this aspect, climate change has an influence on grapevine and on grapevine moths by changing phenology, voltinism and distribution ranges (Martín-Vertedor et al. 2010; Reineke and Thiéry 2016; Svobodová et al. 2014). With regard to changing requirements on pest control due to climate change (Reineke and Thiéry 2016), this innovative monitoring tool could help to adapt pest control strategies on future challenges

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Supplementary material

Fig. S1: Chromatograms of berry wax extracts from V. vinifera 'Regent' at BBCH 77 (above) and BBCH 89 (bottom, mirrored) obtained after derivatization and GC-FID analysis. IS = Internal standard = Tetracosane, OA = Oleanolic acid, MA = Montanic acid. (TIFF 37 kb)
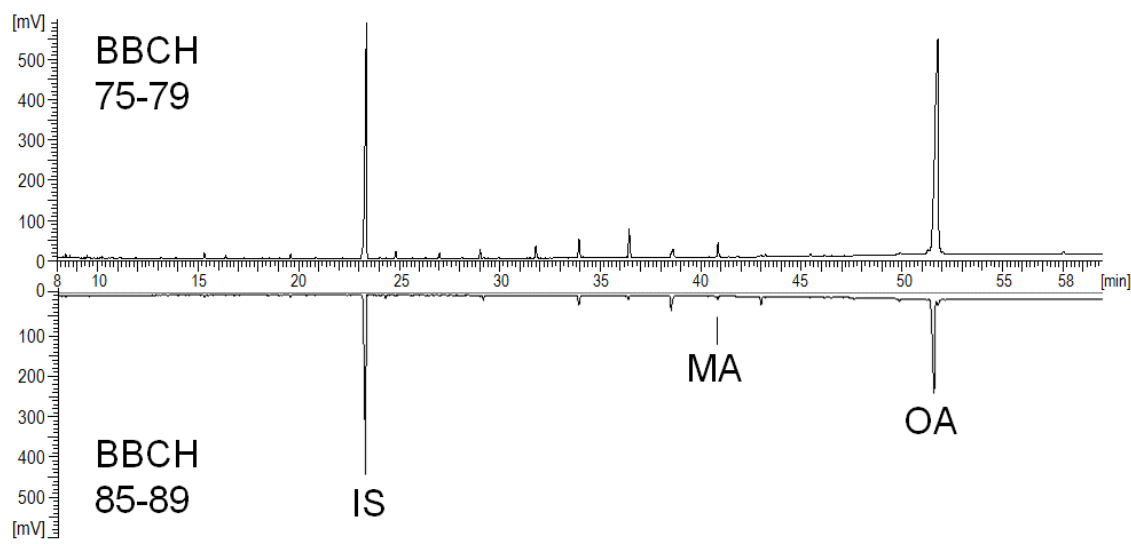
Table S1 Results of the first generalized linear model (GLM) of laid eggs on wax extracts, calculated without standard substances, because standard substance have no levels for BBCH-stage.

Species			D	F-value	p-value
E. ambiguella	Interaction	Variety : BBCH-stage	5	1.15	0.34
	Factor	Variety	3	0.45	0.72
	Factor	BBCH-stage	2	13.13	< 0.001
L. botrana	Interaction	Variety : BBCH-stage	5	1.51	0.19
	Factor	Variety	3	2.66	0.05
	Factor	BBCH-stage	2	19.28	< 0.001

Distribution: Quasi-binominal; Factors: 'variety' (levels: 'Regent', 'Pinot Noir', 'Müller-Thurgau', 'Riesling') and 'BBCH-stage' (levels: BBCH 73, BBCH 77, BBCH 85)

Chapter IV

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Volatiles of several grapevine cultivars emitted at different phenological stages linked to discriminatory ability of grapevine moths

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Abstract

The tortricid moths *Lobesia botrana* (Denis and Schiffermüller) and *Eupoecilia ambiguella* (Hübner) are major insect pests of grapevine *Vitis vinifera*. We compared the pattern of volatile organic compounds in headspace samples of four different grapevine cultivars in dependency of their phenological stage by thermodesorption coupled with gas chromatography (GC) and mass spectrometry. Further, we assessed the detection of compounds by the antenna of both moth species by GC coupled with electroantennographical detection. We detected significant differences between the chemical compositions of headspace samples of the four cultivars based on the odor bouquet. By comparing the principal component linear discriminant analysis pattern of only those chemical compounds that could be actually detected by the moths' antenna, the different cultivars showed similar odor patterns indicating that differences measured by GC may not be detected by the moths' antenna. The supposed reduced discriminatory ability of both moth species was supported by behavioral bioassays using a Y-tube olfactometer. In connection to the recently published impact of the berries' visual appearance (shape and color) and non-volatile compounds of the wax layer, the presented results suggest that reported moth preferences for specific cultivars are not encoded by volatiles only. The results of this study will contribute to the development of an innovative egg-monitoring tool for grapevine moths.

1. Introduction

The grape berry moths *L. botrana* (European grapevine moth) and *E. ambiguella* (European grape berry moth) (Lepidoptera: Tortricidae) are important pest insects causing high economic losses in European viticulture. The larvae of the second and third generation of both species have the greatest economic impact on grapevine, depending on the phenology of grape cultivar and latitude. In Central Europe, larvae of the second generation feed on green unripe grape berries [BBCH-stage 77; after Lorenz et al. (1994)]. Depending on weather conditions a third or fourth generation can emerge, which lay their eggs on ripe berries after veraison (BBCH-stage 83–85). Damage is caused by larval feeding enabling gray mould *Botrytis cinerea* rapidly to develop, causing the entire grape bunch to rot (Fermaud and Giboulot 1992).

In former studies, remarkable differences of damages between several cultivars of *V. vinifera* by *L. botrana* and *E. ambiguella* were reported (Birgücü et al. 2015; Fermaud 1998; Pavan et al. 2018; Sharon et al. 2009; Snjezana 2004; Thiéry et al. 2014). Furthermore, Sharon et al. (2009) reported differences in the number of eggs laid by *L. botrana* on different grapevine cultivars. The behavioral sequence of moths' oviposition behavior on distinct cultivars of grapevine (host finding, landing and oviposition) may be influenced by factors, such as cluster compactness (Fermaud 1998), light (Zahavi et al. 2003), fruit epidermis thickness (Snjezana 2004), contact cues like sugar (Maher et al. 2006; Varandas et al. 2004) and waxes (Rid et al. 2018) or by emitted volatile organic compounds (VOCs) (Tasin et al. 2005).

Vineyards could be highly heterogeneous host sites in time and space, since different grape cultivars may vary in shape and color and alter during the growing season. There are several factors affecting oviposition choice, which are theoretically differentiated into cues for prealighting and postalighting behavior (Knolhoff and Heckel 2014). The prealighting behavior is mainly influenced by visual and volatile cues, while the postalighting behavior is largely affected by gustatory and tactile cues (Knolhoff and Heckel 2014). It was shown that *L. botrana* is attracted by host plant volatiles from a distance (Tasin et al. 2005). By comparing olfactory, visual and contact cues, Tasin et al. (2011) suggested that olfactory cues had the strongest influence on the number of eggs laid by *L. botrana*. Nevertheless, little is known about the contribution of cultivar olfactory cues regarding oviposition preference. Therefore, the aims of this study were (I) to evaluate the role of volatile compounds in determining *L. botrana's* and *E. ambiguella's* preferences for four grapevine cultivars, (II) to investigate the discriminatory ability of moth's antenna between cultivars, and (III) to identify a potentially attractive blend of VOCs contributing to the development of an egg-monitoring tool for grapevine moths ('M-

Ovicard' = moth oviposition card). On the 'M-Ovicard', signals that are necessary for successful oviposition of grape berry moths, such as the physical structure (Markheiser et al. 2018), the non-volatile chemical composition from the berry surface (Rid et al. 2018), and the volatiles emitted by berries (this study) will be mimicked. The number of eggs laid on such a card gives information about grape berry moth infestation level and time of oviposition. This information is useful for determining the necessity and termination of insecticide treatments in a vineyard to control vulnerable early life stages of the moths according to the concept of Integrated Pest Management (IPM) (Gross and Gündermann 2016).

To unravel the differences in odor bouquets of *V. vinifera,* depending on the cultivar and phenological stages, the entire bouquets (= grape bunches + leaves) of three cultivars at six phenological stages were collected in a vineyard and analyzed by TD–GC–MS (Thermal desorber connected to a gas chromatograph coupled to a mass spectrometer). Furthermore, the odor bouquets of the berries of four cultivars at four phenological stages were collected under standardized greenhouse conditions and examined for dissimilarity. Beyond that, volatiles that are detected by the moth antenna were evaluated by electrophysiological measurements. In addition, multivariate statistical methods were used to analyze patterns of emitted volatiles and link them with antennal responses of grapevine moths to determine their ability to discriminate between *V. vinifera* cultivars at a specific phenological stage (BBCH 73–75). For verification of the discrimination ability between the berry scents of different grapevine cultivars, the walking behavior of the moths prior to oviposition was examined in two choice olfactometer experiments.

2. Materials and methods

2.1. Insects

Lobesia botrana and *E. ambiguella* used in experiments originated from a laboratory culture maintained at Julius Kühn-Institut (JKI), Siebeldingen, Germany. The rearing cycles of both moth species are described in Markheiser et al. (2018). Pupae of both species were taken from the permanent rearing and kept in cages (Bugdorm, $30 \times 30 \times 30$ cm, MegaView Science, Taiwan) in a conditioning cabinet (RUMED, Modell 3501, Rubarth Apparate GmbH, Laatzen, Germany) provided with water ad libitum until further treatment. The temperature was 23 ± 0.5 °C during photophase (14 h + 1 h dusk and 1 h dawn) and 19 ± 0.5 °C during scotophase (8 h) adapted from Moreau et al. (2006). The 14 photophase hours were at 2000 lx, and light intensity progression through time to imitate dusk and dawn was performed by a

smooth light/dark transition (every 12 min 20% less or more light, respectively). Mated females (24–72 h post copula) were used in electrophysiological experiments and Y-tube olfactometer bioassays. The mating status was verified by controlling the presence of a spermatophore in female's abdomen after the experiments.

2.2. Plants

VOCs of three different cultivars ('Müller-Thurgau', 'Regent' and 'Pinot Noir') of *V. vinifera* were sampled at the vineyards of S. & W. Stein, Großsachsen, Germany (49°30′44.5″N 8°40′19.6″E), in 2014. Conventional plant protection measures were applied (Table S1, Online Resource). Further, greenhouse experiments were conducted. We studied one red ('Regent') and one white cultivar ('Müller-Thurgau') with early ripening properties, and one red ('Pinot Noir') and one white cultivar ('Riesling') with late ripening properties to get a representative spectrum of grapevines. The four cultivars were grown in individual pots ($Ø = 11.7$ cm, $H = 13.5$ cm, Hermann Meyer KG, Rellingen, Germany) in a greenhouse at JKI, Dossenheim, Germany at 23 ± 5 °C, 16:8 h (L/D) photoperiod and $50 \pm 10\%$ relative humidity. Only grapes with a healthy appearance (visual inspection) were used for experiments. Chemical control of downy mildew (*Erysiphe necator*) by the fungicides Talendo (active ingredient: Proquinazid), Vivando (Metrafenone) and Dynali (Cyflufenamid + Difenoconazole) was applied in a rotating matter once a week. The time between pesticide application and headspace sampling was at least 48 h. In behavioral experiments, we ensured the comparisons of berries in the same phenological stage of the different ripening cultivars (early and late) by shifting pruning and defoliating of 2-year-old grape cultivars.

2.3. Headspace sampling

Volatiles were collected with a headspace sampling device, which ensures an exact collection of the same volume of VOCs in headspace samples for five plants simultaneously according to Rid et al. (2016). Whole branches (field experiment) or single berry clusters (greenhouse experiment) were carefully wrapped in oven plastic bags made of polyethylene terephthalate ($Ø = 20$ cm, Melitta, Minden, Germany). The plant parts were thereby not detached from the plant (in vivo headspace sampling), to prevent an emission of green leaf volatiles (GLV) due to damage of the plants (Fall et al. 1999). Ambient air was purified by passing through washing bottles filled with activated charcoal (granulated 4–8 mm, AppliChem GmbH, Darmstadt, Germany) and streamed with 1 L/min through the bag until it reached a final volume of 100 L.

After each trial, the washing bottles and tubes were rinsed with 70% ethanol (p.a.; Merck Millipore, Darmstadt, Germany) and baked at 230 °C for at least 2 h. Volatiles from headspace sampling were trapped on $1/4'' \times 3.5$ stainless steel, prepacked sample tubes filled with 200 mg Tenax® TA60/80 sorbent (PerkinElmer, Rodgau, Germany; Markes, Neu-Isenburg, Germany). Used tubes were closed with Teflon-coated brass compression caps (Swagelok, PerkinElmer) and stored for a maximum of one week at room temperature before analysis. Headspace samples collected in the field ($N = 112$) were taken from the whole branches at different phenological stages [following BBCH-scale of Lorenz et al. (1994)] of the cultivars 'Müller-Thurgau' ($n_{13-18} = 8$; $n_{55} = 4$; $n_{65-71} = 5$; $n_{73-75} = 6$, $n_{77-79} = 4$, $n_{83-89} = 8$), 'Regent' ($n_{13-18} = 8$; $n_{55} = 8$; $n_{65-71} = 7$; $n_{73-75} = 8$, $n_{77-79} = 4$, $n_{83-89} = 4$), and 'Pinot Noir' ($n_{13-18} = 8$; $n_{55} = 4$; $n_{65-71} = 7$; $n_{73-75} = 8$, $n_{77-79} = 4$, $n_{83-89} = 7$). Sampling started at the beginning of the growing period (without leaves: BBCH 13–15) and ended at berries ripening (BBCH 89). A second set of headspace samples ($N = 266$) were taken from berries of potted grape cultivars 'Müller-Thurgau' ($n_{65-71} = 8$; $n_{73-75} = 19$, $n_{77-81} = 21$, $n_{83-89} = 16$), 'Regent' ($n_{65-71} = 15$; $n_{73-75} = 21$, $n_{77-81} = 12$, $n_{83-89} = 20$), 'Riesling' ($n_{65-71} = 6$; $n_{73-75} = 12$, $n_{77-81} = 27$, $n_{83-89} = 29$), and 'Pinot Noir' ($n_{65-71} = 11$; $n_{73-75} = 16$, $n_{77-81} = 19$, $n_{83-89} = 14$), and were collected in the greenhouse at 20 ± 2 °C at the respective phenological stages. Analytical thermal desorption was selected as a very sensitive and reliable method for volatile analysis, but made it necessary to collect additional headspace samples for GC–EAD experiments. These samples were collected from berries (BBCH 73–75) of potted grape cultivars 'Müller-Thurgau' ($n = 8$), 'Regent' ($n = 8$), 'Riesling' ($n = 4$), and 'Pinot Noir' ($n = 8$), as described for GC–MS above. The headspace sampling parameters as well as the phenological stage of the plants were exactly the same.

2.4. TD–GC–MS

Samples were analyzed using thermal desorption (TD) (TurboMatrix™ Automated Thermal Desorber ATD 650, PerkinElmer) connected to a GC–MS. The TD details were as follows: Tube desorption for 10 min at 250 °C and collection on a cold trap (Tenax TA), which was held at -20 °C throughout the tube desorption process, and afterward heated at a rate of 99 K/s to 250 °C and held for 1 min. The desorbed volatile compounds were separated and identified using a PerkinElmer® Clarus® 680 GC system coupled to a PerkinElmer quadrupole inert mass selective detector. A nonpolar Rxi-5 ms® (Crossbond 5% diphenyl–95% dimethyl polysiloxane, Restek, Bad Homburg, Germany) capillary column ($30\ \text{m} \times 0.25\ \text{mm}$ id $\times 0.25\ \mu\text{m}$ film thickness) was used for the GC separation. Splitless injection was employed using helium as the carrier gas (Helium 6.0, Linde, Munich, Germany) at a flow rate of about

1 mL/min (150 kPa). The initial oven temperature was 40 °C and was held for 1 min, followed by a linear programmed temperature from 40 to 180 °C at a rate of 5 K/min, and a rate of 20 K/min from 180 to 280 °C. The final temperature of 280 °C was held for 6 min. The ion source temperature was 180 °C, and GC inlet line temperature was 250 °C. The quadrupole mass detector was operated in the electron impact (EI) mode at 70 eV. The electron multiplier was set to the autotune procedure. All data were obtained by collecting the full-scan mass spectra within the range of 35–350 m/z.

2.5. Identification and quantification with AMDIS

Identification of volatile compounds was confirmed by matching mass spectra with spectra of reference compounds in the National Institute of Standards and Technology (NIST08) Mass Spectral Search Program (MS-Search). Mass spectral deconvolution, identification and integration were imported in the 'Automated Mass Spectral Deconvolution and Identification System' software (AMDIS, version 2.71) for interpretation of GC–MS data according to Gross et al. (2019). Strict identification criteria were applied. The match factor had to be $\geq 80\%$ and the relative retention index deviation must be $\leq 8\%$ from reference value. The default settings for deconvolution were: component width: 32; adjacent peak subtraction: one; resolution: low; shape requirements: low; level: very strong; maximum penalty: 50 and 'no RI in library': 20. Reference retention indices (RI) values were obtained from NIST Chemistry WebBook (Stein 2010). In case of multiple literature RI values for an individual compound, the mean of the reported values (corresponding to the same column diameter and film thickness as well as a comparable stationary phase material) was used for comparison. In addition, the compounds were identified by comparing the experimental retention times, RI and mass spectra with those from commercially available standards introduced in the same system according to Weintraub and Gross (2013). Where a compound is identified based only on a comparison of the spectra with the NIST library, it is marked in Table S4 (Online Resource). Some compounds stayed unidentified because of low match of the measured mass spectra with the mass spectra from the libraries. They were set as 'known unknowns', named after their RI values, and contributed to the overall spectrum. Compounds that occurred in less than 5% of the total sample numbers were excluded from calculations. The peak areas were integrated and relative proportions were calculated, in which the sum of the selected compounds was set as 100%, and this compositional data set was used for statistical analysis.

2.6.TD–GC–FID/EAD

The samples of headspace volatiles from greenhouse berries of four different grape cultivars were subjected to coupled thermal desorber–gas chromatography–flame ionization detector and electroantennographic detection (TD–GC–FID/EAD) analyses using the antennae of females of both grapevine moth species to identify detectable volatiles. A thermal desorption unit (TurboMatrix™ Thermal Desorber TD 300, PerkinElmer) was coupled to a GC–FID (PerkinElmer® Clarus® 680) and equipped with a Rxi-5 ms® capillary column (Restek). The thermodesorption program was the same as mentioned for TD–GC–MS analysis. The GC-temperature program was as following: 40 °C hold for 1 min, then with 10 K/min to 280 °C that was held for 2 min. Compounds eluting from the GC-column were split between the FID and a heated (280 °C) transfer line (Ockenfels Syntech®, Kirchzarten, Germany), into a continuous, charcoal filtered and humidified air stream (1.5 L/min) directed to the antenna preparation (EAD detector). Moths' antennae were excised with fine scissors. The indifferent, reference electrode was connected to the base of the antenna, whereas the different, recording electrode was connected to the tip of the antenna (with the last segment of antenna cut off). Glass capillaries (0.58 mm I.D., Science Products, Hofheim, Germany) filled with Ringer solution (NaCl 7.5 g, KCl 0.35 g CaCl$_2$ 0.21 g ad 1 L H$_2$O) were used as electrodes and connected to silver wire. Antennal response was confirmed by controlling with a positive reference chemical substance (*E. ambiguella*: Linalool (10 µg, Sigma-Aldrich, Schnelldorf, Germany); *L. botrana*: Farnesene (mixture of isomers, 10 µg, Sigma-Aldrich)). The analog signal was detected with a probe (INR-II, Ockenfels Syntech®), captured and processed with a data acquisition controller (IDAC-2, Ockenfels Syntech®), and analyzed with GC–EAD software (Gc-Ead 2014 v1.2.5, Ockenfels Syntech®). EAD responses to FID peaks were defined as repeatable alterations of the antennal baseline. Corresponding peaks from TD–GC–FID and TD–GC–MS were matched by RI for identification of the EAD-active compounds. GC–EAD experiments were conducted with headspace samples of berries of all four cultivars at the BBCH-stage 73–75, namely 'Müller-Thurgau' (*n* = 4 per species), 'Regent' (*n* = 4 per species), 'Riesling' (*n* = 2 per species), and 'Pinot Noir' (*n* = 4 per species).

2.7.Behavioral experiment

To investigate the olfactory discrimination ability of females of *L. botrana* and *E. ambiguella* to berry odor bouquets of different *V. vinifera* cultivars, Y-shaped olfactometer trials were conducted. This method was used to reflect the behavior of the females, which had alighted at

the grapevine canopy searching mainly by walking for a suitable oviposition site. In every trial, the berry volatiles of two different cultivars were presented simultaneously to perform a dual-choice experiment. The 2.5-cm-i.d. olfactometer had a 14.5-cm-long common arm that split into two 25.5-cm-long side arms angled to 40° from each other. The olfactometer was mounted on an angular board to reach an inclining position of 45° from the horizontal plane. The insects walked upward, little flights were also possible, and a centered uniform light source (LED; PURElite, UK; 60 lx) imitated dusk conditions, but facilitated moth's walk. The experiments were conducted in a 3-h time period, which correlated with the artificial dusk (1 h before dusk + 1 h at dusk + 1 h after dusk) in the insect rearing. Two undamaged berry clusters were each wrapped in an inert plastic bag (Ø = 20 cm, Melitta, Minden, Germany). With this setup, we were able to provide pure berries' scent without leaf volatiles in a non-invasive (in vivo) way from a living grapevine. The cultivars 'Regent', 'Müller-Thurgau', 'Riesling' and 'Pinot Noir' were presented in alternating combinations at BBCH-stage 73–75. A purified and humidified airflow entered each plastic bag, transporting the volatiles of the berries through polytetrafluoroethylene (PTFE) tubes to the respective side arm of the olfactometer. Prior to the experiments, the flow was set at 100 mL/min by flow regulators (PTFE) at each arm of the olfactometer. The moths were released into the entrance of the olfactometer and their behavior was observed for a maximum of 5 min. Moths entering the left or right branch after passing a marking (10 cm behind branching) were counted ($n \geq 30$). Numbers of non-moving moths contributed to the calculation of the response rate, which was defined as the percentage of individuals showing behavioral response (walking) out of all tested ones (shown in Fig. 6). After every fifth replication, the Y-shaped olfactometer was rotated 180° to avoid position effects. After every comparison, the glass tube was rinsed with 70% ethanol and baked out at 100 °C for 2 h.

2.8. Statistical analyses

All statistical analyses and graphs were performed using the software R (version 3.3.1 (2016-06-21) (R Core Team 2016). Multivariate statistical methods were used in order to compare the volatile bouquets. Compositional data from peak areas derived by AMDIS were scaled and mean centered prior to principal component analysis (PCA) using the 'scale' and 'center' function of R. This was done for component reduction and to prevent over-interpreting of highest peaks. Afterward, a linear discriminant analysis (LDA) out of the principal components (PCs) that explained over 80% of the variance, was conducted (MASS package) (Venables and Ripley 2002). LDA analyses were calculated by three discriminant functions (DFs) with

descending explaining variance and were visualized by x–y plots. Each dot represents the full odor profile of a sample. The larger dots indicate the group centroids. To assess the proportion of individual odor bouquets classified correctly, a classification table (CT) of cross-validated (cv) data was performed. Here, the DFs were calculated by excluding one sample from the dataset and using the respective DFs to classify the individual scent pattern of a group according to BBCH-stage and cultivar. This was repeated with all individuals in the dataset and the proportion of false classified individuals (error rate, ER) to each group was returned. To assess the statistical significance of the classification, Pearson's χ^2 for the predicted classification data was calculated. Groups with a low rate of false classifications are easier to discriminate than those with higher ones. This procedure described above was applied separately to overall volatile bouquets and GC–EAD-active odor bouquets. Additionally, for comparisons among single compounds, the relative amount of every compound of each BBCH-stage was compared within the cultivar using *Kruskal–Wallis* followed by the post hoc *Wilcoxon rank-sum* test and *Bonferroni* correction. Determining preferences for one cultivar in olfactometer tests was confirmed by *binominal test*. Comparing the response rate of the two moth species within olfactometer experiments was accomplished with a *Student's t test*. Figures and heatmaps were created using R package ggplot2 (Wickham 2009).

3. Results

3.1. Identification of whole plant volatiles sampled in vineyard under natural conditions

A total of 108 compounds was detected in the headspace of grapevines (berries and leaves) grown under natural conditions ($N = 112$; Table S2, Online Resource). A PCA–LDA of the scaled compositional dataset revealed differences between the odor bouquets depending on grapevine cultivar and phenological stage (Fig. 1, Table 1). The six tested phenological BBCH-stages (13–18, 55, 65–71, 73–75, 77–81 and 83–89) are separable based on their odor bouquet, especially within each cultivar. PCA–LDA DF1 (Fig. 1: y-axis) separated the odor bouquet of phenological stages 55 and 13–18 from the other ones. In addition, DF2 (Fig. 1, x-axis) separated the rest of the phenological stages (65–89) from each other, and DF3 (14.4% variance explained, not shown) separated the first two phenological stages (13–18 and 55).
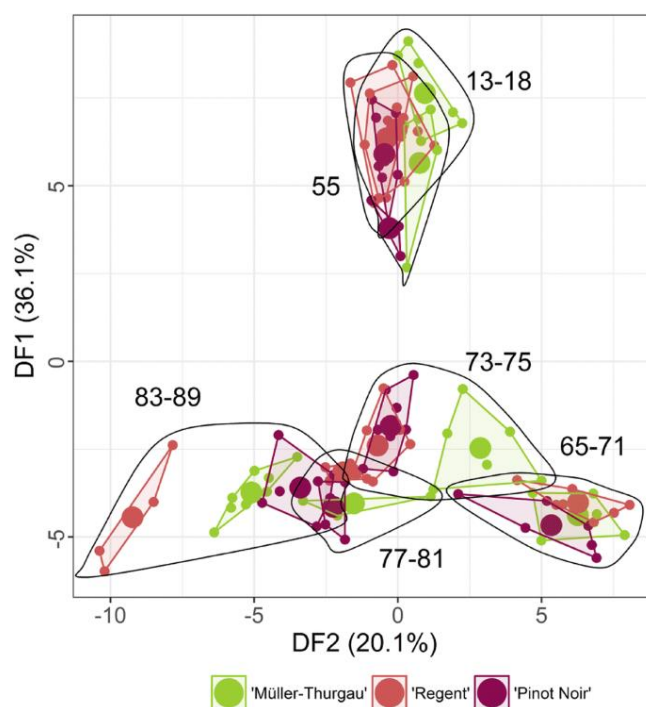
Fig. 1. Linear discriminant plot of the headspace of whole grapevine plants (berries & leaves) sampled in a vineyard in Germany for three cultivars ('Müller-Thurgau', 'Regent', and 'Pinot Noir') at six phenological development stages (BBCH-scale). Small dots represent an odor bouquet and lager group centroids ($N = 112$)

Table 1. Details of statistical analysis

PCA–LDA based on	BBCH	Figures	PCs for 80% exp. variance	Variance explained by DF1 (%)	Variance explained by DF2 (%)	Classification table (CT) of cross-validated (cv) data			
						Pearson's χ^2	df	p	Error rate (%)
107 volatiles (vineyard)	All	1	27	36.1	20.1	1175.9	289	<0.01	27.5
104 volatiles (greenhouse)	All	3a	29	43.4	13.0	1094.1	225	<0.01	39.0
104 volatiles	BBCH 73–75	5a	29	47.6	34.3	41.383	9	<0.001	42.6
24 EAD-active compounds	BBCH 73–75	5b	11	62.1	27.0	7.860	9	>0.5 (ns)	71

Changes in the odor bouquets along phenological stages are presented in Fig. 2 for the five most abundant VOCs of berries and leaves: nonanal, decanal, (Z)-3-hexenyl acetate, ethyl butanoate and β-caryophyllene, representing 33% up to 77% of the total volatile compounds (Fig. 2).

Statistical differences of the relative amount of these five components across the phenological stages within each cultivar were found and marked in Fig. 2 (*Wilcoxon rank-sum* test with *Bonferroni* correction; $p < 0.05$). Whereas β-caryophyllene was a minor component at BBCH 13–18 and BBCH 55, it became a prevailing substance in the subsequent phenological stages in the headspace of 'Müller-Thurgau' and 'Regent'. The same was observed for (Z)-3-hexenyl acetate in 'Pinot Noir'.
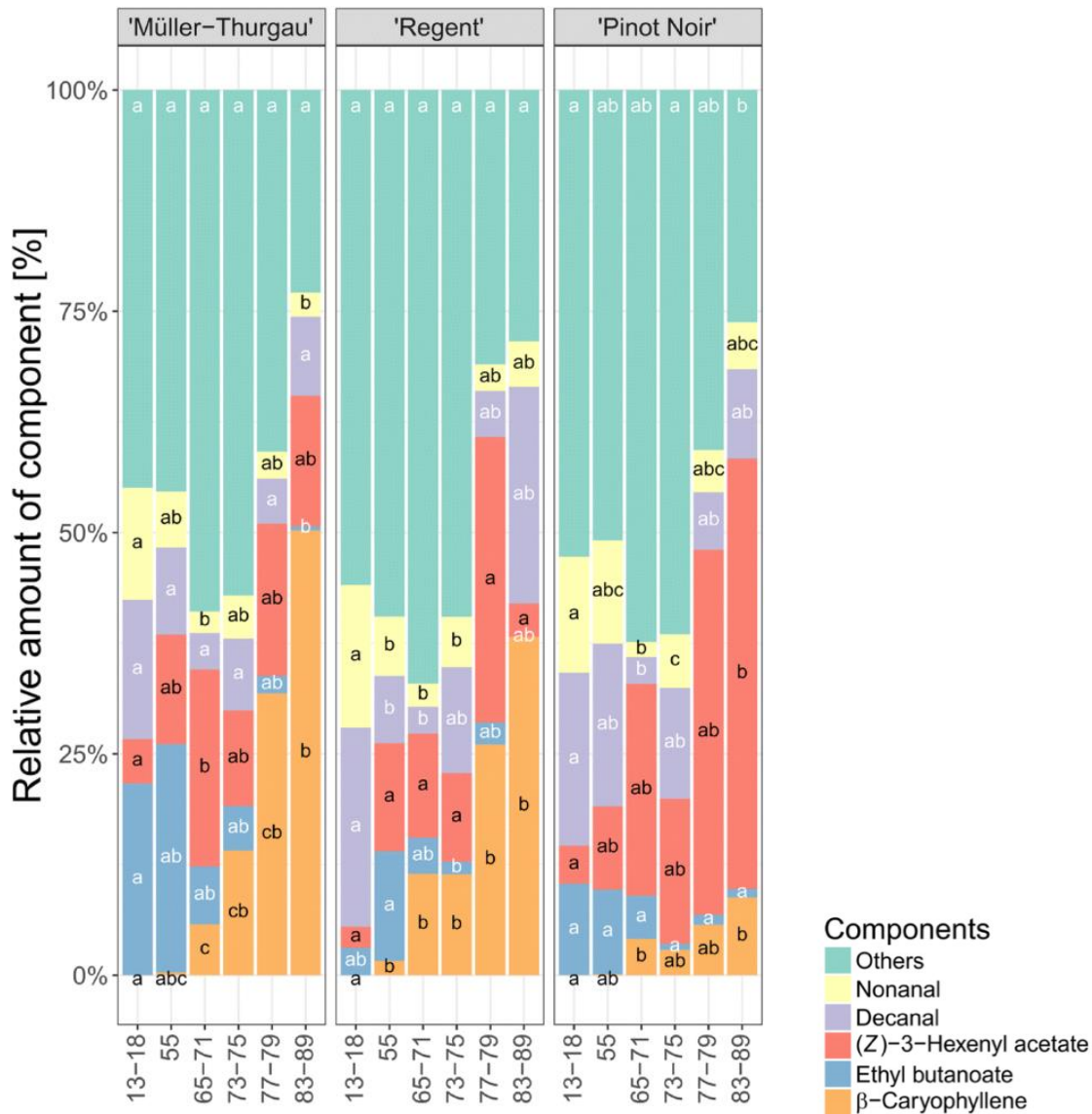
Fig. 2. Relative amounts of the five most abundant volatile organic compounds in *Vitis vinifera* headspace collected from whole plants in vineyard belonging to three cultivars ('Müller-Thurgau', 'Regent', and 'Pinot Noir') at six phenological stages (BBCH-scale). Different letters across BBCH-stages within a variety indicate significant differences for a given compound. (*Wilcoxon rank-sum* test with *Bonferroni* correction, $p < 0.05$, $N = 112$)

3.2. Identification of berry volatiles sampled in greenhouse under standardized conditions

We determined qualitative and quantitative differences between the chemical composition of headspace samples ($N = 266$, Table S3, Online Resource). A total of 104 compounds were detected in headspace samples of grapevine berries from four cultivars. Seventy-four of these volatiles were reliable identified (Table S4, Online Resource), encompassing a wide range of different chemical classes of volatile compounds, including alcohols (6), aldehydes (12), alkanes (8), alkenes (2), esters (8), ketones (5), terpene derivates (2), carotenoid derivates (2), benzenoids (12) and terpenoids (15).

3.3.Comparison of berry odor bouquets

Since there were only few qualitative differences between the cultivars and BBCH-stages, the compositional dataset was used to perform a PCA for component reduction and out of that a LDA was calculated with 29 PCs, which explained 80% of the variance for inspection their grouping behavior. Following results are deduced from PCA–LDA (Fig. 3, Table 1) together with the corresponding confusion matrix (Table 2).
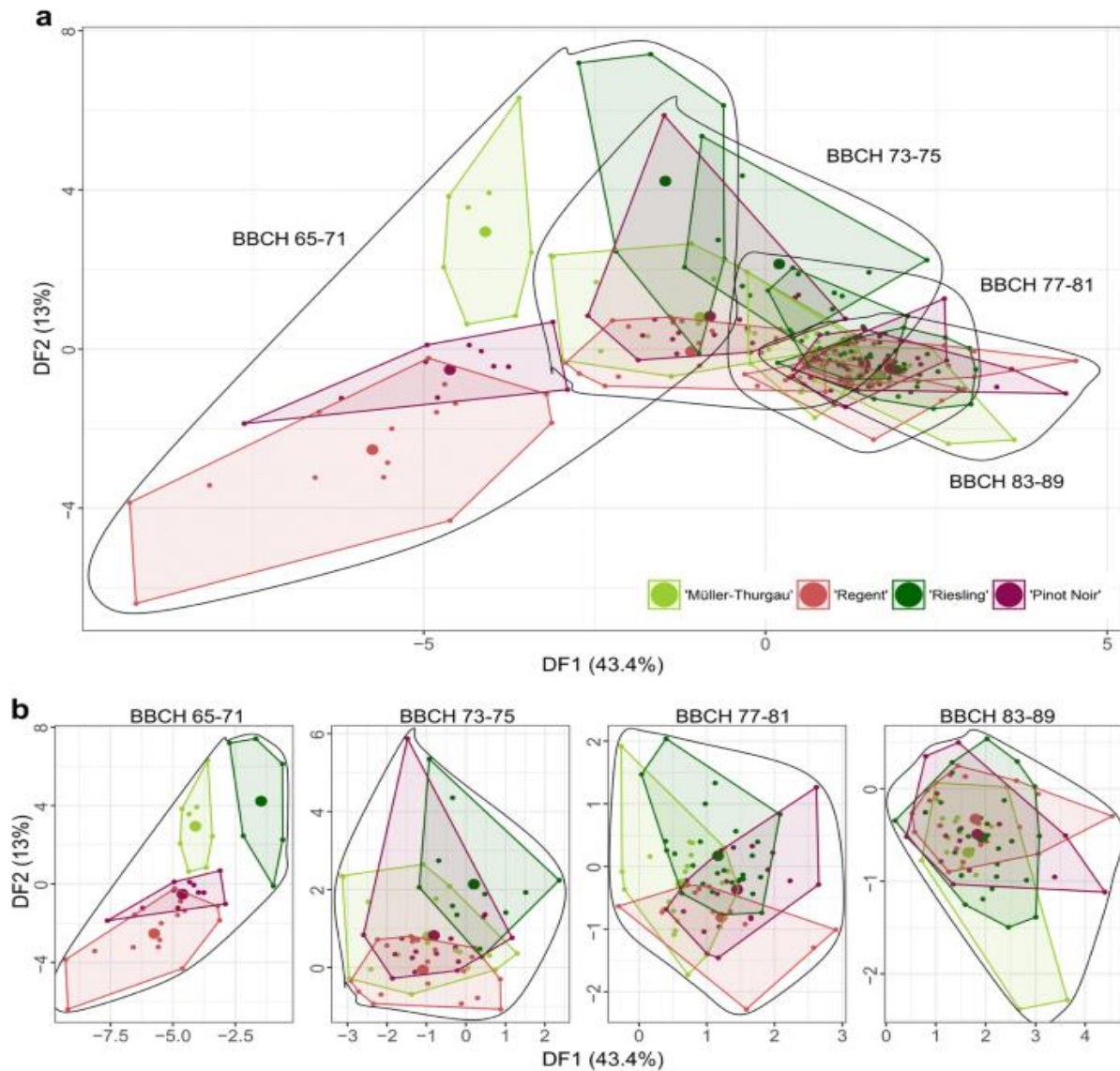
Fig. 3. **a** Linear discriminant plot of headspace of potted *V. vinifera* belonging to four cultivars ('Müller-Thurgau', 'Regent', 'Riesling', and 'Pinot Noir') at four phenological subsequent stages (BBCH-scale). 104 detected VOCs from the berries' odor bouquet were used for the analysis ($N = 266$). **b** Image detail for the four subsequent phenological stages for better visibility. Small dots represent an odor bouquet and lager group centroids. For more details please read the text

The whole odor bouquets of grape bunches differed quantitatively between phenological stages, especially within each of the four cultivars. The flowering stage (BBCH 65–71) and the first

berry formation stage (BBCH 73–75) delineated from each other and from the stages during subsequent berry formation and ripening, based on the DF1 (Fig. 3). The group centroids of all cultivars at BBCH 77–81 and BBCH 83–89 are close to each other, indicating that they are more similar to each other than to volatiles emitted during flowering, but their separation was verified by CT (Table 2). High error rates resulted of more mismatches with other cultivars rather than across the BBCH-stage. (II) Nevertheless, the mismatches between BBCH 73–75 and BBCH 77–81 and between BBCH 77–81 and BBCH 83–89 account for a smooth transition of the odor bouquet along the berry formation and maturation. (III) The four analyzed cultivars showed quantitatively different odor bouquets of their berries. The grouping of each cultivar was rather shown in the CT (Table 2) than in the PCA–LDA plot obtained from the whole dataset.

Table 2 Classification table of LD-analysis of the whole odor spectrum (104 substances) of four varieties, 'Müller-Thurgau' = MT, 'Regent' = R, 'Riesling' = Rie, 'Pinot Noir' = PN at subsequent phenological stages with misclassification rate = error rate

V. vinifera BBCH-stage and varieties	Predicted group membership																Total	Error rate
	BBCH 65–71				BBCH 73–75				BBCH 77–81				BBCH 83–89					
	MT	R	Rie	PN	MT	R	Rie	PN	MT	R	Rie	PN	MT	R	Rie	PN		
BBCH 65–71																		
MT	7	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	8	0.13
R	0	12	0	2	0	1	0	0	0	0	0	0	0	0	0	0	15	0.20
Rie	0	0	3	0	0	0	2	1	0	0	0	0	0	0	0	0	6	0.50
PN	0	0	0	10	0	0	0	1	0	0	0	0	0	0	0	0	11	0.09
BBCH 73–75																		
MT	1	0	0	0	11	1	0	0	2	0	2	1	0	1	0	0	19	0.42
R	0	0	0	0	0	13	0	2	2	0	4	0	0	0	0	0	21	0.38
Rie	0	0	0	0	0	0	8	0	0	0	3	1	0	0	0	0	12	0.33
PN	1	0	0	0	1	1	0	10	1	0	2	0	0	0	0	0	16	0.38
BBCH 77–81																		
MT	0	0	0	0	0	0	0	1	10	4	1	1	0	3	0	1	21	0.52
R	0	0	0	0	0	0	0	0	2	4	2	2	1	1	0	0	12	0.67
Rie	0	0	0	0	0	0	1	0	1	1	16	1	1	0	3	3	27	0.41
PN	0	0	0	0	0	0	0	0	2	0	1	12	2	2	0	0	19	0.37
BBCH 83–89																		
MT	0	0	0	0	0	0	0	0	0	0	2	1	6	3	1	3	16	0.63
R	0	0	0	0	0	0	0	0	0	0	1	0	0	15	1	3	20	0.25
Rie	0	0	0	0	0	0	0	0	1	0	6	0	2	3	15	2	29	0.48
PN	0	0	0	0	0	0	0	0	0	0	0	1	0	3	0	10	14	0.29

Bold numbers indicate correct predicted group memberships

For a clearer observation of the differences of the cultivars, each BBCH-stage is shown in a picture detail (Fig. 3b). During the flowering period (BBCH 65–71) all cultivars were separated based on DF1 and DF2. Additionally, there was a separation of the cultivars at the first berry formation stage (BBCH 73–75) due to their whole berry odor bouquets, but for a proper visualization more than these two DFs would be necessary, but low mismatching rates

accounted for a separation (Table 2). At flowering (BBCH 65–71) and early berry formation (BBCH 73–75), the cultivars showed a clear separation, with an average error of 30% (Table 2). During late formation of fruits (BBCH 77–81) and during berry ripening (BBCH 83–89), the odor bouquets of the cultivars were different, but became more similar. This is underlined by the average mismatches (45%) of the CT table (Table 2). (IV) Due to increasing maturity the similarity of cultivar berry odor bouquets is decreasing.

3.4. Detection of berry volatiles by grape berry moths and calculated discriminatory ability

TD–GC–EAD measurements were conducted with both moth species and all four cultivars of grapevine at the BBCH-stage 73–75, when the second generation of grape berry moths searches for oviposition sites and an olfactory discrimination should be possible. EAD recordings revealed that 24 VOCs of the berry odor were detected by *L. botrana* and *E. ambiguella* (Fig. 4a). To visualize the difference of the odor bouquet at berry formation stage, an extra PCA–LDA was conducted based on all 104 chemical compounds and showed a separation of all cultivars (Fig. 5a, Table 1). Overlapping dots of 'Müller-Thurgau' and 'Pinot Noir' could be separated from each other based on DF3 (18.1% variance explained, not shown in Fig. 5a). In contrast, the cultivars were not separable by the 24 GC–EAD-active compounds (Fig. 5b; Table 1). The ratios of these 24 components do not differ (Fig. 4b). Therefore, we summarized it to a 'cultivar-independent' blend of VOCs (Fig. 4c).
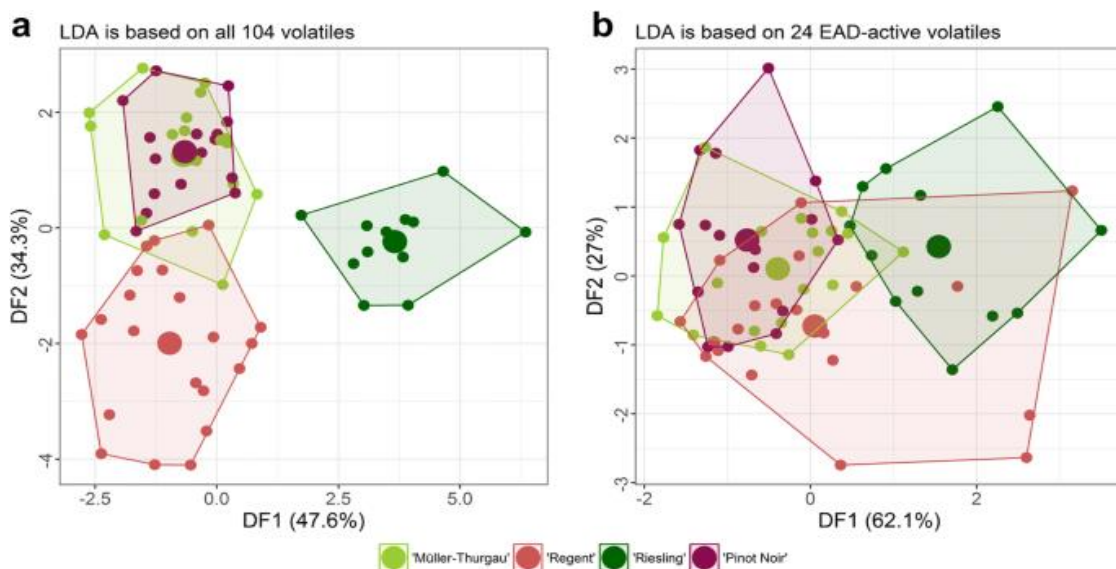
Fig. 5: Linear discriminant plots at BBCH 73–75 comparing the odor bouquets of four cultivars ('Müller-Thurgau', 'Regent', 'Riesling', and 'Pinot Noir', $N = 68$) based on all detected 104 volatiles (**a**) or based on only the 24 GC–EAD-active VOCs (**b**). Small dots represent an odor bouquet and lager group centroids

Fig. 4: **a** Representative TD–GC–EAD recording of electroantennograms of the moth species *L. botrana* (LB) and *E. ambiguella* (EA) including a corresponding chromatogram from berry headspace ('Regent'; BBCH 73–75) (FID). Numbers highlight the 24 detected chemical compounds. The identity and mean ratios are listed in the table. **b** Box-whisker plots with jittered raw values as closed circles of the peak area obtained by GC–MS of the 24 EAD-active substances for each variety at BBCH 73–75. Lines represent median, crosses mean, boxes the IQR and whiskers 1.5 * IQR. **c** Average volatile profile of the 24 EAD-active substances

3.5. Cultivar preferences based on olfactory cues in Y-shaped olfactometer

Crosswise comparison of the berries' scent of four grapevine cultivars at BBCH 73–75 in Y-shaped olfactometer bioassays revealed no preferences for any cultivar of fertilized females of *E. ambiguella* (Fig. 6, *binominal test*, $p > 0.05$). In contrast, fertilized *L. botrana* females preferred statistically significant the scent of 'Regent' berries over 'Riesling' and 'Pinot Noir' berries (Fig. 6, *binominal test*, $p < 0.05$). For the other cultivars, *L. botrana* females showed no preference. The response rate was significantly different between *E. ambiguella* and *L. botrana*. *Eupoecilia ambiguella* showed a higher response rate to walk in the olfactometer setting ($63 \pm 18\%$), than *L. botrana* females ($45 \pm 15\%$, *Student's t test*, $t = 1.898$, $df = 10$, $p < 0.05$).
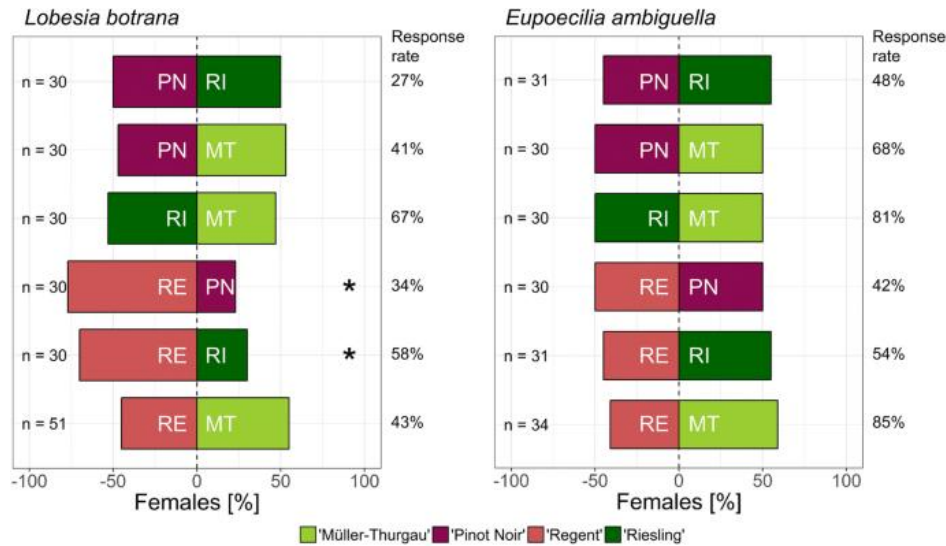
Fig. 6: Response of female *L. botrana* and *E. ambiguella* in Y-shaped olfactometer experiments when presented berry odor blends of different cultivars at BBCH 73–75. Statistically different results are indicated by asterisks (*binomial test*, $p < 0.05$)

4. Discussion

Insects locate and select their host plants largely based on chemosensory cues (Schoonhoven et al. 2005) in synergy with tactile and visual cues. Most moths pass a catenary process leading to oviposition (Knolhoff and Heckel 2014; Renwick and Chew 1994; Schoonhoven et al. 2005), which could be differentiated in pre- and postalighting behavior. Olfactory and visual cues mediate long-range attraction during prealighting behavior. After landing, contact perception of both physical and chemical cues of the plant surface become prevalent for the moths to determine the suitability of the oviposition site (Knolhoff and Heckel 2014). The prealighting behavior of *L. botrana* to find its host plant was mainly triggered by olfactory cues (Tasin et al. 2006). Volatiles, contact and visual cues guide gravid *L. botrana* females to final oviposition sites (Anfora et al. 2009; Markheiser et al. 2018; Tasin et al. 2008, 2011). During searching behavior, the importance of different types of stimulus may change with distance to the plant.

The comparison of the complete odor bouquet of 'Müller-Thurgau', 'Regent' and 'Pinot Noir' during the whole growing season revealed clear differences between the phenological stages and few between the cultivars. According to this data, it is clear that the odor bouquet of vineyard samples can be attributed to the presence of the five typical grapevine leaf volatiles (Lima et al. 2017): nonanal and ethyl butanoate (which exhibited higher intensities during leaf development and inflorescence emergence), β-caryophyllene and (*Z*)-3-hexenyl acetate (which both increase during the growing season) and decanal. Indeed, these volatiles are not restricted to leaves. Tasin et al. (2005) found nonanal in leaves, flowers and berries, β-caryophyllene in

berries, and (*Z*)-3-hexenyl acetate in flowers of grapevine. However, nonanal, (*Z*)-3-hexenyl acetate and β-caryophyllene are not only common volatiles of *V. vinifera,* they are also known to be emitted by, e.g., *Viburnum lantana* or *Olea europaea*, which are alternative host plants of *E. ambiguella* (Schmidt-Büsser et al. 2011). Furthermore, attraction of *L. botrana* was already proven for blends containing β-caryophyllene and (*Z*)-3-hexenyl acetate (Tasin et al. 2007; von Arx et al. 2011) in wind tunnel experiments. In studies with different blends, approximately only half of the number of tested females showed a source contact in wind tunnel experiments (Tasin et al. 2007), leading to the conclusion that other volatiles (resp. ratios of the volatiles) elicit landing/oviposition behavior. Anfora et al. (2009) tested a blend of six components with another composition and revealed an oviposition stimulation of *L. botrana*. Due to the dominance of leaf volatiles in the headspace around the 'vineyard' and the similarity of leaf volatiles between cultivars and other host plants, we suggest that long-range orientation of grapevine moths could be based mainly on leaf volatiles, while the alighting on the plant seems to be triggered by another blend of volatiles together with contact cues, presumably acting synergistically.

The headspace volatiles of just the grape berry clusters differed between the cultivars. We identified in part the same substances as earlier published for other cultivars (Anfora et al. 2009; Schreier et al. 1976; Tasin et al. 2005). These methodologies, which have in common that they injured plant tissue, enabled the excessive emission of GLVs (Fall et al. 1999). In contrast, by using a non-invasive and very sensitive method based on headspace sampling of intact plant tissue followed by thermodesorption in the presented study, we detected five major leaf volatiles emitted by *V. vinifera*, but only (*Z*)-3-hexenyl acetate is a typical C6-GLV (Dudareva et al. 2006; Fall et al. 1999).

VOCs emitted by investigated cultivars of *V. vinifera* berries differed in their odor bouquet, but according to the mathematical calculations of the GC–EAD-active volatiles, these differences may not be detectable by *E. ambiguella* and *L. botrana* females. Further, ovipositional preference for one of the four cultivars was only recognized when visual, olfactory, and contact cues together guided *L. botrana* or *E. ambiguella* to oviposition sites (Rid et al. 2018). Therefore, we suggest that an oviposition discrimination of cultivars is not based on olfactory stimuli alone, while a preference of different host plant species is encoded by volatiles (Maher and Thiéry 2006; Tasin et al. 2010). The diverse susceptibility of grapevine cultivars to *L. botrana* infestation (Birgücü et al. 2015; Fermaud 1998; Pavan et al. 2018; Sharon et al. 2009; Snjezana 2004; Stellwaag 1928; Thiéry et al. 2014) is either based on differences in, e.g., larval colonization and development (Fermaud 1998) than on olfactory cues. This is in consistency

with findings of previous studies, which evaluate oviposition preferences for certain cultivars (Maher et al. 2001). For example, *L. botrana* exhibited an ovipositional preference for 'Cabernet Sauvignon' and 'Chasselas' over 'Merlot' (Maher et al. 2001). But no ovipositional preference of *L. botrana* was found by testing two different susceptible cultivars ('Trebbiano' and 'Sangiovese') based just on olfactory and visual cues (Tasin et al. 2008). Interestingly, we found no qualitative differences between the EAD-profiles of both moth species to volatiles emitted by grapevine as has been demonstrated in other studies investigating reaction to common host plant odors of generalist herbivores (Pers 1981; Ramachandran et al. 1990).

Some VOCs (e.g., farnesene) that were also found in the headspace of *V. vinifera* berries in the presented study, were found to be detectable by *L. botrana* (Tasin et al. 2005) and *E. ambiguella* (Anfora et al. 2008), but did not contribute to the identified 24-components blend (Fig. 4c). This could be due to very low amounts in the headspace eliciting no signal in TD–GC–EAD measurements.

The combination of mathematical predictions and electrophysiological data may explain host plant discrimination abilities (Conchou et al. 2017) or disabilities. Such results must always be verified with behavioral experiments. To verify the data, it was important to study discrimination in dual-choice experiments presenting both choices simultaneously. Olfactory perception for *L. botrana* is located mainly on the antenna (Maher and Thiéry 2004) and antennal discriminatory ability between the cultivars had to be determined. As we focused on analyzing short-range volatile cues, we favored Y-shaped olfactometer trials over wind tunnel experiments. While an olfactometer excludes signals other than volatile (visual, contact), wind tunnel studies should include at least visual cues, which are important for landing (Masante-Roca et al. 2006), because landing is mandatory for interpreting of dual-choice experiments. We were aware that Y-olfactometer experiments have also limitations. The setting is artificial, and usually not suitable for studying flying insects, but appropriate for testing their short-range behavior, which is often expressed by walking after landing. The applicability of this method is reflected by the moths' response rates, which have been in average 63% for *E. ambiguella* and 46% for *L. botrana* (Fig. 6), very similar to response rates in wind tunnel experiments (Masante-Roca et al. 2006). While the results of the behavioral study (no preferences) at BBCH 73-75 may reflect the results of the electrophysiological and analytical measurements in *E. ambiguella*, females of *L. botrana* preferred 'Regent' over 'Riesling' and 'Pinot Noir'. Thus, we may not have considered all minor compounds of 'Regent' that could be actually perceived by *L. botrana* by reducing the detectable odor bouquet to just 24 ones. From an evolutionary point of view*, E. ambiguella* and *L. botrana* are polyphagous insects, with females being able

to oviposit and develop on host plants other than grapevine (Maher and Thiéry 2006). There is no selection pressure, since the fitness of females raised on alternative host plants is even higher than of those developed on *V. vinifera* (Thiéry and Moreau 2005). Therefore, a specialization of the olfactory system toward one cultivar seems not to be necessary.

The aim of the study was to compose an attractive blend based on preferred cultivars, which could be used to enhance the attractiveness of the new monitoring tool 'M-Ovicard' for grapevine moths. We were able to identify 24 VOCs, which may be responsible for the attractiveness of grapevines in general. They may be responsible for short-range attraction and suggested to be tested in future oviposition experiments with both moth species. Anfora et al. (2009) reported that a blend of six components [limonene:(*E*)-DMNT:linalool:(*E*)-caryophyllene:(*E,E*)-α-farnesene:methyl salicylate; 10:1:1:1:1:1] elicited in low doses an oviposition on an artificial substrate for *L. botrana*. Our 24-blend contains the same substances except (*E,E*)-α-farnesene and methyl salicylate, while two aldehydes, nonanal and decanal, are the prevailing substances.

For the development of the egg-monitoring tool 'M-Ovicard' we focus on signals that stimulate moths' oviposition. We abstain from incorporating leaf volatiles for long-range attraction in the oviposition card, because this will be done by the emissions of the vineyard itself. But to compete with the berry for oviposition, we intend to incorporate short-range attractants as identified in this study and combine them with nonvolatile components identified recently (Rid et al. 2018, Markheiser et al. 2018). For practical purposes, less components of a mixture are easier to formulate, due to different chemical properties (e.g., volatilities) (Pickett and Khan 2016), and will reduce the price for the lure. Thus, a selection of components with high attractiveness for gravid moths will be in the focus of future studies.

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Supplementary Material
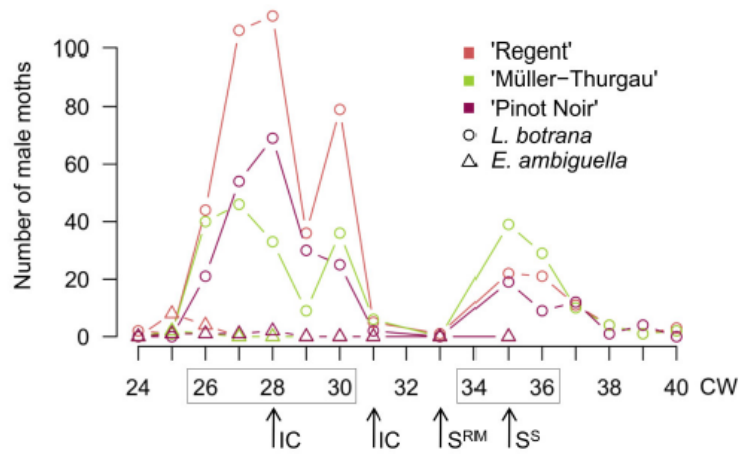
Figure S1 Flight curves of the second and third generations of *L. botrana* and *E. ambiguella* males in a vineyard near Heidelberg while conducting field collections of *V. vinifera* headspace. There was one pheromone trap (BIO-Pherotrap, Temmen GmbH, Hattersheim, Germany) per variety and moth species. Arrows indicate insecticide application according to Table S1 (IC = Indoxacarb; S = Spinosad; [RM] = only on 'Regent' and 'Müller-Thurgau'; [S] only on 'Pinot Noir'). Grey marking = flight activity of males

Table S1 BBCH-stages during the vegetation period 2014 (calendar week = CW) while conducting field collections of *Vitis vinifera* headspace in German vineyards. The applied pesticides are indicated (IC = Indoxacarb; S = Spinosad). Grey marking = flight activity of males of *L. botrana* and *E. ambiguella*

V. vinifera	CW	24	25	26	27	28	29	30	31	32	33	34	35
'Regent'	BBCH-stage		71		73-75		75		81				89
	Insecticide					IC			IC		S		
'Pinot Noir'	BBCH-stage		71		73-75		75		79		83		85
	Insecticide					IC			IC				S
'Müller-Thurgau'	BBCH-stage		71		73-75		75			79		85	89
	Insecticide					IC			IC		S		

Table S2: Means ± standard deviation of the relative proportions of all volatile compounds detected in headspace samples of whole grapevines. Three varieties, 'Müller–Thurgau' (MT), 'Regent' (R), and 'Pinot Noir' (PN) during the growing season (BBCH 13–18 until BBCH 83–89) are shown. For visualisation a heatmap from low contributing components (green) over middle (blue) to high (orange) contributing compounds is added.

Compounds

Compound																		
2–Hexanone																		
Hexanal	4.415±5.96	7.815±2.32	11.18±4.41	2.446±4.63	2.376±2.92	4.578±5.1	1.949±3.56	1.205±2.25	0.718±0.99	0.446±0.28	1.155±1.26	1.312±1.29	0.501±0.59	0.612±0.56	1.238±0.89	0.346±0.4	0.58±0.64	0.99±0.9
Ethyl butanoate	21.66±15.5	3.117±2.06	10.34±6.39	25.80±15	12.376±7.29	9.552±5.35	6.521±4.77	4.07±2.92	4.87±5.24	5.002±3.79	1.44±0.97	0.686±0.95	1.945±2.14	2.485±2.02	1.116±1.41	0.5±0.58		0.974±1.81
Butyl acetate	0.11±0.14	0.252±0.08	0.194±0.06	0.136±0.03	0.255±0.37	0.322±0.1	0.077±0.09	0.062±0.06	0.09±0.06	0.062±0.04	0.091±0.1	0.092±0.07	0.084±0.06	0.127±0.08	0.118±0.08	0.07±0.1		0.176±0.32
Known unknown 850	0.292±0.35		0.052±0.13	0.356±0.48	0.826±1.02	0.1±0.1	0.916±0.79	0.522±0.51	1.122±1.77	2.331±2.93	0.103±0.14	0.098±0.06	0.899±1.02	0.153±0.27	0.034±0.07			0.067±0.15
Trans–2–Hexen–1–al	4.015±2.17	3.909±2.32	5.346±0.69	1.243±0.82	0.92±0.56	2.852±0.32	0.589±0.52	1.357±1.34	0.839±0.86	0.956±0.34	0.442±0.41	0.671±0.86	0.171±0.12	0.536±0.14	0.873±0.57	0.196±0.17	0.619±0.75	3.398±5.46
(Z)–3–Hexen–1–ol	0.071±0.2			1.297±0.99	1.369±1.97		0.889±1.27	1.77±1.42	1.332±2.32		0.334±0.52	0.632±0.92			1.115±1.08			
Known unknown 861	1.65±0.81	3.968±1.12	2.446±1.09	1.109±1.15	0.821±0.82	1.585±1.17	0.275±0.4	0.377±0.87	0.103±0.22	0.883±1.41	2.293±3.05	0.984±1.27	0.275±0.18	0.198±0.13	0.664±1.04	0.72±0.14		0.057±0.11
1–Hexanol	1.964±2.15	1.756±0.92	0.952±0.13	2.492±1.45	1.685±2.07	0.666±0.24	0.116±0.26	0.439±0.27	0.124±0.15									
2–Heptanone				0.004±0.01			0.075±0.1	0.092±0.12	0.05±0.05	0.019±0.04								
1–Nonene	0.214±0.15	0.347±0.23	0.331±0.1	0.234±0.16	0.129±0.14	0.289±0.12	0.13±0.14	0.358±0.75	0.67.5±0.07	0.175±0.14	0.186±0.08	0.24±0.12	0.184±0.09	0.145±0.07	0.201±0.08	0.329±0.35	0.006±0.01	0.067±0.12
Nonane	0.645±0.38	1.596±0.53	0.662±0.21	0.45±0.46	0.326±0.18	0.55±0.14	0.141±0.22	0.13±0.22	0.094±0.06	0.336±0.19	0.535±0.53	0.363±0.32	0.446±0.51	0.254±0.05	0.448±0.23	0.287±0.32	0.051±0.07	0.115±0.21
Heptanal	2.561±0.89	2.629±0.44	2.371±0.37	1.464±0.53	0.436±0.66	2.708±2.07	0.845±0.81	0.325±0.34	0.587±0.57	0.707±0.5	0.483±0.49	0.772±0.44	0.315±0.13	0.2±0.13	0.271±0.16	0.294±0.34	0.179±0.14	0.148±0.17
(E,E)–2,4–Hexadienal	0.115±0.14	0.044±0.07	0.071±0.06	0.139±0.1	0.07±0.09	0.091±0.12	0.025±0.06	0.03±0.06			0.024±0.05	0.096±0.18						0.005±0.01
Known unknown 911	0.013±0.02	0.017±0.03	0.138±0.07	0.06±0.02	0.028±0.02	0.136±0.02	0.459±0.33	0.232±0.13	0.301±0.12	0.271±0.14	0.122±0.08	0.194±0.14	0.246±0.26	0.129±0.07	0.394±0.32	0.083±0.1		0.063±0.1
Cumene		0.012±0.02			0.008±0.02		0.015±0.02	0.012±0.02		0.027±0.03	0.018±0.02		0.039±0.04					
alpha–Pinene	0.071±0.06			0.172±0.15	0.123±0.11		0.021±0.03	0.042±0.04	0.265±0.4	0.002±0	0.046±0.03	0.742±0.85	0.008±0.01	0.038±0.03	1.008±0.94	0.001±0		0.041±0.07
1–Butoxy–2–propanol																		
Ethyl tiglate	0.008±0.02			0.039±0.03	0.103±0.18		0.154±0.3	0.064±0.04	0.015±0.03	0.108±0.11	0.056±0.08		0.042±0.08					
2–Heptenal	0.268±0.27	0.248±0.12	0.374±0.08	0.251±0.14	0.112±0.06	0.322±0.1	0.186±0.37	0.246±0.25	0.067±0.04	0.124±0.05	0.132±0.13	0.141±0.11		0.046±0.04	0.098±0.05	0.003±0.01	0.019±0.04	0.008±0.02
Benzaldehyde	1.761±1.17	4.275±2.17	1.285±0.57	1.182±1.32	1.149±0.65	1.222±0.82	0.214±0.27	0.315±0.49	0.049±0.04	0.369±0.26	0.589±0.61	0.405±0.31	0.087±0.11	0.14±0.06	0.376±0.6	0.09±0.14	0.07±0.12	0.167±0.25
Known unknown 970		0.031±0.06			0.26±0.39	1.146±0.69	0.009±0.02	0.26±0.25	2.833±4.33		0.162±0.18	1.525±3.01		0.145±0.29				
1–Heptanol																		
Known unknown 978																		
beta–Pinene	0.006±0.01	0.105±0.11	0.105±0.13	0.245±0.42	0.671±0.57		0.641±0.53	0.467±0.69	1.57±1.25	0.788±1.16	0.906±1.19	2.715±2.9	0.124±0.19	0.19±0.14	3.233±3.12	0.269±0.31		0.154±0.18
6–Methyl–5–hepten–2–one	0.386±0.75	0.644±0.88	0.836±0.71	2.06±1.77	2.522±1.49	3.784±2.53	1.805±1.94	1.814±1.11	2.006±1.3	4.998±2.69	4.534±3.36	6.687±5.03	7.66±6.24	5.064±1.69	3.809±2.79	5.845±5.66	4.761±3.3	6.575±7.04
Known unknown 990		0.029±0.04		0.141±0.28	0.222±0.31	0.234±0.11	0.06±0.13	0.055±0.14			0.691±1.39							
Pseudocumene	0.091±0.11	0.465±0.14	0.059±0.05		0.401±0.43						0.028±0.06		0.038±0.03	0.018±0.02	0.011±0.02			0.003±0.01
2–Octanone																		
Known unknown 999	5.356±6.04	0.498±0.23	2.709±2.72	2.283±2.4	2.51±1.98	1.07±1.48	0.402±0.33	0.335±0.5	0.907±1.2	0.126±0.11	0.011±0.02	0.676±0.22		0.019±0.03				0.016±0.03
Decane																		
Octanal	3.292±1.86	5.013±0.97	4.115±1.52	1.579±1.65	1.091±0.94	3.102±1.04	0.067±0.15	0.409±0.68	0.064±0.09	1.32±1.73	1.325±1.68	0.994±1.11	0.585±0.54	0.271±0.19	0.488±0.59	0.83±1.13	1.273±0.75	0.09±0.1
(Z)–3–Hexenyl acetate	4.996±4.06	2.966±2.42	4.276±4.3	12.37±6.34	12.23±10.5	9.395±3.73	27.26±8.14	11.76±6.14	29.92±9	10.81±6.68	10±7.71	16.4±8.97	17.21±15.2	32.25±11	41.24±13.9	14.7±15.5	3.791±2.26	48.58±23.3
Hexyl acetate	0.795±0.24	0.784±0.5	1.23±0.81	0.832±0.16	0.812±0.53	1.436±1.24	1.053±1.14	1.282±0.66	1.469±1.06	1.085±0.43	0.76±0.6	1.617±0.61	1.971±1.65	1.07±0.38	2.643±1.38	1.995±1.09	0.264±0.3	3.19±2.5
m–Cymene	0.058±0.06	0.058±0.01	0.122±0.12	0.28±0.26	0.424±0.19	0.612±0.35	0.155±0.11	0.305±0.17	2.081±2.47	0.264±0.13	0.59±0.42	6.786±2.79	0.432±0.52	0.332±0.19	3.104±1.95	0.147±0.19		0.995±0.46
(–)–Limonene	0.049±0.04	0.098±0.05	0.087±0.05	0.618±0.5	1.114±0.41	0.845±0.42	0.933±1.09	1.038±0.69	2.217±2.27	1.671±0.9	2.507±1.64	10.51±9.32	0.656±0.35	0.922±0.26	4.716±3.79	0.411±0.36	0.04±0.05	0.568±0.71
Benzylalcohol																		
(E)–beta–Ocimene	0.028±0.05	0.004±0.01	0.05±0.06	0.336±0.25	0.37±0.22	0.392±0.24	3.086±3.69	1.543±1.91	0.291±0.13	4.834±5.83	3.8±4.1	0.667±0.3	1.113±1.67	0.302±0.24	0.264±0.06	0.16±0.19		0.079±0.12
Benzenacetaldehyde	0.186±0.13	0.338±0.16	0.159±0.06	0.185±0.2	0.114±0.12	0.108±0.08	0.045±0.07	0.051±0.1	0.019±0.01	0.018±0.04	0.063±0.09	0.031±0.05						0.001±0
gamma–Terpinene				0.037±0.04	0.064±0.08	0.029±0.05	0.066±0.08	0.789±1.13	0.097±0.12	0.132±0.12	1.342±1.69	0.02±0.01	0.103±0.07	2.145±2.21	0.008±0.02		0.147±0.17	
(E)–2–Octenal	0.134±0.1	0.085±0.11	0.137±0.04	0.134±0.07	0.069±0.09	0.186±0.05	0.17±0.3	0.106±0.12	0.012±0.03	0.086±0.13	0.073±0.11	0.066±0.13						0.01±0.02
Acetophenone	0.984±0.68	2.234±1.45	0.636±0.42	0.41±0.76	0.431±0.49	0.446±0.47	0.036±0.04	0.056±0.13	0.005±0.01	0.024±0.04	0.177±0.3	0.049±0.08		0.252±0.5				0.042±0.07
Known unknown 1066	0.626±0.27	0.95±0.32	1.038±0.25	0.48±0.34	0.355±0.18	0.827±0.14	0.149±0.12	0.127±0.19	0.12±0.11	0.61±0.33	0.619±0.32	0.486±0.25	0.266±0.04	0.255±0.08	0.325±0.1	0.143±0.2	0.143±0.2	0.136±0.15
1–Octanol	0.429±0.61	0.307±0.27	0.352±0.23	0.159±0.32	0.168±0.27	0.21±0.24		0.06±0.14	0.042±0.06		0.008±0.02							
Known unknown 1072																		
Terpinolen								0.145±0.2	0.047±0.09	0.362±0.49	0.074±0.06	0.07±0.04	0.524±0.57	0.027±0.04			0.038±0.04	
Linalool oxide (furanoid)	0.027±0.06		0.094±0.11	0.74±1.66	0.009±0.03	0.435±0.03	0.165±0.18	0.151±0.15	0.302±0.34	0.641±0.5	0.215±0.2	0.098±0.21		0.317±0.29			0.019±0.05	
DMNT (peak 1)				0.564±0.7	0.189±0.38	0.325±0.27	0.689±0.5	0.951±0.51	0.174±0.24	0.312±0.3	0.331±0.12	0.076±0.03	0.256±0.13	0.117±0.02	0.019±0.03			
Known unknown 1090																		
1–Undecene	0.062±0.15		0.002±0.01		0.007±0.01	0.195±0.3	0.066±0.11	0.01±0.03	0.049±0.04	0.076±0.09	0.046±0.07	0.127±0.07	0.094±0.03	0.061±0.05	0.168±0.05	0.078±0.09		0.032±0.04
Methyl benzoate	0.014±0.04	0.227±0.09	0.009±0.03	0.467±0.29	0.301±0.35	0.369±0.37												
Linalool	0.27±0.76		0.293±0.03	4.594±4.89	1.609±0.95	1.606±1.89	0.42±0.34	0.761±0.54	0.781±0.85	0.166±0.41	0.062±0.09	0.676±0.12	0.193±0.16					
Undecane	0.258±0.17	0.394±0.12	0.192±0.13	0.111±0.22	0.258±0.15	0.072±0.14	0.291±0.22	0.03±0.03	0.076±0.03	0.26±0.2	0.256±0.1	0.373±0.15	0.266±0.1	0.685±0.2	0.126±0.14		0.084±0.12	
Nonanal	12.64±4.19	16.11±1.83	13.09±2.83	6.30±3.98	6.691±2.68	11.65±2.05	2.446±2.74	2.59±2.25	1.703±1.25	4.90±4.9	5.677±4.43	6.035±2.53	3.045±1.05	3.005±0.9	4.762±2.41	2.727±3.06	5.119±2.98	5.301±4.28
DMNT (peak 2)	0.016±0.04	0.018±0.05	0.194±0.16	0.925±0.82	2.351±1.66	1.995±0.87	0.93±0.83	2.536±2.25	2.686±2.2	0.696±0.83	0.987±0.44	1.404±0.87	0.132±0.09	1.324±0.81	0.396±0.22	0.092±0.14		0.24±0.45
Known unknown 1121																		

Compound																		
(E,Z)–2,6–dimethyl–2,4,6–Octatriene	0.004±0.01	0.002±0.01	0.022±0.03	0.228±0.25	0.246±0.23	0.199±0.1	1.227±12.36	3.494±6.11	0.191±0.11	6.441±7.56	6.764±13.33	0.118±0.08	1.508±2.77	0.322±0.24	0.07±0.01	0.101±0.15		0.008±0.01
Known unknown 1134	0.042±0.04	0.069±0.02	0.053±0.02	0.076±0.05	0.086±0.05	0.084±0.02	0.045±0.06	0.583±1.36	0.01±0.02	0.037±0.03	0.06±0.07	0.088±0.06	0.028±0.07	0.098±0.04	0.033±0.07	0.017±0.03		0.026±0.04
Camphor	0.07±0.13		0.029±0.05		0.037±0.04		0.004±0.01			0.972±1.61		0.005±0.01	0.025±0.03	0.026±0	0.004±0			
Known unknown 1160a																		
Known unknown 1160b		0.054±0.06	0.017±0.03		0.09±0.23	0.096±0.1	0.105±0.1	0.067±0.19	0.043±0.07	0.039±0.06	0.04±0.06	0.021±0.04	0.006±0.01	0.143±0.18	0.842±1.6			
2–Nonenal	0.246±0.39	0.181±0.06	0.157±0.05	0.096±0.03	0.027±0.05	0.113±0.05	0.074±0.11	0.099±0.12	0.045±0.01	0.069±0.1	0.048±0.07	0.108±0.06	0.096±0.15		0.037±0.04			
Known unknown 1167	1.028±0.5	1.675±0.63	1.879±0.5	0.755±1.05	0.367±0.50	1.801±0.36	0.505±0.49	0.709±0.98	0.431±0.47	1.171±1.46	1.6±1.32	1.856±0.87	0.608±0.2	0.67.5±0.2	0.812±0.48	0.332±0.41	0.877±0.46	0.31±0.33
Ethyl benzoate	3.186±2.36	4.46±1.23	1.301±0.98	5.022±4.07	11.62±6.25	1.566±1.87	5.46±9.85	2.168±1.61	0.837±0.98	0.562±0.31	0.36±0.3	0.129±0.26	0.098±0.06	0.849±1.03	0.034±0.05	0.016±0.05		0.109±0.18
(Z)–3–Hexenyl butyrate	1.239±1.19	0.13±0.21	0.265±0.25	1.154±0.67	1.044±0.36	0.401±0.22	4.983±5.29	1.79±1.36	0.768±0.67	1.039±0.96	0.701±0.87	0.319±0.29	3.484±4.33	1.799±0.99	0.464±0.32	0.458±0.62		0.414±0.61
Hexyl butyrate	1.584±0.99	0.59±0.63	0.332±0.3	1.489±0.4	0.705±0.99	0.263±0.09	0.161±0.15	0.096±0.04	0.12±0.02	0.01±0.02		0.024±0.05	0.02±0.02	0.07±0.08	0.006±0.02			0.005±0.01
Methyl salicylate	0.783±0.68	2.531±1.25	5.413±1.44	1.817±0.89	5.93±4.33	3.872±0.97	0.501±0.32	1.038±0.58	0.915±1.03	0.084±0.06	0.147±0.17	0.129±0.08	0.014±0.03	0.033±0.03	0.063±0.1	0.006±0.02		0.008±0.02
n–Dodecane	0.119±0.09	0.207±0.06	0.057±0.06	0.109±0.07	0.125±0.11	0.153±0.02	0.753±0.59	0.046±0.06	1.97.5±2.03	0.086±0.06	0.161±0.08	0.134±0.04	0.359±0.15	0.065±0.02	0.256±0.12	0.066±0.07		0.037±0.06
Decanal	15.75±5.9	22.5±4.35	19.56±4.61	9.86±6.68	7.609±3.72	18.40±3.21	4.101±2.91	3.069±2.84	3.02±2.27	8.102±8.35	11.98±10.25	12.54±6.54	5.084±1.32	5.209±1.36	6.521±3.9	9.959±9.23	24.46±22.5	10.12±11.3
Known unknown 1210																		
Benzothiazole																		
(Z)–3–Hexenyl–methyl butyrate	0.054±0.08		0.024±0.04	0.065±0.05	0.068±0.05	0.02±0.01	0.727±0.27	0.393±0.33	0.113±0.12	0.142±0.15	0.065±0.06	0.088±0.09	0.18±0.15	0.02±0.01	0.002±0.01		0.014±0.02	
(E)–2–Undecen–1–ol	0.153±0.13	0.467±0.81	0.241±0.08		0.057±0.11	0.276±0.07		0.045±0.06	0.061±0.06	0.107±0.12	0.117±0.1	0.124±0.06		0.031±0.04	0.052±0.04			
Ethyl salicylate	0.153±0.14	0.406±0.3	0.178±0.14	0.051±0.06	0.656±0.46		0.047±0.06	0.186±0.15		0.043±0.04		0.008±0.02						
Known unknown 1271	1.006±0.54	1.439±0.38	1.529±0.71	0.58±0.35	0.523±0.26	1.304±0.23	0.306±0.24	0.213±0.21	0.213±0.17	0.705±0.73	0.836±0.61	0.884±0.45	0.292±0.1	0.258±0.09	0.395±0.19	0.218±0.36	0.195±0.23	0.124±0.12
Bornyl acetate																		
Nonanoic acid	0.517±0.59	1.065±0.72	0.576±0.57		0.13±0.26	0.155±0.31				0.126±0.29	0.101±0.29	0.01±0.03		0.048±0.1				
1–Tridecene	0.013±0.02	0.026±0.02	0.038±0.09	0.099±0.04	0.023±0.03	0.084±0.03	0.088±0.04	0.085±0.05	0.145±0.08	0.186±0.1	0.12±0.12	0.218±0.12	0.144±0.1	0.073±0.03	0.289±0.13	0.196±0.25		0.035±0.06
Tridecane	0.212±0.07	0.39±0.1	0.166±0.05	0.398±0.26	0.084±0.09	0.264±0.04	0.34±0.25	0.31±0.12	0.353±0.1	0.291±0.18	0.33±0.12	0.409±0.27	0.23±0.1	0.145±0.06	0.406±0.14	0.181±0.23		0.074±0.13
Known unknown 1306				0.06±0.09	0.39±0.39	0.006±0.01	0.065±0.05	0.308±0.43	0.01±0.01	0.151±0.13	0.234±0.16	0.026±0.02	0.014±0.02	0.025±0.02	0.013±0.01	0.005±0.02		
Undecanal	1.098±0.34	1.393±0.33	1.121±0.46	0.522±0.35	0.55±0.4	1.196±0.27	0.399±0.3	0.42±0.03	1.281±0.22	0.355±0.4	0.4±0.47	0.772±0.42	0.289±0.17	0.131±0.2	0.238±0.29	0.086±0.1		
Known unknown 1345							0.044±0.08	0.02±0.03	0.05±0.06	0.55±0.58	0.231±0.14	0.388±0.3	0.39±0.53	0.081±0.02		0.115±0.18		0.042±0.05
Known unknown 1346	0.01±0.03		0.049±0.06	0.046±0.09	0.122±0.12	0.086±0.05	0.068±0.04	0.138±0.05	0.201±0.13	0.52±0.43	0.262±0.08	0.184±0.22	0.215±0.15	0.02±0.02	0.068±0.04	0.015±0.04		0.01±0.02
Known unknown 1349a				0.008±0.02			0.07±0.04	0.046±0.06		0.36±0.29	0.296±0.2	0.213±0.24	0.68±0.1	0.524±0.16	0.434±0.35	1.1±1.09	0.344±0.31	0.284±0.46
Known unknown 1349b		0.03±0.06		0.034±0.05	0.026±0.06							0.463±0.26						
2–Undecenal	0.255±0.11	0.304±0.24	0.245±0.13	0.032±0.04	0.076±0.09	0.066±0.08	0.067±0.07	0.058±0.07	0.011±0.02	0.058±0.07	0.072±0.09	0.029±0.04		0.016±0.03				0.003±0.01
Known unknown 1364				0.003±0.01	0.179±0.3	0.053±0.03	0.165±0.07	0.372±0.18	0.289±0.14	0.59±0.33	0.955±0.67	0.467±0.36	0.329±0.12	0.101±0.01	0.345±0.12	0.159±0.1	0.262±0.35	0.05±0.06	2.059±2.39	0.124±0.17
Known unknown 1374	0.082±0.09		0.162±0.13	0.24±0.26	0.425±0.2	0.498±0.3	0.165±0.07	0.372±0.18	0.289±0.14	0.59±0.33	0.955±0.67	0.467±0.36	0.329±0.12	0.101±0.01	0.345±0.12	0.159±0.1	1.965±0.74	0.105±0.12
Known unknown 1381	0.024±0.10	0.016±0.05	0.057±0.05	0.802±0.94	0.947±0.65	0.841±0.47	0.418±0.16	0.727±0.31	0.324±0.22	0.464±0.35	1.004±0.35	0.334±0.19		0.609±0.32	0.148±0.07	0.12±0.18	2.264±0.35	0.173±0.07

Area

Table S3: Means ± standard deviation of the relative proportions of all volatile compounds detected in headspace samples of grapevine berries. Four varieties, 'Müller–Thurgau' (MT), 'Regent' (R), 'Riesling' (Rie) and 'Pinot Noir' (PN), during the growing season (BBCH 65–71 until BBCH 83–89) are shown. For visualisation a heatmap from low contributing components (green) over middle (blue) to high (orange) contributing compounds is added.

Compound																
2–Hexanone	1.288±0.54	1.509±0.75	1.409±0.93	1.743±0.99	1.946±0.8	1.369±0.85	0.93±1.21	1.652±0.62	2.785±3.08	1.406±0.81	1.2±1.24	1.316±0.9	4.737±7.08	1.712±0.94	1.129±2.91	1.613±0.7
Hexanal	4.443±0.75	3.825±2.15	7.615±2.2	3.63±1.83	5.706±2.03	5.789±3.37	6.117±3.93	6.55±3.87	5.491±2.63	5.554±2.41	6.454±3.92	5.101±2.04	6.168±2.68	5.622±2.39	7.265±5.19	6.108±2.63
Ethyl butanoate	0.221±0.37	0.38±0.65	0.048±0.08	0.159±0.26	0.071±0.19	0.004±0.02			0.097±0.41	0.248±0.67	0.281±0.95	0.434±1.31	0.666±1.99	0.106±0.21	0.082±0.25	0.014±0.05
Butyl acetate	0.552±0.35	0.537±0.31	0.306±0.17	0.572±0.59	1.773±1.99	0.698±0.47	1.301±1.96	1.252±1	0.958±0.75	1.074±0.86	0.972±1.94	1.147±0.93	1.474±1.88	0.465±0.23	1.077±1.34	0.707±0.39
Known unknown 850																
Trans–2–Hexen–1–al	2.59±2.26	2.929±3.51	3.804±2.77	3.452±4.55	1.877±2	1.906±2.97	1.109±0.74	2.315±3.57	0.828±0.61	0.593±0.47	1.567±1.26	0.924±0.96	2.745±3.3	0.46±0.64	5.844±8.65	1.605±1.66
(Z)–3–Hexen–1–ol	3.366±4.25	0.168±0.33	0.389±0.27	0.265±0.44	0.281±0.82	0.761±2.84	0.379±0.61	0.462±1.27	0.029±0.1	0.207±0.46	0.913±1.72	0.359±0.71	0.073±0.2	0.148±0.58	0.76±1.18	0.07±0.15
Known unknown 861	1.031±0.71	0.537±0.35	1.183±0.95	0.713±0.44	0.74±0.8	1.03±1.04	1.011±1.08	1.032±0.97	1.247±0.93	0.73±0.46	1.079±1.09	0.884±0.63	1.419±0.87	1.645±1.24	0.815±0.98	2.061±1.26
1–Hexanol	3.878±2.98	1.572±1.14	2.043±1.01	1.585±0.75	0.996±1.13	1.969±2.62	2.354±1.81	0.896±1.08	1.039±1.32	1.476±1.18	1.734±1.26	1.302±1.5	1.813±1.16	0.872±0.87	2.86±3.44	1.358±1.12
2–Heptanone	1.469±0.49	1.341±0.88	1.53±0.67	1.613±0.91	1.619±0.62	1.672±0.81	2.206±1.36	1.771±0.45	2.458±1.37	2.235±1.16	1.412±1.12	1.959±1.3	1.843±0.53	1.721±0.78	1.132±0.56	1.656±0.61
1–Nonene																
Nonane	0.291±0.59	0.629±1.1	0.143±0.23	1.175±0.77	1.172±1.06	2.07±2.49	0.224±0.4	0.396±0.42	0.61±0.52	0.553±1.24	0.449±0.64	0.912±1.06	0.457±0.2	0.336±0.21	0.334±0.33	0.414±0.18
Heptanal	2.733±0.62	2.301±1.17	2.894±0.95	2.596±1.3	3.592±1.6	3.142±1.25	3.115±2.58	4.181±1.95	3.317±1.78	3.287±1.09	2.809±1.74	3.237±1.17	4.208±1.09	3.505±1.22	2.317±1.44	3.504±0.96
(E,E)–2,4–Hexadienal	0.014±0.04	0.01±0.02	0.025±0.06	0.034±0.06			0.005±0.02		0.009±0.03	0.031±0.1	0.044±0.09		0.011±0.04	0.01±0.05	0.097±0.18	
Known unknown 911																
Cumene	0.059±0.04	0.038±0.05	0.056±0.04	0.035±0.05	0.17±0.14	0.147±0.13	0.134±0.17	0.126±0.1	0.156±0.1	0.146±0.1	0.092±0.12	0.198±0.1	0.121±0.08	0.081±0.08	0.105±0.12	0.174±0.09
alpha–Pinene	0.068±0.08	0.060±0.07	0.747±0.6	0.127±0.12	0.454±0.77	0.092±0.13	0.647±0.63	0.101±0.09	0.163±0.13	0.16±0.17	0.18±0.44	0.202±0.29	0.009±0.12	0.131±0.13	0.144±0.16	0.151±0.1
1–Butoxy–2–propanol	1.264±0.58	3.505±2.51	0.09±0.2	3.672±1.65	0.558±0.74	0.678±0.94	0.294±0.46	0.62±0.78	0.568±0.71	0.795±0.8	0.568±0.68	0.689±0.73	1.203±1.51	0.577±0.59	0.398±1.04	0.828±0.77
Ethyl tiglate																
2–Heptenal			0.038±0.05			0.033±0.08	0.044±0.06	0.013±0.04	0.041±0.09	0.068±0.12	0.075±0.16	0.012±0.05			0.119±0.23	0.139±0.23
Benzaldehyde	3.829±1.71	3.298±1.76	4.345±0.97	4.247±1.61	5.832±2.08	6.019±3.25	7.718±2.97	5.408±2.25	7.702±2.91	8.357±2.4	6.483±3.44	9.657±3.77	7.413±3.08	7.595±3.52	6.356±3.01	8.401±1.81
Known unknown 970			0.164±0.2%			0.001±0.01	0.242±0.61		0.001±0	0.009±0.03					0.012±0.03	
1–Heptanol	0.152±0.17	0.194±0.19	0.038±0.07	0.179±0.3	0.117±0.22	0.124±0.25	0.163±0.27	0.122±0.22	0.112±0.2	0.273±0.34	0.106±0.18	0.229±0.34	0.173±0.32	0.062±0.14	0.048±0.09	0.08±0.17
Known unknown 978	0.483±0.2	0.509±0.3	0.34±0.28	0.63±0.2	0.763±0.49	0.612±0.47	0.48±0.46	0.632±0.35	0.379±0.18	0.401±0.26	0.305±0.28	0.534±0.24	0.406±0.15	0.399±0.21	0.215±0.14	0.408±0.16
beta–Pinene			0.624±0.45		0.042±0.1	0.007±0.03	0.271±0.19	0.004±0.01	0.037±0.05	0.035±0.06	0.007±0.08	0.106±0.21	0.01±0.03		0.06±0.09	0.012±0.02
6–Methyl–5–hepten–2–one	4.436±1.63	4.196±1.8	5.26±0.99	4.918±3.05	4.675±3.41	5.073±3.39	4.809±2.9	4.825±2.36	5.057±3.1	5.916±3.52	8.202±5.57	5.609±3.98	6.261±3.78	5.32±4.15	8.258±4.38	5.349±3.43
Known unknown 990		0.082±0.12			0.369±0.47	0.214±0.36	0.269±0.3	0.178±0.32	0.332±0.39	0.155±0.34	0.076±0.14	0.129±0.26			0.068±0.11	
Pseudocumene	1.888±1.1	2.515±2.25	1.261±1.05	2.412±1.39	3.006±2.55	2.063±1.79	1.824±2.05	2.589±1.69	1.546±0.96	1.254±1.15	1.148±1.22	2.042±1.14	1.325±0.96	1.382±1.4	0.839±0.81	1.566±1.01
2–Octanone	5.349±1.91	4.522±3.53	4.248±1.99	6.191±2.83	6.209±2.41	5.514±3.19	4.483±3.48	7.16±1.76	6.739±2.67	5.975±2.64	4.686±4.05	5.311±2.59	7.724±2.36	8.186±2.81	3.628±3.07	8.233±2.01
Known unknown 999																
Decane	0.661±0.3	0.876±0.47	0.516±0.14	0.959±0.43	1.172±0.94	1.593±2.12	0.618±0.52	0.746±0.41	0.634±0.2	0.764±0.51	0.495±0.48	0.937±0.61	0.685±0.24	0.529±0.19	0.499±0.46	0.568±0.16
Octanal	2.458±0.18	2.069±1.28	2.612±1.73	2.77±1.41	3.077±1.47	2.655±1.34	2.116±0.75	3.318±1.33	2.642±1.11	2.55±1.09	2.839±1.01	2.67±1.33	3.812±0.82	4.243±1.04	2.703±1.19	4.293±1.08
(Z)–3–Hexenyl acetate	6.944±3.56	5.577±0.57	1.462±0.44	2.057±2.61	2.552±2.85	1.956±2.34	5.973±4.54	4.629±5.16	2.196±4.82	2.344±2.05	6.336±7.83	2.572±3.19	1.582±1.89	1.263±3.63	4.611±5.99	0.6±0.3
Hexyl acetate	0.606±0.32	0.186±0.29	0.372±0.07	0.26±0.27	0.33±0.21	0.225±0.24	0.521±0.29	0.599±0.68	0.222±0.27	0.206±0.2	0.448±0.53	0.139±0.11	0.17±0.08	0.142±0.09	0.215±0.31	0.108±0.08
m–Cymene	0.124±0.07	0.135±0.1	2.973±1.97	0.18±0.09	0.619±0.63	0.512±0.42	1.008±0.87	0.415±0.38	1.196±1.95	0.925±1.93	0.296±0.16	0.756±1.18	0.148±0.1	0.486±1.5	0.195±0.13	0.143±0.08
(–)–Limonene	1.167±0.62	1.596±2.34	5.395±2.65	1.136±0.71	2.504±2.48	3.805±8.21	3.131±3.16	1.03±0.39	8.605±14.18	8.407±13.45	1.24±1.49	5.831±10.65	0.926±0.62	4.074±6.39	2.12±2.85	6.882±0.39
Benzylalcohol	2.246±1.52	2.855±2.06	3.018±3.4	2.864±1.74	1.963±2.77	4.296±5.21	8.499±6.62	1.494±2.64	4.65±5.75	8.23±10.46	8.066±7.94	8.081±8.41	3.879±6.53	2.823±3.98	7.039±8.81	4.611±5.94
(E)–beta–Ocimene	0.081±0.1	0.045±0.09	0.311±0.31		0.152±0.25	0.181±0.32	0.167±0.18	0.042±0.06	0.036±0.12		0.104±0.21	0.001±0.01	0.019±0.05	0.509±2.26	0.033±0.14	
Benzenacetaldehyde	0.016±0.05	0.02±0.04	0.029±0.04	0.028±0.07	0.013±0.05	0.047±0.08	0.047±0.04	0.028±0.06	0.019±0.04	0.031±0.05	0.042±0.08	0.04±0.1	0.008±0.03	0.019±0.05	0.087±0.31	0.014±0.05
gamma–Terpinene																
(E)–2–Octenal																
Acetophenone	0.861±0.39	0.883±0.55	0.606±0.09	1.159±0.27	1.061±0.86	0.992±0.69	0.774±0.42	1.117±1.04	0.893±0.56	0.702±0.3	0.719±0.53	0.678±0.52	0.709±0.72	0.768±0.5	0.545±0.39	0.882±0.49
Known unknown 1066	0.347±0.12	0.349±0.25	0.376±0.28	0.624±0.27	0.496±0.36	0.353±0.24	0.332±0.15	0.511±0.19	0.466±0.29	0.372±0.2	0.492±0.29	0.449±0.32	0.668±0.3	0.704±0.21	0.482±0.27	0.76±0.26
1–Octanol		0.04±0.11			0.072±0.16	0.103±0.33					0.024±0.04				0.017±0.04	
Known unknown 1072	0.645±0.32	0.126±0.15		0.045±0.08	0.227±0.57	0.263±0.33	0.431±0.64	0.217±0.26	0.202±0.3	0.254±0.28	0.171±0.29	0.198±0.32	0.178±0.21	0.056±0.13	0.031±0.12	0.248±0.38
Terpinolen																
Linalool oxide (furanoid)	0.291±0.3		0.078±0.12		0.069±0.21	0.047±0.16	0.148±0.14	0.025±0.1			0.109±0.14			0.009±0.05		
DMNT (peak 1)																
Known unknown 1090	0.429±0.14	0.442±0.31	0.384±0.12	0.64±0.25	0.726±0.5	0.518±0.2	0.481±0.54	0.822±0.45	0.691±0.58	0.595±0.25	0.332±0.37	0.481±0.33	0.511±0.24	0.48±0.27	0.268±0.26	0.454±0.27
1–Undecene																

Compounds (y-axis, top to bottom):

- Methyl benzoate
- Linalool
- Undecane
- Nonanal
- DMNT (peak 2)
- Known unknown 1121
- (E,Z)-2,6-dimethyl-2,4,6-Octatriene
- Known unknown 1134
- Camphor
- Known unknown 1160a
- Known unknown 1160b
- 2-Nonenal
- Known unknown 1167
- Ethyl benzoate
- (Z)-3-Hexenyl butyrate
- Hexyl butyrate
- Methyl salicylate
- n-Dodecane
- Decanal
- Known unknown 1210
- Benzothiazole
- (Z)-3-Hexenyl-methyl butyrate
- (E)-2-Undecen-1-ol
- Ethyl salicylate
- Known unknown 1271
- Bornyl acetate
- Nonanoic acid
- 1-Tridecene
- Tridecane
- Known unknown 1306
- Undecanal
- Known unknown 1345
- Known unknown 1346
- Known unknown 1349a
- Known unknown 1349b
- 2-Undecenal
- Known unknown 1364
- Known unknown 1374
- Known unknown 1381
- Known unknown 1387
- 1-Tetradecene
- Tetradecane
- Known unknown 1404
- Dodecanal
- Known unknown 1411
- alpha-Cedrene
- beta-Caryophyllene
- alpha-Ionone
- Known unknown 1426
- Known unknown 1430
- Geranylacetone
- beta-Farnesene
- alpha-Caryophyllene
- 2-Dodecenal
- Known unknown 1474
- Known unknown 1475
- Known unknown 1477
- Known unknown 1481
- beta-Ionone
- Known unknown 1485
- Known unknown 1498
- Known unknown 1499
- Pentadecane
- Known unknown 1504
- alpha-Farnesene
- Known unknown 1509
- Known unknown 1520
- Known unknown 1527
- Known unknown 1536
- Known unknown 1554
- (Z)-3-Hexen-1-ol benzoate
- Caryophyllene oxide
- Known unknown 1583
- Hexadecane
- Tetradecanal
- Known unknown 1605
- Known unknown 1616
- Known unknown 1644
- Known unknown 1671
- 1-Heptadecene
- 2-Pentadecanone

X-axis (Variety & BBCH-stage): Varieties MT, R, Rie, PN grouped under BBCH 65–71, BBCH 73–75, BBCH 77–81, BBCH 83–89.

Legend: Area % (scale 0, 4, 8, 12, 16)

Variety & BBCH-stage

Table S4 Overview of all volatiles identified in headspace samples of four grapevine varieties with retention index (RI) and the way of identification.

Chemical compound	RI	Origin	Chemical compound	RI	Origin
2-Hexanone	791	SA	Undecane	1100	SA
Hexanal	796	SA	Nonanal	1105	Aldrich
Ethyl butanoate	801	SA	DMNT (peak 2)	1115	Enamine
Butyl acetate	815	Aldrich	(E,Z)-2,6-dimethyl-2,4,6-Octatriene	1134	MS
Trans-2-Hexen-1-al	855	Aldrich	Camphor	1145	Aldrich
(Z)-3-Hexen-1-ol	860	Aldrich	2-Nonenal	1161	SA
1-Hexanol	867	Fluka	Ethyl benzoate	1171	Fluka
2-Heptanone	890	SA	(Z)-3-Hexenyl butyrate	1186	Sigma
1-Nonene	894	MS	Hexyl butyrate	1188	Aldrich
Nonane	900	SA	Methyl salicylate	1195	Sigma
Heptanal	901	SA	n-Dodecan	1200	SA
(E,E)-2,4-Hexadienal	911	MS	Decanal	1208	Sigma
Cumene	914	Aldrich	Benzothiazole	1224	SA
α-Pinene	927	Fluka	(Z)-3-Hexenyl-methylbutyrate	1231	SA
1-Butoxy-2-propanol	940	SA	(E)-2-Undecen-1-ol	1254	MS
Ethyl tiglate	949	MS	Ethyl salicylate	1265	Roth
2-Heptenal	955	Aldrich	Bornyl acetate	1280	Sigma
Benzaldehyde	961	SA	Nonanoic acid	1290	MS
1-Heptanol	971	SA	1-Tridecene	1294	MS
β-Pinene	980	Fluka	Tridecane	1300	Aldrich
6-Methyl-5-hepten-2-one	988	SA	Undecanal	1310	SA
Pseudocumene	991	Fluka	2-Undecenal	1360	MS
2-Octanone	995	Roth	1-Tetradecene	1391	MS
Decane	1000	SA	Tetradecane	1400	SA
Octanal	1004	Aldrich	Dodecanal	1406	Sigma
(Z)-3-Hexenyl acetate	1007	SA	α-Cedrene	1413	SA
Hexyl acetate	1012	Fluka	β-Caryophyllene	1420	SA
m-Cymene	1023	SA	α-Ionone	1425	Aldrich
(-)-Limonene	1030	SA	Geranylacetone	1453	Fluka
Benzylalcohol	1035	RH	β-Farnesene	1454	Aldrich[a]
(E)-β-Ocimene	1040	SA[a]	α-Caryophyllene	1455	Sigma
Benzeneacetaldehyde	1043	SA	2-Dodecenal	1466	MS
γ-Terpinene	1055	Fluka	β-Ionone	1484	SA
(E)-2-octenal	1058	Aldrich	Pentadecane	1500	SA
Acetophenone	1065	SA	α-Farnesene	1508	Aldrich[a]
1-Octanol	1069	Fluka	(Z)-3-Hexen-1-ol benzoate	1560	Aldrich
Terpinolen	1085	Roth	Caryophyllen oxide	1579	Sigma
Linalool oxide (furanoid)	1086	SA	Hexadecane	1600	Fluka
DMNT	1090	Enamine	Tetradecanal	1600	MS
1-Undecene	1091	MS	1-Heptadecene	1676	Sigma
Methyl benzoate	1095	RH	2-Pentadecanone	1696	MS
Linalool	1099	Sigma			

Identification of components by comparing with mass spectra and retention indices of authentic standard substances purchased from Sigma-Aldrich (SA)-group (Schnelldorf, Germany; SA, Fluka, Sigma, Aldrich, and Riedel-de-Haën (RH)), Enamine (Kyiv, Ukraine) and Carl Roth (Karlsruhe, Germany) or identification without comparing with standard substance just because of mass spectra (MS; match > 90 %).

[a] Identification confirmed by mix of isomers as standard substance only; DMNT = 4,8-dimethyl-1-(E)-3,7-nonatriene

Tracking short-range attraction and oviposition of European grapevine moths affected by volatile organic compounds in a four-chamber olfactometer

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Abstract

The identification of volatile organic compounds (VOCs) leading to short-range attraction and oviposition of the European grapevine moth *Lobesia botrana* and European grape berry moth *Eupoecilia ambiguella* (Lepidoptera: Tortricidae) is crucial in order to establish bait-based decision support systems for the control of these pests. Therefore, we developed a method to measure the real-time behavioral response of female moths to VOCs using a four-chamber olfactometer coupled with a video tracking system. Ten synthetic VOCs were selected for this study: (S)-(-)-perillaldehyde, (E)/(Z)-linalool oxide, (±)-limonene, linalool, (E)-β caryophyllene, α/β-farnesene, (-)-α-cedrene, methyl salicylate and cumene. The effect of VOCs on egg deposition was determined using a dual-choice oviposition test, whereas perception by female antennae was verified using electroantennography (EAG). During video tracking, females responded to volatile compounds emitted by grapevine with higher antennae and ovipositor activity than to air control. (E)/(Z)-linalool oxide, cumene and (S)-(-)-perillaldehyde released ovipositor activity of *L. botrana*, while the latter provoked oviposition. (R)/(S)-limonene affected ovipositor activity of *E. ambiguella*, whereas none of the VOCs tested attracted for oviposition. The results suggest that females have the ability to perceive specific VOCs by the antennae but also by the ovipositor, which could attract or repel for egg deposition.

1. Introduction

The selection of actual host plants by herbivorous insects for reproduction is amongst others triggered by the emitted host plant bouquet, specified as volatile organic compounds (VOCs). In this regard, Lepidoptera belong to a well-studied order of insects [1], in which the European grapevine moth *Lobesia botrana* and the European grape berry moth *Eupoecilia ambiguella* are also included. They are two of the most important insect pests threatening European viticulture [2], whereas *L. botrana* has been reported as a new grape pest in the Americas: California, Chile and Argentina [3–5]. Both species are crepuscular [6,7] and mainly oviposit on the surface of fructiferous organs of plants, whereas several families were identified as hosts (e.g., Vitaceae, Oleaceae, Thymeleaceae and Rosaceae) [8–12]. Most notably, they are multivoltine on grapevine. *Lobesia botrana* is able to complete up to four generations per year under favorable climatic conditions [13]. The first generation is considered to be exclusively anthophagous (flower-feeding) and may cause direct yield losses to a greater extent than the following carpophagous (fruit-feeding) generations [14,15]. Direct damage is caused by feeding of larvae on immature, ripening and ripe berries, which indirectly promotes an infection by the grey mould fungus *Botrytis cinerea* Persoon: Fries [16,17]. In certain cases, the direct damage favors the colonization of acetic acid bacteria and yeasts, which may cause greater incidence of sour rot in presence of *Drosophila* spp. [18,19].

Several studies have investigated the oriented flight of females and males to host plants [20–24], whereas less evidence has been given on short-range attraction. Especially for females, VOCs are assumed to initiate the crucial step of oviposition [25,26], which finally triggers pest infestation in vineyards. It has already been proven that *L. botrana* females have sensory structures, which allow the contact detection of physical and chemical stimuli that show no or reduced volatility after settlement on a plant [26,27]. Mainly non-porous sensilla and terminal pore sensilla, located at the legs, the ovipositor, the proboscis and the antennal tip of females are assumed to evaluate particular plants as hosts [27]. In former studies, the behavioral effect of single compounds identified in the scent bouquet of grapevine was evaluated by the flight activity of females in wind tunnel studies [28–30]. The perception of VOCs by female's antennae was measured using electroantennography (EAG) [20,24], while a comparable method to evaluate the influence of VOCs on short-range attraction and oviposition activity is still missing. In particular, receptors on the ventral surface of the ovipositor are assumed to allow the detection of plant allelochemicals and/or deterrents [27,31].

Therefore, we developed a method to measure essential behavioral patterns, which reflect female's orientation to suitable egg laying sites. The aim was to (i) quantify female short-range attraction induced by VOCs and (ii) determine the effect of volatiles on oviposition behavior. The general perceptibility of single VOCs by *E. ambiguella* and *L. botrana* female antennae was verified using EAG. To assess short-range orientation to host plants and VOCs, a four-chamber olfactometer assay was developed, which allows female behavior to be tracked, like settlement near volatile sources, flight activity and movement of the antennae or ovipositor. Especially the observation of ovipositor movement can be used as evidence for compounds, which are recognized by the ovipositor's sensilla.

Furthermore, the results contribute to the identification of VOCs, which are responsible for females' oviposition decisions. They can be used for the development of bait-based tools for the control of these pests.

2. Materials and Methods

2.1. Insect Rearing

Moths used in the bioassays were taken from an insect culture established at Julius Kühn-Institut, Siebeldingen, Germany. They were reared according to Markheiser et al. [32] on a semi-artificial diet. Pupae were separated by sex [12], individually transferred into 15 mL falcon tubes (VWR International GmbH, Darmstadt, Germany) and closed using a moisturized cellulose-plug. One male and one female moth (age < 24 h) were coupled 48 h before the bioassay was carried out. One hour before starting the experiments, at the beginning of dusk, couples were separated and females, which deposited >10 eggs during copulation period, were used for the study. The insect rearing was conducted under controlled climatic conditions of 14:8 h (light:dark) photoperiod, 1 h each of dusk and dawn, 23:19±2 °C and 70±5% relative humidity. The two moth species were kept separated from each other in climatic chambers 'Fitotron type SGR233' (Weiss Technik UK Ltd., Loughborough, UK). The day-night setback within the chambers was shifted by 8 h in advance to ensure higher oviposition activity during the studies, which preferably appears during dusk as observed by Stellwaag [7].

2.2. Volatile Organic Compounds (VOCs)

Potted grapevine plants, cv. 'Regent', were used for the validation of the behavioral studies. They were propagated by wood cuttings and cultured in a greenhouse at Julius Kühn-Institut,

Siebeldingen, Germany, under controlled conditions of 23±5 °C and 30±10% relative humidity. A fungicide treatment against powdery mildew (*Erysiphe necator*) was conducted once a week with either Vivando (500 g/L metrafenone, BASF SE, Ludwigshafen, Germany), Talendo (200 g/L propynazide, DuPont, Wilmington, DE, USA) or Dynali (60 g/L difenoconazole +30 g/L cyflufenamide, Syngenta, Basel, Swizerland). No insecticides were applied. Fourteen days before the start of the experiment, fungicide treatments were stopped.

Synthetic VOCs examined in this study (Appendix A) were selected from literature [28–30,33,34]. All volatiles were identified as components of the scent of the host plants grapevine (*Vitis vinifera*) or flax-leaved daphne (*Daphne gnidium*). Except from the components mentioned in Rid et al. [34] and Cattaneo [35], VOC blends differ in their chemical composition and induced an attraction of *L. botrana* or *E. ambiguella* females in wind tunnel studies. The following ten substances were tested individually: (S)-(-)-perillaldehyde, (E)/(Z)-linalool oxide (furanoid), (E)/(Z)-linalool oxide (pyranoid), (+/-)-limonene, linalool, (E)-β-caryophyllene, α/β -farnesene (mixture of isomers), (-)-α-cedrene, methyl salicylate and cumene. All chemical substances except (E)/(Z)-linalool oxide (pyranoid) (Nippon Terpene Chemicals Inc., Kobe, Japan) were purchased from Merck, Darmstadt, Germany.

2.3. Electroantennography (EAG)

The response of mated females' antennae of *L. botrana* and *E. ambiguella* to volatiles was studied using EAG. Moths' antennae were excised with fine scissors. The reference electrode of the EAG device was connected to the base of the antenna, whereas the recording electrode was connected to the tip of the antenna, while the last segment of antenna was cut off. Glass capillaries (0.58 mm I.D., Science Products, Hofheim, Germany) filled with Ringer solution (NaCl 7.5 g, KCl 0.35 g, CaCl2 0.21 g ad 1 L H2O) were used as electrodes and connected to silver wire. The analogue signal was detected with a probe (INR-II, Ockenfels Syntech®, Kirchzarten, Germany), captured and processed with a data acquisition controller (IDAC-2, Ockenfels Syntech®) and analyzed with EAG software (EAGpro, Ockenfels Syntech®). The air passing over the antenna was filtered using activated charcoal and humidified. To prepare the odor sources, a piece of filter paper (type 413, VWR International bvba, Belgium) was placed into the wide end of standard glass Pasteur pipettes. One µL of each test substance was pipetted onto the filter paper. The odor-loaded pipette was immediately placed in-line with the puff apparatus. For stimulation, an air puff (1 s, flow = 1.4 L/min) was passed through the pipette transporting the respective VOC to the continuous airflow (1.5 L/min) that passed over

the antenna. The order of a set of puffs was as following: control (only filter paper), solvent control (only dichloromethane (DCM), Merck, Darmstadt, Germany), test substance (diluted in DCM), control, solvent control, and reference substance (diluted in DCM). This set was repeated three times per antenna while the substances were pipetted on a fresh filter paper for each set immediately before use. A refractory phase of 5 s was kept between the single puffs.

Response of the antenna was confirmed by controlling with a reference substance (*E. ambiguella*: linalool (10 µg/µL), *L. botrana*: α/β-farnesene (mixture of isomers, 10 µg/µL)). The test substances were puffed onto the antenna, and differences of the sum of the receptor potentials from individual olfactory receptor neurons [mV] were analyzed. Amplitudes statistically significantly higher than amplitudes derived from solvent control (DCM puff, eliciting mechanoreceptors and others) represent perceivable substances.

2.4. Four-Chamber Olfactometer Assays

The influence of volatiles on the behavior of mated *L. botrana* and *E. ambiguella* females was proven in a four-chamber olfactometer system (CADS-4CCP, Sigma Scientific LLC, Micanopy, FL, USA). It consisted of a five-port system (30 x 30 x 2.5 cm) with a removable lid out of glass (0.6 cm thickness) and wing nuts for air-tight sealing. The main body and the inlet and outlet ports were made from solid ultra-high-molecular-weight polyethylene (UHMW-PE).

The arena, where the insects remain during inspection, was shaped like a four-pointed star (Figure 1). Each point of the star was connected to an inlet port, which enabled the insertion of either an external volatile source (plant headspace or synthetic volatile) or a reference source (clean air or solvent). Each inlet was connected to a set of borosilicate glass elements and consisted of an insect isolation trap (IIT) and a Teflon-tube adapter. The volatiles were provided via an inline odor source adaptor (IOA), which was connected to the IIT. The IIT collected insects responding to an odor source and prevented them from returning into the arena or reaching the odor source. Moths were separately introduced into the olfactometer arena via a fifth port, a bottom fed insect inlet adapter (IIA) made of borosilicate glass and equipped with a glass frit, to stop them from moving into the connected vacuum system.

To operate the system, a four push with one pull clean air delivery system (CADS, Sigma Scientific LLC, Micanopy, FL, USA) was connected via Teflon-tubes (OD: 0.635 cm, ID: 0.396 cm) to the ports of the arena. The CADS was coupled to a compressed air source of 1.5 bar

pressure. Four output (push) flowmeters controlled the flow of clean air into each port of the system and the vacuum (pull) side was connected to the IIA.
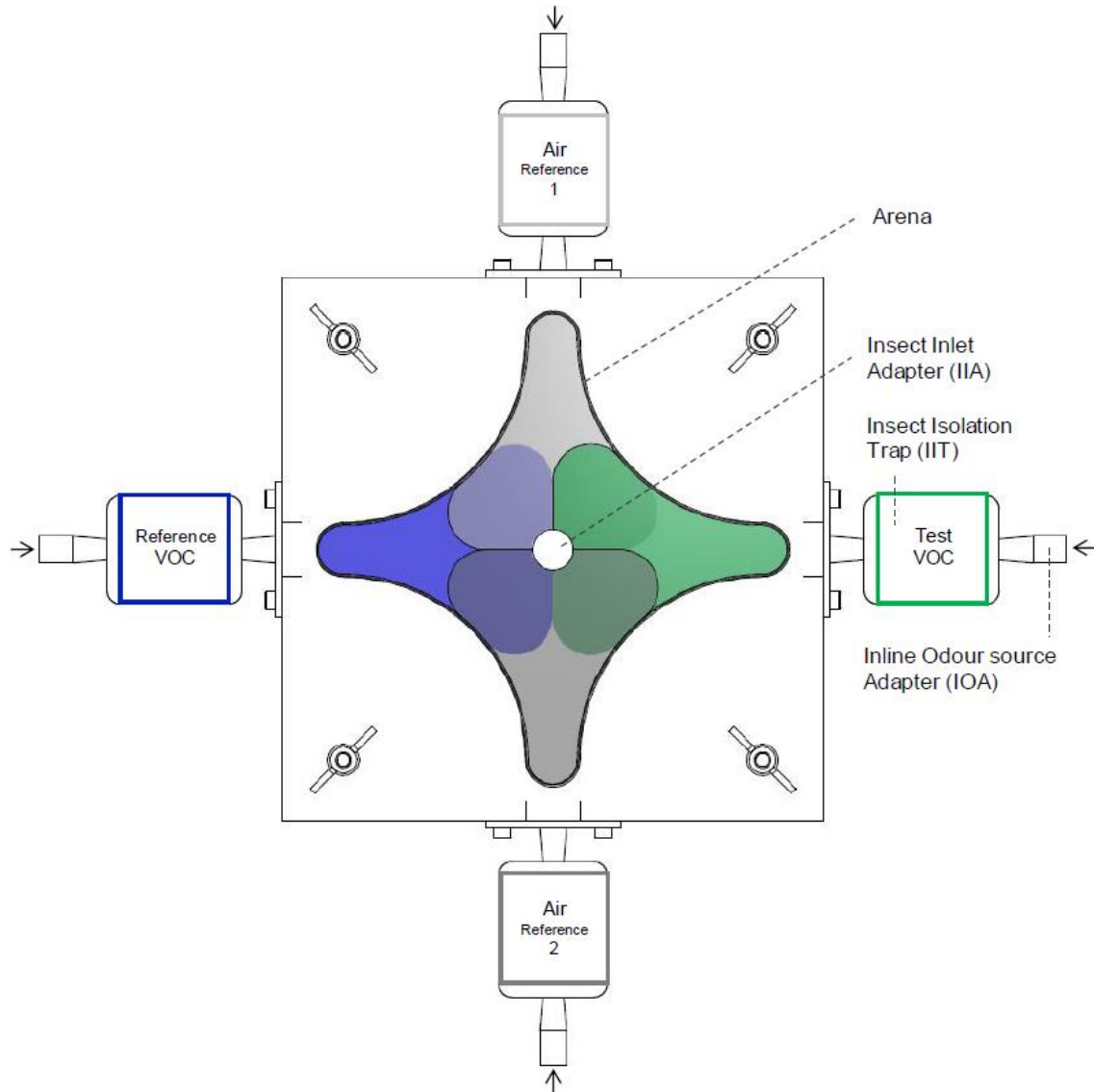
Figure 1. Top view of the four-chamber olfactometer. Insect arena is divided in four overlapping volatile organic compound (VOC) sectors, marked by colors. Arrows mark the connection to the clean air delivery system (CADS).

The flowmeters of the CADS were adjusted to values, which enable a uniform spread of the volatiles within the olfactometer arena. To prevent a high or low atmospheric pressure within the system, the vacuum must be equal to the sum of the four single air pressures ($pvac = p1 + p2 + p3 + p4$). Smoke-pens (Björnax AB, Nora, Sweden), placed inside the IITs, were used to visualize the dispersal of volatiles within the system under different airflow adjustments. The arena was divided into four identical odor sectors (color markings in Figure 1). For each

pressure source at the CADS, a value of 0.2 mbar was adjusted to provide a laminar flow within the arena and hence used for the choice experiments (and 0.8 mbar for the vacuum source). To validate the attraction of moths within the four-chamber olfactometer in general, males known to be attracted by species-specific pheromones were introduced into the system. The IIT was equipped with a pheromone source of a delta-trap (Bio-Pherotrap, Temmen GmbH, Hattersheim, Germany), specific for alluring males of *L. botrana* ((E,Z)-7,9-dodecadienyl acetate) and *E. ambiguella* ((Z)-9-dodecenyl acetate). The pheromone source was unpacked and stored in a fume cupboard 98 h before the beginning of the experiments to reduce high concentration levels within the system.

In contrast to males, the behavior of females within the system was evaluated by providing grapevine headspace. A visual healthy grapevine cluster (plant reduced to one grape cluster and four leaves 24 h before the experiment) was wrapped in an oven plastic bag (Toppits®, Cofresco Frischhalteprodukte GmbH & Co. KG, Minden, Germany) according to [34,36]. Cut surfaces were sealed with Parafilm® to prevent an evaporation of green leaf volatiles produced by injuries. Two Teflon-tubes were, via a 50 mL Falcon tube, airproof fixed at the oven bag. One was connected to the CADS to pass air into the system and the other was connected to one port of the olfactometer system (IOA) to enable overpressure to pass off. Synthetic volatiles were provided to the females in volumes of 5 µL (1:100 dilutions in DCM; Alfa Aesar, Karlsruhe, Germany), presented on filter paper (10 x 10 mm, Schleicher & Schuell, Dassel, Germany) and inserted in the IOA. Twenty-five moths (age < 72 h) were observed per volatile source over a period of five minutes each.

Females and males were evaluated in different experimental approaches. After each trial (moth), the IOA, the arena and the glass lid of the arena were rinsed with 70% ethanol and the chemical component was renewed. At the end of the experiment, all glass elements and Teflon-tubes were cleaned with 70% ethanol and baked out at 130 °C for at least 12 h. The UHMW- PE components (arena system and IOA connecting elements) were rinsed with 70% ethanol.

2.5. Video Tracking System

For a uniform recording of the female's position within the olfactometer arena, a digital camera (Basler GenICam acA1300-30um, Basler AG, Ahrensburg, Germany) was installed 1 m above the center of the arena and coupled with an EthoVision®XT (version 10) video tracking software (Noldus Information Technology, Wageningen, The Netherlands).

The software tracks the moth (an automatic detection) within a specific sector of the arena. Four volatile sectors were generated within the software system based on the previously observed distribution of the smoke within the arena (Figure 1). The following detection settings were adjusted: method: static subtraction; video sample rate: 25,000/s (video pixel smoothing: none; track noise reduction: off); subject is: darker (than background); dark contrast: 0–200 px; subject size: 0–750 px; subject contour: erode first, than dilate (4 px (erosion); 3 px (dilation)). During the tracking mode of static subtraction, the software calculates differences between a life image (recorded with moth) and a reference image (recorded prior to the introduction of the moth into the olfactometer system). As the tracking mode needs uniform and indirect illumination during the whole experiment, four dimmable LED lamps (Purelite 4 in 1 Crafters Magnifying lamp, Groves, Aylesbury, UK) were installed at the corners of the arena. Given that female moths are crepuscular, low intensities of illumination were necessary during the behavioral experiments. In our setup we observed 90 lx as the lowest level at which moths could be grabbed by the software system. The illumination intensity at the four exits (entry to IIT) of the arena was measured with a portable luxmeter (model 93560D, Beha Amprobe, Glottertal, Germany) and adjusted to 90 ± 2 lx, whereas the room temperature was set at 21 ± 2 °C.

Based on the predefined trial control settings, the tracking mode was initiated automatically as soon as the moth entered one of the four volatile sectors and stopped after five minutes. Confirmed by the observed distribution of the smoke within the system, a longer period resulted in a spread of the VOC to further volatile sectors. Females were discarded if more than three minutes passed until they entered the arena. The duration (in seconds) spent in each volatile sector was calculated. Conspicuous behavior of the moth during the experiment was, after a visual registration by the operator, manually recorded by the aid of a wireless touchpad keyboard (model E2700, Rapoo Europe BV, Bergschenhoek, The Netherlands). The record of the behavior was switched on/off via a predefined button ("a" = antennae activity, "f" = flight activity and "o" = ovipositor activity) on the keyboard. The software subsequently assigned the behavior to a specific volatile sector and its duration (in seconds) and frequency for each sector was calculated.

2.6. Oviposition Bioassay

The effect of VOCs on oviposition was quantified using a dual choice oviposition assay in comparison to a solvent (DCM). The volatiles were offered via a dispenser system, a 2 mL Eppendorf vial filled to one third with an unscented cotton wick (Ebelin, dm-Drogerie Markt

GmbH + Co. KG, Karlsruhe, Germany). The cotton wick was loaded with the test substance (1:100 dilution in DCM; Alfa Aesar, Karlsruhe, Germany) and covered by 150 µL paraffin oil (Sigma-Aldrich Chemie GmbH, München, Germany) to enable a slow release of the volatile.

Each volatile was checked in two volumes (vial either loaded with 5 µL or 10 µL). Each dispenser was fixed with a two-sided tape at the lid inside a transparent polypropylene cup (100 mL, Kastelplast GmbH, Mainz-Mombach, Germany) to offer an artificial surface for oviposition. Following [25], the cups were perforated with a needle (60 holes per cup, Ø = 1.1 mm), so volatiles could evaporate. The two prepared dispenser cups (test substance in DCM and DCM) were placed in the middle of a gauze cage (60 x 40 x 40 cm, The Caterpillar Castle, Live Monarch Foundation, Boca Raton, FL, USA), 20 cm apart from each other.

Analogous to Rid et al. [34], a grapevine cluster cv. 'Regent' (BBCH 77), containing 10 berries of *V. vinifera* 'Regent', was used as positive control to validate the experimental setup. As negative control, in order to exclude any influence of the solvent or paraffin oil on oviposition of *E. ambiguella* or *L. botrana*, the solvent was checked against a cup containing a vial filled exclusively with a cotton wick. Four couples (sexed < 48 h) were introduced into one cage to enable adequate egg amounts. Eight repetitive cages were provided for each volatile, volume and species, changing the position of the solvent and the VOC between cages in order to avoid a position effect on egg deposition. The cages were kept in climatic chambers 'Fitotron type SGR233' (Weiss Technik UK Ltd., Loughborough, UK) at 23:19 ± 2 °C, 70 ± 10% relative humidity and a 14:8 h photoperiod with either 1 h dusk or dawn. Moth species were kept in separated chambers. The experiment was stopped after 72 h by removing the couples from the cage and counting the eggs deposited outside the cups. After each experiment, the climatic chambers were warmed at 60 °C for at least 3 h to enable remained odors to volatilize.

2.7. Statistical Analyses

All data were analyzed using the software R—version 3.6.0—'Planting of a Tree' [37]. Statistical differences of the response of the antennae (EAG experiments) by different chemical compounds compared to the respective DCM control were achieved using linear mixed models (LMs) for each species using package 'lme4' [38] with 'substance' as fixed, 'antenna-ID' as random factor and 'compound-DCM pair' nested in antenna-ID. Post hoc comparisons between the responses were obtained from estimated marginal means (EMM) using function 'emmeans' [39].

For olfactometer assays, the frequency per moth (FPM) and duration (in seconds) per moth (DPM) spent in each volatile sector were recorded in total and during conspicuous behavioral traits (flight-,antennae- and/or ovipositor-activity). Statistically significant differences between the four volatile sectors in the arena were calculated using generalized linear models (GLMs) with 'behavior', 'moth species' and 'volatile source' as fixed factors. Statistical models were compared using AICs (Aikaike information criterions) and simplified by removing non-significant factors with the function 'drop1' and F-test. Time measurements and frequencies were transformed by $\log(y + 1)$. The experiment was set as valid, if total settlement duration in both air references was not statistically different. In cases of a settlement of specimens in two overlapping sectors, the time and frequency were counted to both sectors. Post hoc comparisons between durations (alternative frequencies) spent by the species in single volatile sectors were generated using EMMs and Tukey's method for p-value adjustment. Significance level was set at $p < 0.05$.

Relative attractiveness induced by volatiles in the oviposition assay was calculated using the oviposition discrimination index (ODI) mentioned in [40]: ODI = [(number of eggs on cup A (test VOC) - number of eggs on cup B (solvent))/total number of eggs] x 100%. The value varies from -100 (negative effect on oviposition) to +100% (positive effect on oviposition). Statistical analysis was carried out by applying non-parametricWilcoxon signed rank test for paired data sets (egg counts). Box-whisker plots were created using R-package 'ggplot2' [41], whereby lines represent the median, dots the mean, boxes the interquartile range (IQR), whiskers 1.5 x IQR and dots outside boxes the outliers. Heat maps were plotted using EthoVision®XT (version 10) video tracking software (Noldus Information Technology, Wageningen, The Netherlands).

3. Results

3.1. Perception of VOCs by EAG

The absolute response [mV] differed between antennae and between sets of antennae due to their viability and lifetime. For that reason, the antenna ID was used as random factor and the response to the test substance was compared to its respective DCM control. The mean absolute response to ten chemical compounds (10 µg) was calculated both for *E. ambiguella* and *L. botrana* (Table 1) and compared to the DCM control. All 10 test substances can be perceived by the antenna of both moth species.

Table 1. Average absolute responses of antennae of both *E. ambiguella* and *L. botrana* females to ten chemical compounds and to air and solvent control (dichloromethane (DCM)) obtained using EAG experiments

Compound		E. ambiguella					L. botrana		
	n	Response [mV] ± SD	p-Value [1]		n	Response [mV] ± SD	p-Value [1]		
(E)-β-caryophyllene	3 × 5	−1.14 ± 0.22	9.30×10^{-12}	***	3 × 5	−0.63 ± 0.23	3.30×10^{-9}	***
(−)-α-cedrene	3 × 5	−0.63 ± 0.22	2.35×10^{-7}	***	3 × 5	−0.41 ± 0.18	1.13×10^{-3}	**
cumene	3 × 5	−0.67 ± 0.35	1.39×10^{-8}	***	3 × 5	−0.37 ± 0.10	5.85×10^{-8}	***
α/β-farnesene	3 × 5	−1.45 ± 0.61	9.94×10^{-7}	***	3 × 5	−1.12 ± 0.40	7.58×10^{-10}	***
(+)-limonene	3 × 5	−0.82 ± 0.39	1.79×10^{-7}	***	3 × 5	−0.58 ± 0.24	1.69×10^{-8}	***
(−)-limonene	3 × 5	−0.96 ± 0.35	1.25×10^{-7}	***	3 × 5	−0.82 ± 0.26	6.47×10^{-8}	***
linalool	3 × 5	−1.88 ± 0.46	8.82×10^{-14}	***	3 × 5	−1.29 ± 0.41	1.35×10^{-10}	***
(E)/(Z)-linalool oxide (furanoid)	3 × 5	−1.40 ± 0.51	2.77×10^{-11}	***	3 × 5	−0.69 ± 0.22	1.89×10^{-7}	***
(E)/(Z)-linalool oxide (pyranoid)	3 × 5	−0.98 ± 0.67	1.68×10^{-5}	***	3 × 5	−0.80 ± 0.26	1.62×10^{-10}	***
methyl salicylate	3 × 5	−1.10 ± 0.43	1.07×10^{-8}	***	3 × 5	−1.06 ±0.46	2.39×10^{-7}	***
DCM	3 × 50	−0.44 ± 0.26			3 × 50	−0.36 ± 0.18		
air	3 × 50	0.48 ± 0.34			3 × 50	0.38 ± 0.22		

[1] Statistical differences between the response to the compound and to DCM control by linear mixed model (LM), post hoc estimated marginal mean (EMM) (*** $p < 0.001$; ** $p < 0.01$).

3.2. Responsiveness of Moths in the Olfactometer

3.2.1. Males

Pheromones were used to quantify the general attraction of moths within the olfactometer system. Males of *E. ambiguella* (n = 22 of 25) and *L. botrana* (n = 23 of 25) almost entirely entered the olfactometer system within a period of three minutes. During the observation period of five minutes, flight activity was observed in 27.3% of *E. ambiguella* and in 60.9% of *L. botrana* specimens. The frequency and duration spent by males in each volatile sector was analyzed (Table 2). Due to the frequent flight interruption, the males' antenna activity could not be recorded during the tracking mode.

Table 2. Frequency per moth (FPM) and duration per moth (DPM) spent by males of E. *ambiguella* (EA) and *L. botrana* (LB) in the four volatile sectors of the olfactometer system during stay (total observation period of 300 s) and flight activity after introducing a pheromone source (pher).

Species	Behavior	n	FPM in Sector (Mean ± SE) [n]				DPM in Sector (Mean ± SE) [s]			
			Air	Reference 1 (Air)	Pher	Reference 2 (Air)	Air	Reference 1 (Air)	Pher	Reference 2 (Air)
EA	stay	22	41.4 ± 6.3	55.6 ± 9.9	47.3 ± 6.9	58.2 ± 10.4	80.5 ± 12.2	82.8 ± 9.5	124.2 ± 14.9	90.8 ± 13.0
	flight	6	5.2 ± 3.6	9.6 ± 6.7	7.8 ± 4.9	10.7 ± 7.6	2.6 ± 2.1	8.8 ± 6.3	18.3 ± 13.6	7.5 ± 4.8
LB	stay	23	30.7 ± 7.0	29.0 ± 4.2	31.4 ± 4.7	37.6 ± 7.4	75.9 ± 16.6	113.2 ± 17.0	123.8 ± 16.9	90.2 ± 16.2
	flight	14	0.5 ± 0.4	1.6 ± 0.7	1.4 ± 0.5	2.0 ± 1.1	1.0 ± 0.7	3.7 ± 2.0	3.7 ± 1.9	2.3 ± 0.9

The moth species had no significant influence on the duration of stay within single sectors (GLM: $F_{1,254} = 0.93$; $p = 0.33$), hence data of *E. ambiguella* and *L. botrana* were merged for

further analyses. The duration of males' flight activity in single sectors was short (4.6 ± 1.3 s, n = 80) if compared to the duration of stay in the single sectors of the total recording time (97.7 ± 5.3 s, n = 180) (GLM: $F_{1,254} = 419.82$; $p < 0.001$). The volatile sector had a statistically significant influence on the settlement duration of males (GLM: $F_{3,254} = 3.41$; $p = 0.016$, Figure 2).
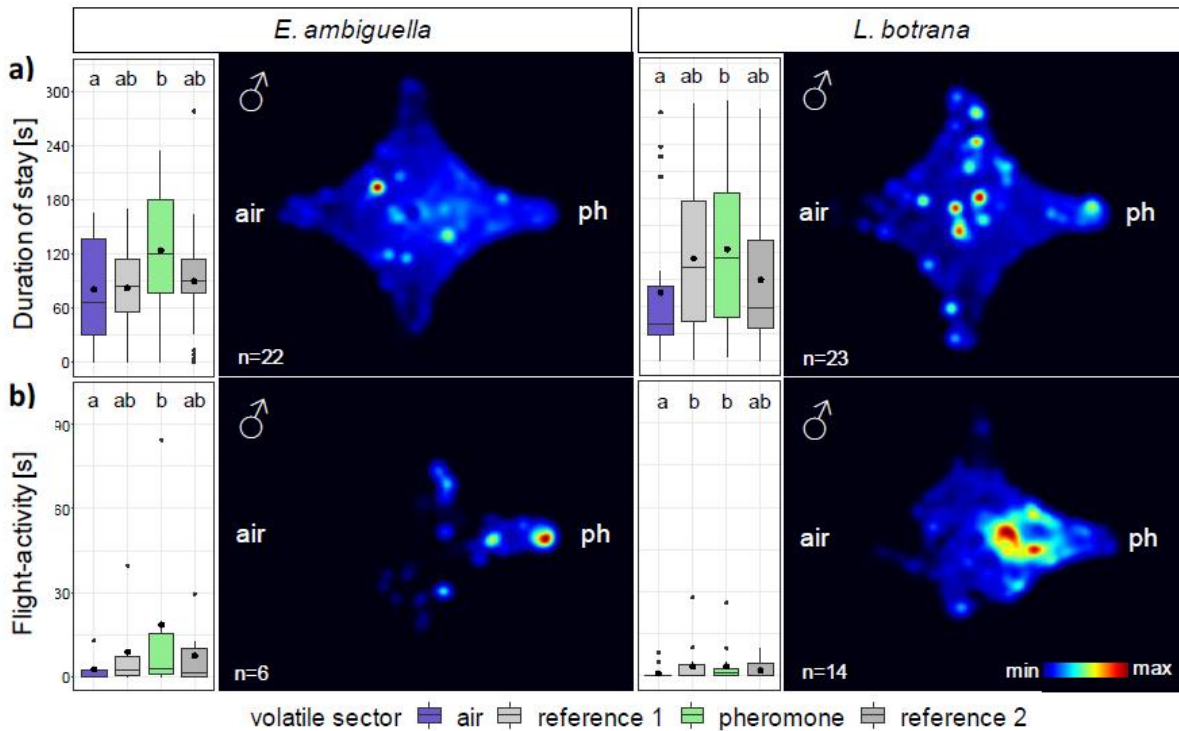

Figure 2. Box-whisker plots and merged heat maps visualizing the duration of (**a**) stay and (**b**) flight activity of (left) *E. ambiguella* and (right) *L. botrana* within the four-chamber olfactometer arena after introduction of a pheromone source (ph). Different letters indicate statistical differences between volatile sectors according to generalized linear model (GLM) and post hoc comparisons using estimated marginal means (EMMs) ($p < 0.05$).

Independent from the observed behavior (stay (n = 45) or flight activity (n = 20)), the cumulative time spent by *E. ambiguella* and *L. botrana* in the pheromone sector was statistically ($z_4 = 3.14$, $p < 0.01$) longer (stay: 124.0 ± 11.2 s; flight activity: 8.1 ± 4.3 s) than in the air sector (stay: 78.1 ± 10.2 s, flight activity: 1.5 ± 0.8 s).

The moth species influenced the frequency of volatile sector entries (GLM: $F_{1,254} = 13.03$; $p < 0.001$), with males of *E. ambiguella* switching more frequently between volatile sectors than males of *L. botrana* ($z_2 = 3.61$, $p < 0.001$). In contrast, the volatile source had no significant influence on the frequency of entering a volatile sector (GLM: $F_{3,254} = 0.96$; $p = 0.41$).

3.2.2. Females

The behavior of mated females near volatile compounds emitted by grapevine (*V. vinifera* 'Regent'; BBCH 77) served as model for female moths, which are in search of oviposition sites. Activities of the female's antennae were characterized by a pivoting up and down movement of the antennae while standing still. The ovipositor activity was recognizable by palpation of the olfactometer surface with the ovipositor, whereby the abdomen was swinging back and forth. Flight activity of females was rare, so that this behavior was not recorded in our experiments (Table 3).

Table 3. Frequency per moth (FPM) and duration per moth (DPM) spent by females of *E. ambiguella* (EA) and *L. botrana* (LB) in the four volatile sectors of the olfactometer system during stay (total observation period of 300 s) and specific behaviors (antennae- and ovipositor-activity) after introducing a grapevine headspace (grape).

Species	Behavior	n	FPM in Sector (Mean ± SE) [n]				DPM in Sector (Mean ± SE) [s]			
			Air	Reference 1 (Air)	Grape	Reference 2 (Air)	Air	Reference 1 (Air)	Grape	Reference 2 (Air)
EA	stay	21	32.8 ± 9.8	25.3 ± 9.1	35.5 ± 10.5	25.8 ± 7.4	42.7 ± 19.1	45.5 ± 19.0	82.3 ± 25.4	72.6 ± 22.7
	antennae	12	0.3 ± 0.3	0.6 ± 0.3	1.6 ± 0.5	1.0 ± 0.4	0.3 ± 0.3	0.4 ± 0.2	1.7 ± 0.8	1.8 ± 0.9
	ovipositor	3	0.7 ± 0.3	1.0 ± 0.6	4.7 ± 2.9	1.3 ± 0.9	1.6 ± 1.2	2.7 ± 1.5	32.7 ± 24.1	31.8 ± 30.6
LB	stay	19	11.9 ± 2.5	11.2 ± 2.4	28.0 ± 12.1	11.5 ± 3.0	73.8 ± 15.4	75.4 ± 17.6	132.6 ± 22.1	100.5 ± 21.8
	antennae	17	0.7 ± 0.3	0.6 ± 0.2	1.4 ± 0.5	1.1 ± 0.5	1.9 ± 0.9	1.5 ± 0.7	5.3 ± 2.2	4.6 ± 2.2
	ovipositor	14	2.4 ± 0.7	3.1 ± 0.8	3.2 ± 0.8	2.2 ± 0.6	12.1 ± 5.4	27.5 ± 10.2	27.9 ± 8.6	21.0 ± 7.5

The duration spent by females in a volatile sector of the olfactometer arena (Table 3) was influenced by the factors behavior (GLM: $F_{1,333} = 121.38$; $p < 0.001$), moth species (GLM: $F_{1,333} = 8.46$; $p < 0.01$) and volatile source (GLM: $F_{3,333} = 4.50$; $p < 0.01$). Females of *E. ambiguella* spent significantly less time (84.6 ± 1.3 s) within the arena than females of *L. botrana* (95.6 ± 9.0 s) ($z_2 = -2.91$, $p < 0.001$), which is a consequence of moths moving back to the IIA. Considering the sum of DPM over all four volatile sectors, both species had a significantly shorter duration ($z_3 = -5.29$, $p < 0.001$) of antennae activity (*E. ambiguella*: 1.0 ± 0.3 s; *L. botrana*: 3.3 ± 0.8 s) than ovipositor activity (*E. ambiguella*: 17.2 ± 9.5 s; *L. botrana*: 22.1 ± 4.0 s).

The duration of stay within single volatile sectors could not be explained by the volatile source, neither for *E. ambiguella* (GLM: $F_{3,76} = 2.17$; $p = 0.10$, Figure 3a left) nor for *L. botrana* (GLM: $F_{3,72} = 1.64$; $p = 0.19$, Figure 3a right). For both moth species, the duration of antennae (Figure 3b) and ovipositor activity (Figure 3c) was triggered by the volatile source. Antennae ($z_4 = -2.01$, $p < 0.05$) and ovipositor activities ($z_4 = -3.44$, $p = 0.01$) were statistically shorter in the air sector than in the grapevine sector.
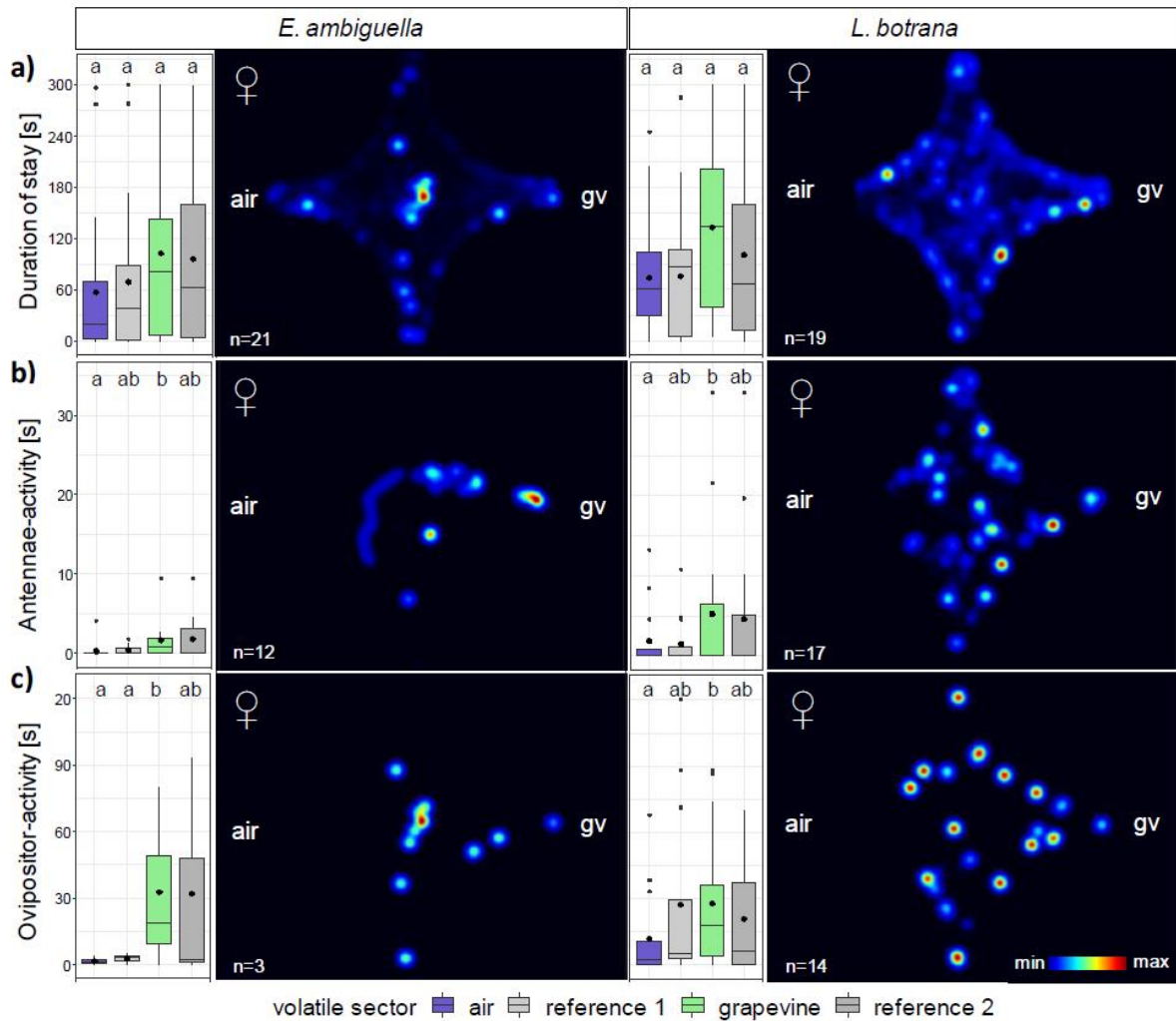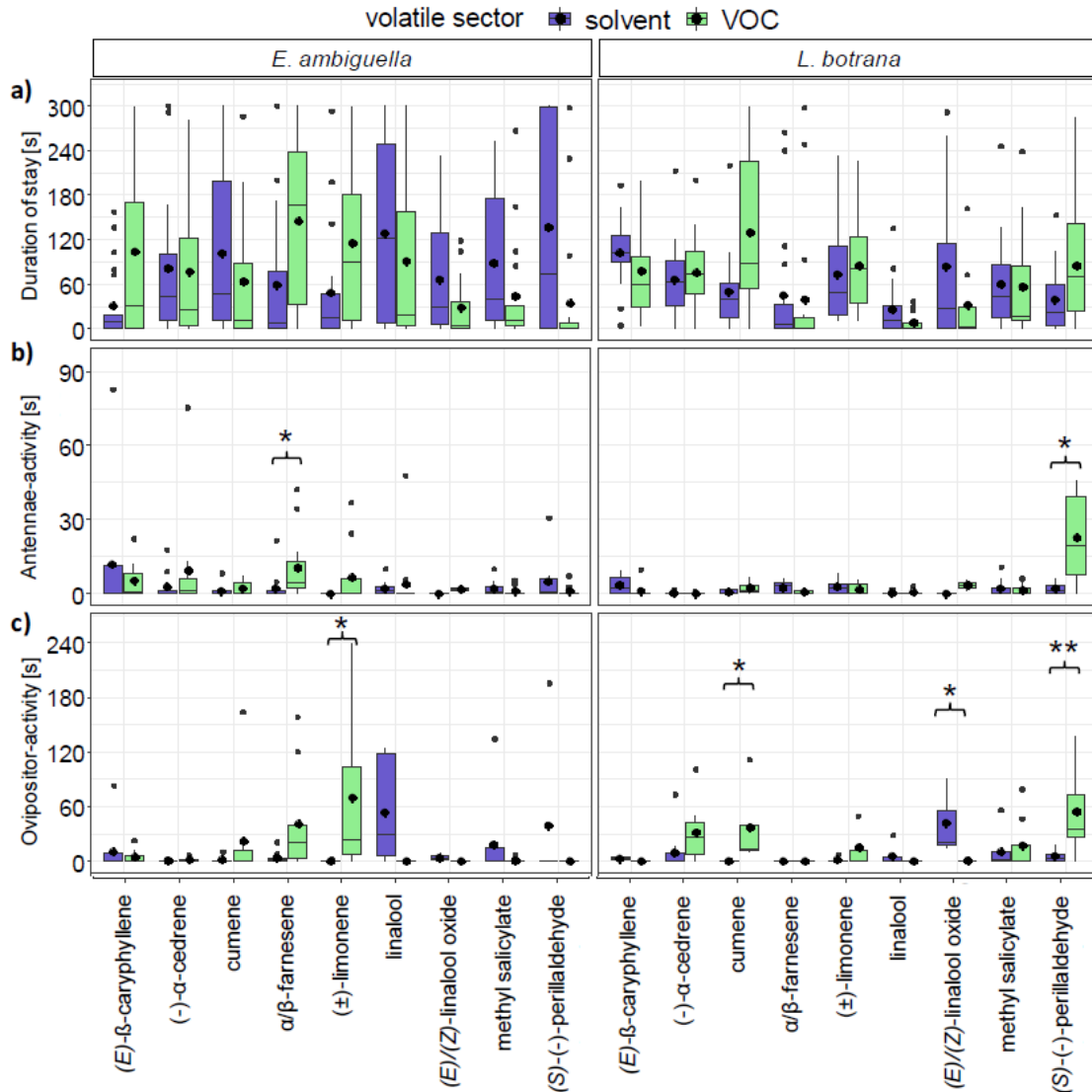
Figure 3. Box-whisker plots and merged heat maps visualizing the duration of (**a**) stay (**b**) antennae activity and (**c**) ovipositor activity of (left) *E. ambiguella* and (right) *L. botrana* females within the four-chamber olfactometer arena after introduction of a grapevine headspace (gv). Different letters indicate statistical differences between volatile sectors according to generalized linear model (GLM) and post hoc comparisons using estimated marginal means (EMMs) ($p < 0.05$).

Differences in the frequency of volatile sector entries (Table 3) were a result of the behavior (GLM: $F_{2,333} = 91.30$; $p < 0.001$) rather than the insect species (GLM: $F_{1,333} = 0.22$; $p = 0.64$) or volatile source (GLM: $F_{3,333} = 1.75$; $p = 0.15$).

3.3. Short-Range Attraction of Females by VOCs

Due to the fact that females previously did not enter the grapevine sector more frequently than the air sector, we focused in the further studies on the duration spent by the females in the single volatile sectors (full dataset in Table S1). Although behavior was a factor explaining differences in the duration in all VOCs tested in this study (statistics in Table S2), there were no statistically significant differences between the duration of antennae and ovipositor activity (data not

shown). None of the VOCs ((±)-limonene and (E)/(Z)-linalool oxide (pyranoide/furanoide) tested as isomere mixture 1:1) had a statistically significant influence on duration of stay within single volatile sectors during the total observation period (GLM; $p > 0.05$; Figure 4a).

Figure 4. Box-whisker plots visualizing the duration of (**a**) stay, (**b**) antennae activity and (**c**) ovipositor activity of (left) *E. ambiguella* and (right) *L. botrana* females within the four-chamber olfactometer arena after introduction of different volatile organic compounds (VOCs). Asterisks indicate statistical differences between VOC and solvent control according to generalized linear model (GLM) and post hoc comparisons using estimated marginal means (EMMs) (* p <0.05, ** < 0.01, n = 25).

The volatiles α/β-farnesene (mixture of isomers) and (±)-limonene influenced the behavior of *E. ambiguella* significantly (Table S2). The antennae activity of *E. ambiguella* was significantly higher ($z_4 = 3.1$; $p < 0.05$, Figure 4b) in the farnesene sector than in the sector of the solvent control. Further, (±)-limonene had a positive effect on the ovipositor activity, which was higher in the volatile sector ($z_4 = 2.63$; $p < 0.05$, Figure 4c) than in the solvent control.

The behavior of *L. botrana* was influenced by cumene, (E)/(Z)-linalool oxide (pyranoide/furanoide) and (S)-(-)-perillaldehyde (Table S2). In comparison to the solvent control, the ovipositor activity was significantly higher in the sector of cumene ($z_4 = 2.72$; p < 0.05, Figure 4c) as well as (S)-(-)-perillaldehyde ($z_4 = 3.35$; p < 0.01, Figure 4c) and significantly lower ($z_4 = -2.62$; *p* < 0.05, Figure 4c) in the sector of (*E*)/(*Z*)-linalool oxide (pyranoide/furanoide). The compound (*S*)-(-)-perillaldehyde promoted the antennae activity of *L. botrana* in comparison to DCM significantly ($z_4 = 2.16$; *p* < 0.05, Figure 4b).

3.4. Oviposition Induced by VOCs

The influence of VOCs (1:100 dilutions in DCM) on oviposition behavior of *E. ambiguella* and *L. botrana* was dose dependent (Figure 5). None of the compounds tested in our studies attracted *E. ambiguella* females for oviposition (Figure 5, left) when compared to the solvent control, whereas dispenser systems equipped with (S)(-)-perillaldehyde significantly (Wilcoxon signed rank test, p < 0.05) attracted females of *L. botrana* for oviposition in a volume of 5 µL (Figure 5a, right).

The compounds (*E*)-β-caryophyllene, (±)-limonene and methyl salicylate (5 µL) were significant in reducing the egg deposition of *E. ambiguella*, whereas a comparable effect was observed in the case of cumene and (±)-limonene (5 µL) for *L. botrana* (Figure 5a).

Increasing the volume to 10 µL (Figure 5b) resulted in none of the VOCs tested having a positive effect on oviposition. Dispenser systems were either avoided or no longer discriminable to the solvent control. Cups equipped with the compounds cumene and α/β-farnesene in a volume of 10 µL were decreasing the egg deposition of *E. ambiguella*, whereas (±)-limonene, (*E*)-β-caryophyllene, cumene, α/β-farnesene and methyl salicylate repelled oviposition of *L. botrana*.
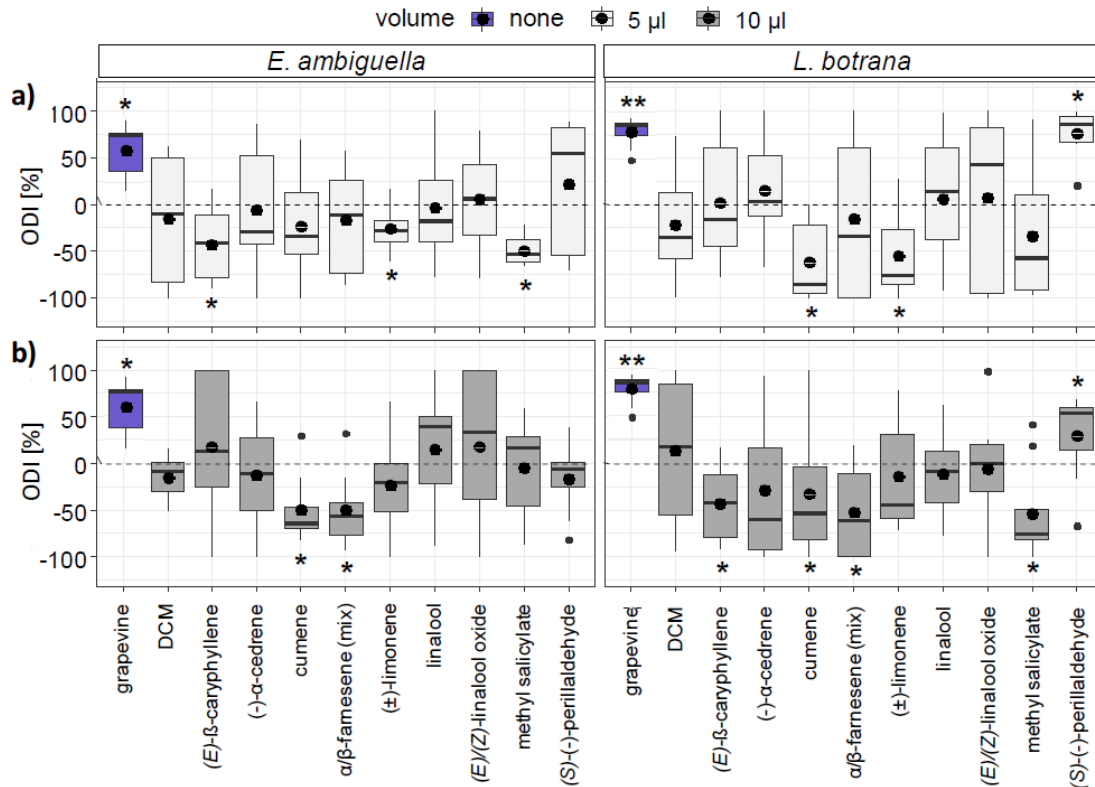
Figure 5. Oviposition preferences of (left) *E. ambiguella* and (right) *L. botrana* induced by volatile organic compounds (VOCs) (1:100 dilution in dichloromethane (DCM)) in a volume of (**a**) 5 µL and (**b**) 10 µL (grapevine = positive control, DCM = negative control). Preferences expressed by oviposition discrimination indices (ODI) (negative ODI = repellent; positive ODI = attractant). Asterisks indicate statistical differences between solvent control (DCM) and VOC according to Wilcoxon signed rank test (* $p < 0.05$, ** $p < 0.01$; n = 8).

4. Discussion

Grapevine moths do not require the stimulus of a natural plant to accept surfaces for oviposition [25,32,40]. *L. botrana* is essentially nocturnal and mating flight is initiated during night, while oviposition starts with sunset and lasts until night. In contrast, *E. ambiguella* is flight active during dawn and egg laying was observed at noon until night [7,42]. Hence, any visual component in hostseeking behavior, which is known to play a key role in some insects that are active by day [43] is assumed to be less significant, at least for *L. botrana* [25,32]. Therefore, response to volatile plant odors and fruit surface condition are probably the most likely mechanisms for Lepidoptera species like *E. ambiguella* and *L. botrana* for locating suitable host plants for reproduction [21,24,26,27,30,44–46]. Plant odors enhance the attraction to pheromones in many species, having the potential as low-cost attractants in traps [47,48]. The synthetic VOCs selected for this study were confirmed to be perceivable by the antennae of *E. ambiguella* and *L. botrana* females in EAG experiments and possibly attract female moths for reproduction. Some of them were derived from plants other than grapevine. A single

compound, (S)-(-)-perillaldehyde, a volatile emitted by the non-host plant *Perilla frutescens* [33], was found to promote oviposition of *L. botrana*. Further VOCs may trigger the female's attraction for reproduction in a similar way. Oviposition response to VOCs is often assessed over longer periods of time and relies on passive dispenser systems [25,40,44,49,50], which possibly allow a rapid degradation of the chemical compounds [48]. As consequence, it is difficult to estimate concentration levels, which operate as attractant or repellent for egg deposition.

Hence, this study aimed at developing a four-chamber olfactometer assay to measure the real-time behavioral responses of adult *E. ambiguella* and *L. botrana* to VOCs over a short range. Within a four-chamber olfactometer, the airflow builds up sectors of different odor levels, visualized by the distribution of the smoke emitted by the pens. Thus, insects were allowed to select within the arena sectors of preferred odor concentrations, an advantage in comparison to a Y-olfactometer, where moths can only respond to one odor level. Furthermore, it is possible to quantify further behavioural patterns such as sensory probing of the surface by the ovipositor, egg deposition, antennae and flight movements or female calling behavior. This enables to analyze the role of the respective substances in the behaviour of the grapevine moths.

In case of males, we observed increased flight activity and duration of stay near the pheromone sector within the olfactometer arena for both species and conclude that the system is suitable to quantify volatile compounds. Based on previous results, which confirmed the attraction of females of *L. botrana* to grape odors in wind tunnels and/or Y-olfactometer experiments [21,24,30,34,51], both species were exposed to the headspace of *V. vinifera* 'Regent'. Independently of the moth species, we observed increased ovipositor and antennae activity in the arena's sector which was enriched with grapevine odor. Regarding the duration of stay, the lack of response of females could be a consequence of the plant material tested (e.g., grape variety, amount of plant material and the release rate of plant volatiles) [52] or the lack of required supplementary signals necessary in locating host plants [25]. Possibly, the settlement position of the moth within the arena system, which is known to trigger oviposition [32], has a higher priority in the stimulus cascade relevant for oviposition than olfactory stimuli. The behaviour of females, which try to locate suitable egg-laying sites, is reviewed by Galet [53] citing various authors. According to this review, females of both species fly agitatedly between grape clusters and lay eggs on different widespread grapes. We assume that this behavior is reflected by females in the olfactometer system, resulting in duration of stay being evenly distributed over volatile sectors, while orientation to directions (antennae activity) and tasting (ovipositor activity) is influenced by volatiles.

The positive effect of (S)-(-)-perillaldehyde on egg deposition and ovipositor activity could be confirmed for *L. botrana* in our studies. Ovipositor activity was also higher in the volatile sector than in the solvent control of the compounds (±)-limonene (*E. ambiguella*), (E)/(Z)-linalool oxide (pyranoide/furanoide) and cumene (*L. botrana*), whereas none of these compounds operated as attractant for oviposition. This highlights that increased egg deposition is not necessarily a result of increased ovipositor activity. It may rather reflect a gustatory perception of the VOC by the ovipositor receptors as hypothesized by Maher and Thiéry [27]. Cumene, a compound recently identified in the bouquet of grapevine [34], was perceived by the female moth's antennae with lower electrical responses (*E. ambiguella*: -0.67 ± 0.35 mV; *L. botrana*: -0.37 ± 0.10 mV) when compared to α/β-farnesene (*E. ambiguella*: -1.45 ± 0.61 mV; *L. botrana*: -1.12 ± 0.40 mV), while *L. botrana* females showed increased ovipositor activity within the volatile sector of cumene in the olfactometer system. We may assume a higher detection with organs other than antennae resulting in a behavioral output.

None of the VOCs tested in this study provoked an increased duration of stay within the volatile sector in comparison to the solvent control. This may be a result of the VOC dose or the kind of compound tested. In the olfactometer experiments, the dose was adjusted to the same as in our EAG experiments (10 µg/µL), which conforms to other studies with grapevine moths [21,22,24]. Deviating VOC doses may result in changes of the behavioral response of females as observed for *L. botrana* in Y-olfactometer experiments [54] or oviposition tests in this study.

Furthermore, moth species such as *L. botrana* possibly avoid artificial and natural oviposition sites treated with particular VOCs as, e.g., shown by Thiéry et al. [55] for the substance methanol or Silva et al. [54] for essential oils of the non-host plant *Schinus molle* L. In this study, some compounds (methyl salicylate, α/β-farnesene and (E)-β-caryophyllene) had a negative effect on egg deposition while presenting no increased ovipositor activity in comparison to the solvent. These compounds are probably to a greater extent perceived by antennae rather than other extremities of females as suggested by Maher and Thiéry [27].

The compound methyl salicylate, derived from salicylic acid, is released in larger amounts by many plants after damage, infection or abiotic stress [56–59]. In some cases, this VOC acts as plant chemical defense to attract natural enemies [59–61]. However, such plants may be less preferred by gravid females for oviposition due to the lack of berry persistence and/or the nutritional conditions necessary for offspring development. For instance, the odor of *Botrytis cinerea*-inoculated fruits reduced oviposition of *L. botrana* [62]. This may explain the

repellent effect on oviposition in this study. Furthermore, Ulland et al. [63] found this compound to reduce the oviposition in the cabbage moth *Mamestra brassicae*.

The isomer mixture of α/β-farnesene had no influence on the attraction of both species. This may be a result of the dose and/or the kind of isomere mixture tested. It may contain sesquiterpenes, (E)-β-farnesene, (Z,Z)-α-farnesene, (Z,E)-α-farnesene and bisabolene. Two of the four stereoisomers of α-farnesene, (E,E)-α-farnesene and (Z,E)-α-farnesene, attracted larvae of the codling moth *C. pomonella*, while (E)- and (Z)-isomers of β-farnesene had no effect on larvae under controlled conditions [64–66]. The isomer (E,E)-α-farnesene was additionally found to promote oviposition of *C. pomonella* [67]. Furthermore, *C. pomonella* responded with walking in Y-olfactometer assays at a higher rate to 0.01 µg of a (E,E)- and (Z,E)-α-farnesene isomer mixture than to other doses tested (0.001, 0.1, 1, 10 µg) and solvent control [68]. Ongoing studies should therefore rely on testing a variety of VOC doses and all isomers.

Future studies aim to decode essential VOCs affecting host plant acceptance for reproduction of grapevine moths. The method developed in this study could be used to validate the suitability of VOCs for short-range attraction and measure behavioral patterns related to oviposition. This will finally support the development of a so-called 'm-ovi-card', a specific egg-monitoring tool, which aims at reflecting critical threshold levels of pest infestations in vineyards to prohibit immoderate insecticide applications [26,32,34]. Therefore, a combination of attractive VOCs and an artificial surface that fulfils the visual and tactile requirements necessary for grape moth females to accept surfaces for oviposition is still under investigation.

5. Conclusions

This study indicates that the short-range attraction of female grapevine moths *E. ambiguella* and *L. botrana* can be triggered by VOCs. We assume that VOCs emitted by grapevine are essential for short-range orientation due to the female's antennae activity that was longer in the grapevine than in the air sector. The four-chamber olfactometer system enables to track important behavioral patterns, like ovipositor activity, which are often disregarded in the evaluation of physical and chemical stimuli for oviposition. Increased durations of ovipositor activities suggest that females have the ability to recognize specific VOCs by the ovipositor, which could attract or repel females for egg deposition.

Appendix

Table A1. Evaluated volatile organic compounds selected from synthetic volatile blends attracting females of *L. botrana* in wind tunnel studies [28–30] and/or identification from grapevine headspace [34].

Compound	Tasin et al., 2007		Anfora et al., 2009	Tasin et al., 2010		Rid et al., 2019
	L *	B *	PS 33 *	GM *	DM *	Grapevine *
Monoterpenes						
(*E*)/(*Z*)-linalool oxide (furanoid)	○	●	○	●	●	●
(*E*)/(*Z*)-linalool oxide (pyranoid)	○	○	○	○	●	○
(±)-limonene	○	○	●	○	○	●
linalool	○	●	●	●	●	●
Sesquiterpenes						
(*E*)-β-caryophyllene	●	●	●	●	●	●
(*E*)-β-farnesene	●	○	○	●	○	●
(*E,E*)-α-farnesene	○	●	●	●	●	●
(−)-α-cedrene	○	○	○	○	○	●
Benzenoids						
methyl salicylate	○	●	●	●	●	●
cumene	○	○	○	○	○	●

● Compound present; ○ Compound absent. * Name of the volatile mixture used in the literature cited.

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Supplementary Material

Table S1. Frequency (FPM) and duration (DPM) spent by *E. ambiguella* and *L. botrana* females in each of the four volatile sectors of the olfactometer system during specific behaviors (stay, antenna- and/or ovipositor-activity) after introducing a volatile organic compound (VOC) and a solvent (DCM) source in opposite sectors.

female species	VOC	behavior	n	FPM in sector (mean ± SE) [n]				DPM in sector (mean ± SE) [s]			
				DCM	reference 1 (air)	VOC	reference 2 (air)	DCM	reference 1 (air)	VOC	reference 2 (air)
E. ambiguella	*(E)*-β-caryo-phyllene	stay	21	15.5 ± 11	13.6 ± 9.4	5.2 ± 2.1	10.3 ± 3.8	30.8 ± 10.6	60.8 ± 20.5	103.8 ± 25.9	105.7 ± 25.1
		antennae	9	1.9 ± 1.2	1.6 ± 0.9	1.4 ± 0.8	0.2 ± 0.2	11.9 ± 9	5.7 ± 3.1	5.1 ± 2.6	0.1 ± 0.1
		ovipositor	10	5 ± 4.2	1.4 ± 0.8	1.8 ± 0.8	0.2 ± 0.2	10.9 ± 8.2	5.1 ± 2.8	4.8 ± 2.3	0.1 ± 0.1
	(-)-α-cedrene	stay	17	6 ± 1.2	4.4 ± 0.9	7.5 ± 2.3	7 ± 2.3	80.8 ± 22.8	97.3 ± 22.7	76.4 ± 24.2	83.7 ± 21.1
		antennae	11	1.2 ± 0.6	1.6 ± 0.6	2.5 ± 1.1	2 ± 0.8	2.7 ± 1.7	9.4 ± 5.7	9.6 ± 6.7	4.5 ± 2.1
		ovipositor	4	0.8 ± 0.2	0.8 ± 0.5	2 ± 1.7	1.8 ± 1.4	0.6 ± 0.4	34.5 ± 34.2	1.8 ± 1.6	2.4 ± 1.6
	cumene	stay	20	5.3 ± 1.3	3.5 ± 1.1	7.7 ± 3.5	16.2 ± 12.8	101.2 ± 25.4	127.4 ± 27.9	63.8 ± 19.6	98.1 ± 24.7
		antennae	12	0.5 ± 0.2	1 ± 0.3	0.9 ± 0.3	0.8 ± 0.4	1.1 ± 0.7	1.8 ± 0.8	2 ± 0.8	3.9 ± 2.4
		ovipositor	9	0.9 ± 0.5	1.6 ± 0.4	1.7 ± 1	1.1 ± 0.6	1.5 ± 1.1	14.4 ± 9.7	22.2 ± 17.9	6.5 ± 4.9
	α/β-farnesene (mixture of isomers)	stay	19	4.5 ± 1.9	2.7 ± 0.8	2.9 ± 0.9	2.6 ± 0.7	59.4 ± 19.9	144.1 ± 28.7	145.4 ± 26	74.3 ± 26.4
		antennae	14	1.1 ± 0.6	3.1 ± 0.8	4.1 ± 1.3	2.1 ± 1.4	2.2 ± 1.5	9.4 ± 3.3	10.4 ± 3.5	3.7 ± 2.9
		ovipositor	10	1.2 ± 0.4	2.1 ± 0.4	2.6 ± 0.7	1.4 ± 0.9	4 ± 2.1	19.2 ± 6.6	41 ± 17.3	21.3 ± 14.6
	(±)-limonene	stay	17	4.7 ± 1.7	102.3 ± 97.7	30.3 ± 26.7	2.6 ± 0.7	48.3 ± 20.5	138.9 ± 27	115.7 ± 28	64 ± 23.8
		antennae	11	0.1 ± 0.1	1.3 ± 0.5	1.6 ± 0.9	1.3 ± 0.9	0	4.5 ± 1.8	6.8 ± 3.7	5.3 ± 3.3
		ovipositor	6	0.3 ± 0.3	1.8 ± 1	76.2 ± 74	0.5 ± 0.3	0.4 ± 0.4	78.6 ± 45.3	69.7 ± 39.1	5.8 ± 4.2
	linalool	stay	20	10.3 ± 4.6	14.9 ± 9.4	6.4 ± 2.9	2.6 ± 0.6	128.3 ± 27.3	98.9 ± 28	90.3 ± 27	152 ± 30.4
		antennae	15	2.2 ± 1	2.2 ± 1.5	1 ± 0.5	1.4 ± 0.4	2.3 ± 0.9	0.9 ± 0.7	4 ± 3.2	2.1 ± 0.8
		ovipositor	8	1.8 ± 0.4	0.6 ± 0.4	0.5 ± 0.4	1.6 ± 0.5	53.9 ± 20.4	38.6 ± 33.8	32.9 ± 32.7	35.7 ± 17.2
	(E)/(Z)-linalool oxide (fur/pyr)	stay	12	44.7 ± 19.1	17.4 ± 8.3	24.5 ± 14.1	13.3 ± 8.2	66 ± 23.4	46.6 ± 20.8	28.5 ± 12.7	84.1 ± 31.4
		antennae	2	0	0	1.5 ± 0.5	0.5 ± 0.5	0	0	1.9 ± 1.1	0.9 ± 0.9
		ovipositor	2	1 ± 1	1 ± 0	0	0	4.1 ± 4.1	66.7 ± 64.4	0	0
	methyl salicylate	stay	17	8.2 ± 1.6	4.3 ± 0.8	7.3 ± 1.4	7.9 ± 1.9	88.7 ± 22.8	116.6 ± 31.5	44.1 ± 17.8	93.4 ± 20.8
		antennae	16	1.1 ± 0.4	0.8 ± 0.4	1 ± 0.5	2.1 ± 0.7	1.9 ± 0.7	1 ± 0.5	1 ± 0.5	3.4 ± 1.3
		ovipositor	10	1 ± 0.5	1.5 ± 1.2	0.4 ± 0.2	1.2 ± 0.5	17.9 ± 13.3	4.8 ± 4	1.2 ± 0.8	9.6 ± 4.7
	(S)-(-)-perillaldehyde	stay	20	3.3 ± 0.6	2.5 ± 0.9	3.1 ± 0.8	2.5 ± 0.7	136.4 ± 31.9	120.1 ± 30.2	33.7 ± 18.4	74.9 ± 25.8
		antennae	11	1.2 ± 0.6	1.1 ± 0.6	0.3 ± 0.2	0.9 ± 0.4	5 ± 2.7	5.2 ± 2.9	0.9 ± 0.7	2.6 ± 1

			n								
		ovipositor	5	0.8 ± 0.6	1 ± 0.8	0.6 ± 0.6	2 ± 0.9	39.5 ± 39.2	19.7 ± 12.1	0.3 ± 0.3	73.4 ± 43.8
	(E)-β-caryophyllene	stay	16	40.4 ± 6.9	42 ± 9.6	29.8 ± 5	34.1 ± 8.1	103 ± 11.7	134.1 ± 18.6	78 ± 15.7	98.6 ± 15.1
		antennae	11	0.7 ± 0.2	0.9 ± 0.3	0.2 ± 0.2	0.6 ± 0.2	3.5 ± 1.2	3.4 ± 1.1	0.9 ± 0.9	2.7 ± 1.2
		ovipositor	2	1 ± 0	1 ± 1	0 ± 0	2 ± 2	3.1 ± 2.9	20.7 ± 20.7	0 ± 0	2.4 ± 2.4
	(-)-α-cedrene	stay	19	15.5 ± 2.6	21.2 ± 4.7	14 ± 2.4	16.3 ± 3.1	66 ± 12	75.6 ± 11.8	75.3 ± 11.6	67.4 ± 11.5
		antennae	9	0.4 ± 0.2	0.3 ± 0.2	0.1 ± 0.1	0.8 ± 0.2	0.4 ± 0.2	0.6 ± 0.4	0.1 ± 0.1	0.7 ± 0.2
		ovipositor	11	1.5 ± 0.8	2.6 ± 1.3	5.7 ± 1.7	2.4 ± 0.8	10 ± 6.6	16.3 ± 11.1	31.3 ± 8.9	8.5 ± 2.9
	cumene	stay	14	22.1 ± 10.7	46.5 ± 22.1	35.6 ± 17.4	18.7 ± 9.8	49.9 ± 15.1	57.2 ± 17	129.2 ± 27.3	72.9 ± 20.9
		antennae	5	0.6 ± 0.4	0.2 ± 0.2	1.2 ± 0.5	0.4 ± 0.4	0.7 ± 0.4	0.3 ± 0.3	2.4 ± 1.2	0.3 ± 0.3
		ovipositor	4	0.5 ± 0.5	17.2 ± 14.1	3.8 ± 1.8	1 ± 1	0.5 ± 0.5	18.3 ± 16.8	37.6 ± 24.9	10.4 ± 10.4
	α/β-farnesene (mixture of isomers)	stay	18	17.6 ± 5.1	2.2 ± 0.7	1.7 ± 0.7	6.8 ± 3.7	44.3 ± 19.4	40 ± 20.2	39.1 ± 20.9	44 ± 22.2
		antennae	5	2.2 ± 1.2	1 ± 0.3	1.4 ± 1	3 ± 1.9	2.6 ± 1.2	1.9 ± 1.1	0.8 ± 0.5	1.2 ± 0.7
L. botrana		ovipositor	2	0 ± 0	1.5 ± 1.5	0.5 ± 0.5	0.5 ± 0.5	0	11.7 ± 11.7	0.2 ± 0.2	0.2 ± 0.2
	(±)-limonene	stay	20	40.7 ± 9.8	40.8 ± 8.8	34.1 ± 8.7	29.1 ± 6.7	73.2 ± 14.8	62.6 ± 13.3	84.3 ± 13.8	93.1 ± 18.6
		antennae	6	0.8 ± 0.3	0.3 ± 0.2	0.5 ± 0.2	1.2 ± 0.5	2.8 ± 1.2	0.7 ± 0.5	1.9 ± 1.1	3.4 ± 1.3
		ovipositor	5	0.2 ± 0.2	0.2 ± 0.2	2 ± 1.1	2.8 ± 1.7	1.6 ± 1.6	0.4 ± 0.4	14.9 ± 9.2	27.7 ± 16.7
	linalool	stay	16	4.8 ± 2.5	3.4 ± 0.8	2.4 ± 0.8	23.2 ± 18.4	26.4 ± 9.8	32.4 ± 10	7.7 ± 3	44.5 ± 17.9
		antennae	6	0.2 ± 0.2	0.3 ± 0.2	0.2 ± 0.2	3.8 ± 2.3	0.3 ± 0.3	0.9 ± 0.8	0.5 ± 0.5	7.6 ± 3.1
		ovipositor	6	0.5 ± 0.3	1 ± 0.5	0.2 ± 0.2	0.3 ± 0.3	5.9 ± 4.8	32.7 ± 21.3	0.1 ± 0.1	0.9 ± 0.9
	(E)/(Z)-linalool oxide (fur/pyr)	stay	8	14.1 ± 7.8	14.4 ± 7.9	20.9 ± 12.8	18.6 ± 12.7	84 ± 42.8	20 ± 11.5	32 ± 20.6	78.7 ± 36.9
		antennae	2	0 ± 0	1.5 ± 1.5	2 ± 0	1.5 ± 0.5	0	2.3 ± 2.3	3.4 ± 2.2	3.1 ± 2.4
		ovipositor	3	3.3 ± 0.9	0 ± 0	0.3 ± 0.3	3.3 ± 3.3	42.1 ± 24.5	0	0.5 ± 0.5	38.3 ± 38.3
	methyl salicylate	stay	19	32.3 ± 7.8	30.1 ± 6.2	32.1 ± 8.7	52.5 ± 17.6	59.9 ± 14	70.2 ± 19.1	56.2 ± 15.3	82.5 ± 14.9
		antennae	8	0.5 ± 0.3	0.2 ± 0.2	2.1 ± 1.7	2.5 ± 1.1	2.2 ± 1.4	0.6 ± 0.5	1.5 ± 0.9	4.1 ± 1.5
		ovipositor	8	1.6 ± 0.8	2.1 ± 1	2.2 ± 1	1.2 ± 0.6	10.4 ± 6.8	13.8 ± 9.1	17.1 ± 10.6	5.4 ± 3.8
	(S)-(-)-perillaldehyde	stay	15	29.8 ± 5	42 ± 9.6	40.4 ± 6.9	34.1 ± 8.1	56.3 ± 19.4	73.2 ± 21.2	80.3 ± 22	72.3 ± 20.9
		antennae	7	0.2 ± 0.2	0.9 ± 0.3	0.7 ± 0.2	0.6 ± 0.2	1.2 ± 0.5	0.8 ± 0.8	1 ± 0.7	2.9 ± 1.5
		ovipositor	3	0	1 ± 1	1 ± 0	2 ± 2	0	0.6 ± 0.6	1.3 ± 1.3	1.1 ± 1.1

Table S2. Summary of statistical parameters explaining the duration spent by *E. ambiguella* or *L. botrana* within the olfactometer arena according to GLM and F-test (*p<0.05; ***p<0.001). Durations may be explained by the factors 'volatile sector'[1] or 'behavior'[2]

VOC	factor	*E. ambiguella*					*L. botrana*				
		Df1	Df2	F-value	p-value		Df1	Df2	F-value	p-value	
(E)-ß-caryophyllene	sector	3	154	0.63	0.6	n.s.	3	110	3,68	0.05	n.s.
	behavior	2	154	26.08	1.76×10^{-10}	***	2	110	193,98	2.00×10^{-16}	***
(-)-*α*-cedrene	sector	3	116	0.47	0.7	n.s.	3	144	1,05	0.37	n.s.
	behavior	2	116	30.37	2.47×10^{-11}	***	2	144	106,02	2.00×10^{-16}	***
cumene	sector	3	152	0.55	0.64	n.s.	**3**	**80**	**4,03**	**0.01**	*
	behavior	2	152	37.54	5.62×10^{-14}	***	2	80	47,12	3.00×10^{-14}	***
α/β-farnesene (mixture)	sector	**3**	**166**	**7.84**	**6.36×10^{-5}**	***	3	138	0,72	0.53	n.s.
	behavior	2	166	21.53	4.88×10^{-9}	***	2	138	3,32	0.04	*
(±)-limonene	sector	**3**	**130**	**6.68**	**3.01×10^{-4}**	***	3	118	2,84	0.05	n.s.
	behavior	2	130	26.95	1.62×10^{-10}	***	2	118	99,66	2.00×10^{-16}	***
linalool	sector	3	166	2.93	0.05	n.s.	3	106	2,71	0.05	n.s.
	behavior	2	166	38.28	2.13×10^{-14}	***	2	106	9,01	2.04×10^{-4}	***
(E)/(Z)-linalool oxide (pyr/fur)	sector	3	58	0.94	0.43	n.s.	**3**	**46**	**11,47**	**0.02**	*
	behavior	2	58	7.68	1.10×10^{-3}	***	2	46	2,47	0.09	n.s.
methyl salicylate	sector	3	166	2.73	0.05	n.s.	3	134	0,54	0.66	n.s.
	behavior	2	166	73.69	2.00×10^{-16}	***	2	134	65,16	2.10×10^{-16}	***
(S)-(-)-perillaldehyde	sector	3	116	3.50	0.06	n.s.	**3**	**106**	**6,67**	**3.59×10^{-4}**	***
	behavior	2	116	11.77	1.90×10^{-5}	***	2	106	5,62	4.78×10^{-3}	***

Bold values represent VOCs influencing *E. ambiguella* or *L. botrana*.

[1]4 levels: DCM, reference 1 (air), reference 2 (air) and VOC

[2]3 levels: stay, antennae-activity and ovipositor-activity

May alternative plants contribute to the spread of the European grapevine moth _Lobesia botrana_ and European grape berry moth _Eupoecilia ambiguella_ in vineyards?

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Abstract

Under the aspects of climatic change, insect pests are capable to shorten their life cycle, tend to appear earlier in the season and develop more generations per year than usual. Consequently, insects may have to seek out alternative hosts in case of their main host plant not being available. It was assessed whether or not alternative plant species in the surrounding vegetation of vineyards are capable of providing the first generation of the European grapevine moth *Lobesia botrana* and European grape berry moth *Eupoecilia ambiguella*, two major insect pests in viticulture, distinguishable stimuli from those of their main host, grapevine *Vitis vinifera*. The effect of volatiles, emitted by alternative plant species, on short-range attraction was evaluated under laboratory conditions in a four-chamber olfactometer. Although a higher number of females were attracted to the odour of *Vitis vinifera* cv. 'Regent' inflorescences compared to the odour of the alternative hosts, females of *L. botrana* were significantly attracted to volatiles emitted by the Common hawthorn *Crataegus monogyna*. However, accompanying oviposition assays showed that this host does not appear to be suitable for initiating reproduction if compared to grapevine. While none of the investigated plants was able to compete against grapevine in case of *E. ambiguella*, *L. botrana* preferred Common ivy (*Hedera helix)* berries as well as dandelion (*Taraxacum sect. Ruderalia)* and Common privet (*Ligustrum vulgare)* inflorescences over inflorescences of grapevine for oviposition. This confirms that alternative plant species may act as reservoir hosts for the spread of these insect pests.

1. Introduction

The impact of climatic change on the phenology of grapevine (Biasi et al., 2019) as well as the development of grapevine diseases and pests (Biasi et al., 2019; Caffarra et al., 2012; Schneider et al., 2022) is well known. Indeed, climatic change can affect crop-pest interactions through various ways (Schneider et al., 2022), although insect species may react faster to climatic adaptions than plants as consequence of their generally short life cycle (Vitasse et al., 2021).

Such adaptions are also reported for the species *Lobesia botrana* (Denis & Schiffermüller) and *Eupoecilia ambiguella* (Hübner) (Lepidoptera: Tortricidae), two of the most important insect pests threatening Western European viticulture (Benelli et al., 2023; Ioriatti et al., 2011). Under Central European conditions, *E. ambiguella* develops two generations per year, while *L. botrana* is able to build up to three generations (Benelli et al., 2023; Lucchi, 2015), while diapause is facultative (Ioriatti et al., 2023). Climatically favourable conditions, e.g. an increase in the mean daily air temperature, cause a shift in the flight activity of the first generation of *E. ambiguella* and *L. botrana*, resulting in an occurrence earlier in the season (Blümel et al., 2020; Comsa et al., 2022). It is evident that this shift does not necessarily correlate with the developmental stage of their actual host grapevine, as suspected by Gabel (1992). Consequently, it is possible that moths of the first generation are in search of alternative host plants to reproduce instead of inflorescences of grapevine.

Although grapevine is reported as major host for both species (EPPO, 2023a, 2023b), *L. botrana* has already been found on over 40 plant species from 27 families as reviewed by Lucchi (2015) and more recently Benelli et al. (2023), most of them belonging to the genera Vitaceae, Thymelaeaceae, Rosaceae, Oleaceae, Ranunculaceae, Polygonaceae, Apiaceae, Asteraceae, Convolvulaceae and Rhamnaceae (Balachowsky & Mensil, 1935; Bovey, 1966; Galet, 1982; Roditakis, 1989; Stoeva, 1982). The first generation is anthophagous (flower-feeding), while the others are carpophagous (fruit-feeding). Some hosts are cultivated plants e.g. *Olea europaea* (Stoeva, 1982), currant (*Ribes uva-crispa*), cherry (*Prunus avium*) and plum (*Prunus domestica*) (Balachowsky & Mensil, 1935; Bovey, 1966), while the flax-leaved daphne (*Daphne gnidium*) (Thymelaeaceae) is reported as main wild host of *L. botrana* (Balachowsky & Mensil, 1935; Maher & Thiéry, 2006): The species was observed e.g. on *Clematis* spp., *Ligustrum vulgare, Medicago sativa, Syringa vulgaris, Solanum tuberosum, Tanacetum vulgare,* and *Cornus alba* (EPPO, 2023a, 2023b; Thiéry & Moreau 2005; Stoeva, 1982 Balachowsky & Mensil, 1935; Bovey, 1966;). Little to no information is available regarding the damage of *E. ambiguella* on alternative plants, although the species was already spotted on

Prunus spp. and *Crataegus* spp. (Lucchi, 2015). The wide host plant range of at least *L. botrana* highlights that there is a possible risk of an infestation of other plants in proximity to vineyards. This does not necessarily lead to economically significant damage to these crops, but may favour the establishment of the pest within the vineyard. Especially in herbivorous insects, certain plant species may be favoured over other plants.

The aim of the study was therefore to evaluate if vineyard companion crops present in Germany may (i) attract *L. botrana* and/or *E. ambiguella* females and (ii) provide an alternative habitat for initiating reproduction. Thereby, it is assumed that volatiles, emitted by a host plant, had the strongest influence on female orientation to a plant (Tasin et al., 2011), while gustatory, visual and tactile stimuli provided by the plant play an important role for selecting suitable surfaces for egg deposition (Maher & Thiéry, 2004; Maher et al., 2006; Markheiser et al., 2018; Rid et al., 2018). Hence, the effect of volatiles, emitted by grapevine-competing plant species, on short-range attraction of *L. botrana* and *E. ambiguella* was evaluated under laboratory conditions in a four-chamber olfactometer. Their potential for reproduction was estimated by oviposition assays, while grapevine was selected as competing host. The results achieved herein should contribute to assess the risk posed by alternative hosts, being situated close to the vineyard plot, for the spread of the insect pests in case of an absence of inflorescences of the actual host plant.

2. Materials and methods

2.1. Insects

Adults of *L. botrana* and *E. ambiguella* used in the studies were taken from a laboratory culture established at the Julius Kühn-Institut in Siebeldingen, Germany. Moths were mass-reared as described in Markheiser et al. (2018) on a semi-artificial diet. Pupae were removed from the culture, sexed and individually, separated into 15 ml falcon tubes which were closed with a moistened cellulose plug. Approximately one week after adults hatch, single female and male moths were allowed to copulate for 48 hours. Single gravid females, depositing >10 eggs within the copulation period, were used for the olfactometer assays. Four couples were used for the oviposition assays to enable sufficient quantities of eggs. The two insect cultures were kept separated from each other under controlled climatic conditions of 23:19 ± 2°C (day: night setback), 70 ± 10% relative humidity and a 14:8 h photoperiod (+1 h each of dusk and dawn) in plant growth chambers "Fitotron type SGR233" (Weiss Technik UK Ltd, Loughborough, UK).

2.2. Plant material

Six host plant species were selected (Table 1) as grapevine competing candidates for this study, as all of them could be detected in close proximity to the vineyard plots at Julius Kühn-Institute, Siebeldingen, Germany, and/or were reported host of at least one of the two moth species. They were either flowering or fruiting at the time of investigation (beginning of April - end of June).

Table 1: Plant species used in the studies and their phenological development stage during the investigation period. Plant fructiferous organs examined in the bioassays are highlighted in bold. Plant genera were described as host of *Lobesia botrana* (LB) and/or *Eupoecilia ambiguella* (EA) according to Balachowsky & Mensil (1935), Bovey (1966), Roditakis (1989), Stoeva (1982), Stavridis & Savopoulou-Soultani (1998) and EPPO (2023a, 2023b).

Common name	Scientific name	Flowering (Begin-End)	Fruiting (Begin – End)	Reported host of
Grapevine	*Vitis vinifera*	**May - June**	Sept. – Nov.	EA, LB
European ivy	*Hedera helix*	Sept.—Oct.	**March - April**	LB
Common dandelion	*Taraxacum sect. Ruderalia*	**April - May**	-	LB
Common hawthorn	*Crataegus monogyna*	**May - June**	Aug. – Sept.	EA
European privet	*Ligustrum vulgare*	**June - July**	Sept. – Oct.	LB
European blackberry	*Rubus fructicosus*	**May - Aug**	Aug. – Sept.	LB
Common dogwood	*Cornus sanguinea*	**May - June**	Sept.	EA, LB

Grapevine inflorescences cv. Regent were taken from potted greenhouse plants at Julius Kühn-Institute, Siebeldingen, Germany. They were raised at different phenological stages at 25±5°C, 20-40% relative humidity and a photoperiod of 16:8 h with a minimum of 2.500 lux light intensity and supplementing artificial light if levels fall below. In order to provide inflorescences over a longer experimental period, cuttings were potted delayed in time.

The candidate host plants were inspected in the field at least once a week in order to take plant samples within the optimum period for the bioassays. In case of *Hedera helix,* berries instead of inflorescences were used as competing fructiferous plant organ (stage 80-81 according to BBCH Biologische Bundesanstalt für Land- und Forstwirtschaft, Bundessortenamt und CHemische Industrie scale of Hack et al. (1992)), as this stage corresponded to the seasonal development of the grape inflorescences during this study (compare Table 1). In all other cases of alternative plants, inflorescence clusters were used (stage 59 according to extended BBCH scale of Hack et al. (1992): first petals visible, flowers still closed and end of spike or panicle emergence). Grapevines organs were collected in stage 57-59 (inflorescences fully developed) of BBCH scale of Lorenz et al. (1994).

2.3. Four-chamber olfactometer assays

The short-range attraction of females of either *E. ambiguella* or *L. botrana* to the plant odor, emitted by the plant organs, was investigated according to the method of Markheiser et al. (2020) by using a 4-chamber olfactometer (Sigma Scientific LLC, Micanopy, FL, USA). The olfactometer system essentially consists of two elements: a 4-armed insect arena and a clean air delivery system, which ensures a uniform flow of purified air within the arena.

The inflorescences of the alternative hosts and grapevine were weighed using a precision scale type XS204 Delta Range (Mettler Toledo GmbH, Gießen, Germany) and cut to the same weight of fresh mass of approximately 35 g. Cutting sites were sealed with Parafilm, type M (Bemis, Neenah, USA) to reduce the release of volatiles released upon damage. The plant material was introduced into the olfactometer system via an inline odor source adaptor. Plant organs were placed in two opposite directions following Markheiser et al. (2020) in order to inhibit an overlap of the two volatile sectors. As control, the odor of clean air was compared to volatiles emitted by inflorescences of *V. vinifera* cv. Regent. The six alternative plant species (ivy, dandelion, hawthorn, privet, blackberry and dogwood) were exclusively compared to grapevine as competing host. Clean air was provided in the remaining two sectors.

Single gravid females were introduced into the olfactometer system via the insect inlet adapter below the arena's centre. A digital camera (Basler GenICam acA1300-30um, Basler AG, Ahrensburg, Germany), linked to the video tracking software EthoVision®XT - version 10 (Noldus Information Technology, Wageningen, Netherlands), was installed above the arena in order to visualize and define the volatile sectors within the arena and track the insects behaviour following Markheiser et al. (2020). The arena was uniformly illuminated (90 lx at the four sector entrances), allowing moths to be recognized within the arena by the software.

Video-tracking started as soon as the insect entered the arena and stopped automatically after a period of 5 min. The cumulative duration in seconds spent by each insect in the individual four volatile sectors (two air controls, *V. vinifera* and alternative plant species) was calculated. 24 trials were carried out per insect species and host plant resulting in a total number of 336 trials. The arena was rotated by 180° after 12 trials to exclude any influence of the position of the plant on the short-range attraction. After each experiment (host plant and/or insect species), the arena and all connected glass and Teflon elements were rinsed with 70% ethanol.

2.4. Oviposition assays

The suitability of alternative hosts for oviposition of *E. ambiguella* and *L. botrana* was tested in comparison to grapevine following Rid et al. (2018) and Tasin et al. (2011), while enabling the access to olfactory, visual and contact stimuli. Inflorescences or berries (compare Table 1) were cut off and assigned to equal weights in fresh mass, as reported in chapter 2.3. To enhance the shelf life of the plant material, inflorescences or berries were placed at the top of 30 ml plastic cups with perforated lids (Huthamaki GmbH, Alf, Germany) providing a water source. The cut plant parts were placed into the cups in order to reduce an effect of volatiles emitted by the injury. The cups were placed in a gauze cage (60x40x40 cm, The Caterpillar Castle, Live Monarch Foundation, Boca Raton, USA), at a distance of 20 cm from each other. 10 reciprocal cages per moth species and comparative plant were set up. An empty water source was tested against grapevine inflorescences as control. The plant position within the cages was rotated between cages in order to avoid a position effect on oviposition. Four coupled males and females of either *L. botrana* or *E. ambiguella* were added to each cage.

The cages were kept in walk-in climatic chambers 'Fitotron type SGR233' (Weiss Technik UK Ltd, Loughborough, United Kingdom) at 23:19±2 °C, 70±10 % relative humidity and a 14:8 (light:dark) photoperiod with 1 h each of dusk and dawn. The experiment was finished after 72 h by removing the couples from the cage and counting the eggs deposited on the single plants. Different oviposition sites within the inflorescence of the plants (e.g. flower stalk or flower bud) were recorded. After each experiment, the climatic chambers were heated to 60 °C for at least 3 hours and the cages were wiped out with 70 % ethanol to remove remaining odors.

2.5. Statistical analyses

The effect of plant species on short-range attraction was assessed by generalized linear models (GLMs) performed for each insect taxon separately. The response variable was the cumulative duration spent within a plant volatile sector in seconds; the categorical explanatory variables was the volatile sector (plant species). Post hoc comparisons between durations spent by the species in single volatile sectors were generated using estimated marginal means with Tukey's p-value adjustment. Olfactometer assays were considered valid if the two air controls did not differ statistically significant to each other. Significance level was set at $p<0.05$.

To estimate the oviposition competing effect of alternative host plants against grapevine, the oviposition discrimination index (ODI) was calculated according to Maher et al. (2004) in order to compensate the variable egg deposition rates of single females:

$$ODI \% = \frac{\text{no. of eggs on alternative plant} - \text{no. of eggs on grapevine}}{\text{total no. of eggs}} \times 100 \%$$

The value might range from -100 to +100%. Negative ODIs imply a preference of grapevine, whereas positive ODIs represent a preference of the alternative host for oviposition. Statistically significant differences in ODIs between grapevine and alternative host were calculated by the nonparametric Wilcoxon signed rank test for paired data sets (egg counts) due to a non-parametric dataset. Analyses were performed in R version 4.2.2 (R Core Team, 2022). GLMs were carried out with 'lme4' (Bates et al., 2015) and 'emmeans' package (Russell, 2018). Boxplots were generated using packages 'ggplot2' (Wickham, 2016) and 'ggstatsplot' (Patil, 2021).

3. Results

3.1. Short-range attraction induced by grapevine inflorescences

Gravid female moths of *E. ambiguella* as well as *L. botrana* were attracted to volatiles emitted by inflorescences of the main host *V. vinifera* in the 4-chamber olfactometer (Figure 1). For simplicity, the two air controls adjacent to the test plants in the olfactometer system are generally not illustrated in the following figures.
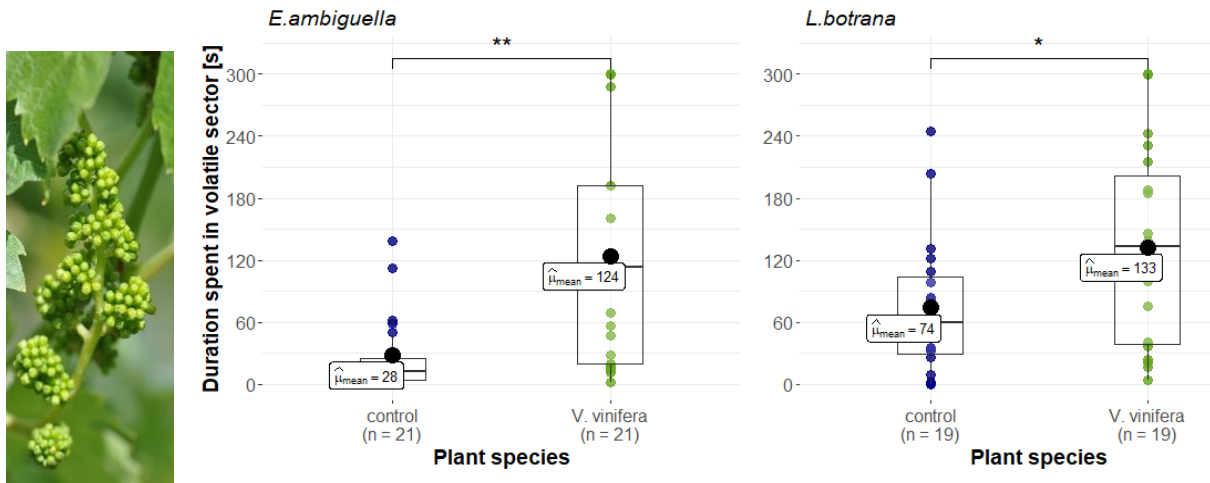
Figure 1: Mean (μ_{mean}) cumulative duration (CD) spent by (left) *Eupoecilia ambiguella* and (right) *Lobesia botrana* in the volatile sector of inflorescences of (blue) clean air as control compared to (green) inflorescences of grapevine *Vitis vinifera* cv. Regent. Statistically significant differences in the CD between control and grapevine were estimated by GLM and post-hoc comparisons by emmeans (*p<0.05, **p<0.01; in boxplots: black dots = means, colored dots = individual observations).

The mean cumulative duration spent within the volatile sector of *V. vinifera* cv. Regent for both female moth species (EA: *E. ambiguella*; LB: *L. botrana*) significantly higher (GLM$_{EA}$: $F_{3,84}$=-8.2, p<0.01), GLM$_{LB}$: $F_{3,76}$ =-5.4, p<0.05) than in the air control (no plant source).

3.2. Short-range attraction induced by alternative hosts

The potential of alternative plant species (ivy, dandelion, hawthorn, privet, blackberry and dogwood) to attract females of *E. ambiguella* and *L. botrana* was investigated in comparison to *V. vinifera* 'Regent inflorescences. Time periods in which the insects were not in any of the two plant volatile sectors, they were either in the overlapping control sectors or retracted the insect inlet adapter. Over all the 4-chamber olfactometer assays, none of the two air control sectors, located in the periphery of the plant sectors, was more attractive to the females than one of the two host plants (p>0.05, data not shown).

3.2.1. Berry clusters

Common ivy *H. helix* was the only plant species in this study, whose fruiting organs were in berry stage during the period of investigation (compare Figure 2, left).
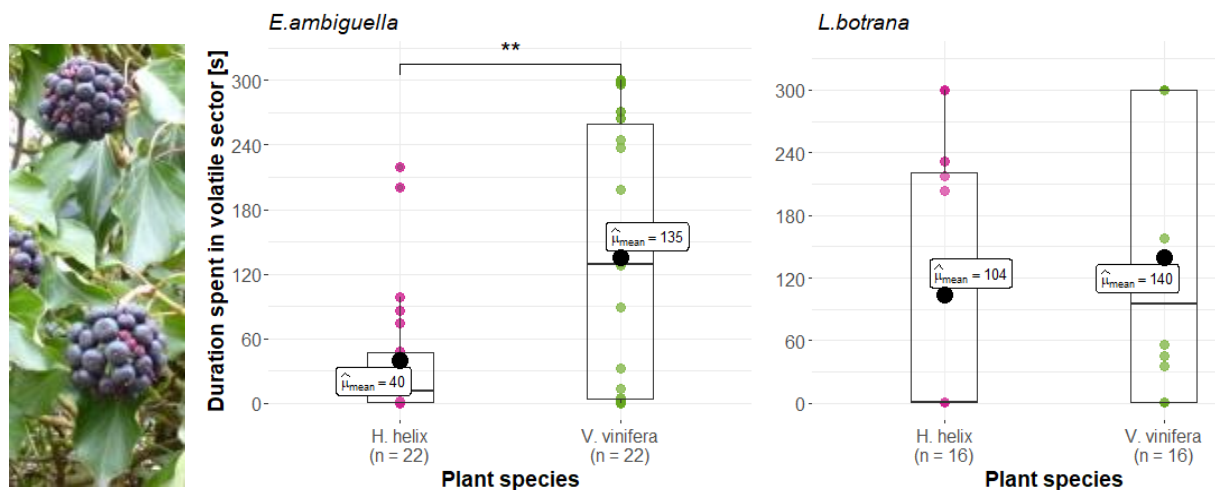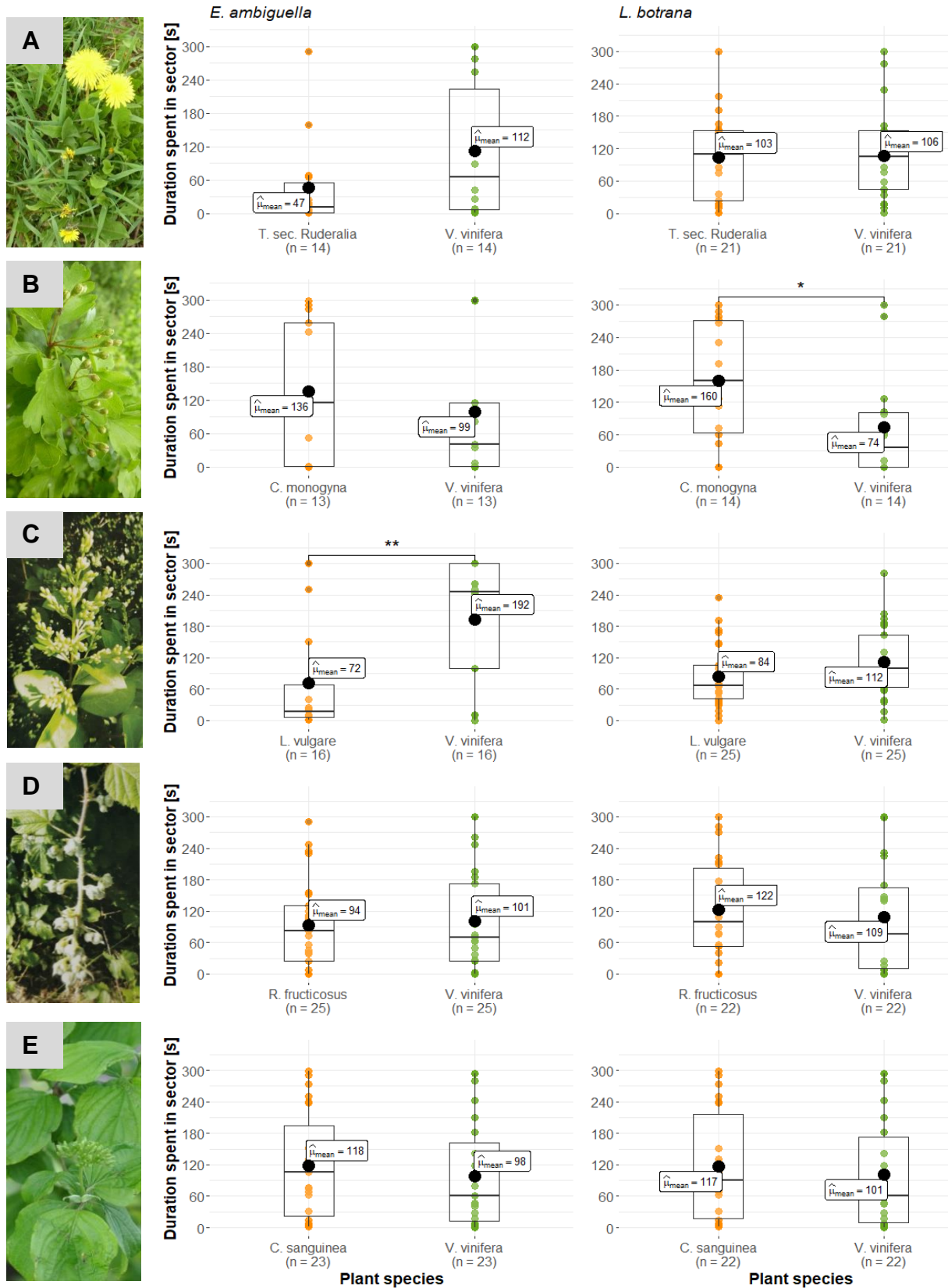

Figure 2: Mean (μ_{mean}) cumulative duration (CD) spent by (left) *Eupoecilia ambiguella* and (right) *Lobesia botrana* in the volatile sector of (violet) berry clusters of ivy *Hedera helix* compared to (green) inflorescences of grapevine *Vitis vinifera* cv. Regent. Statistically significant differences in the CD between ivy and grapevine were estimated by GLM and post-hoc comparisons by emmeans (*p<0.05, **p<0.01; in boxplots: black dots = means, colored dots = individual observations).

While females of *L. botrana* spent similar amount of time under the odor of the two plant species (GLM$_{LB}$: $F_{3,64}$=-0.2, p>0.05), females of *E. ambiguella* were significantly (GLM$_{EA}$:

$F_{3,88}$=-3.1, p<0.01) more attracted to grapevine inflorescences if compared to *H. helix* berries, while the motivation to enter the arena was higher in *E. ambiguella* (92 % of the females) than in *L. botrana* (67 % of the females).

3.2.2. Inflorescences

Five alternative host plants were tested in pre-flowering stage for the competing effect against grapevine inflorescences (Figure 3). In terms of short-range attraction, volatiles emitted by *T. sec.* Ruderalia (figure 3A) could not be distinguished from those of *V. vinifera*, neither in case of. *E. ambiguella* (GLM_{EA}: $F_{3,56}$=-2.1, p>0.05) nor *L. botrana* (GLM_{LB}: $F_{3,84}$=-0.1, p>0.05), although *E. ambiguella* tended to prefer grapevine over this plant species.

Volatiles emitted by *C. monogyna* (Figure 3B) tend to attract females of both moths species more than those of grapevine, although this effect was only statistically significant for *L. botrana* (GLM_{LB}: $F_{3,56}$=2.4, p<0.05). In contrast, volatiles emitted by *L. vulgare* (Figure 3C) tended to be less suitable for short-range attraction of the two species than those of grapevine, although this was only statistically significant for *E. ambiguella* (GLM_{LB}: $F_{3,64}$=3.2, p<0.01).

In case of *R. fructicosus* (Figure 3D; GLM_{EA}: $F_{3,96}$=0.1, p>0.05; GLM_{LB}: $F_{3,88}$=0.3, p=>0.05) and *C. sanguinea* (Figure 3E; GLM_{LB}: $F_{3,92}$=0.4, p>0.05; GLM_{LB}: $F_{3,88}$=0.2, p=>0.05), females of both moth species do not differentiate statistically significant between volatiles of the alternative host plant and grapevine, evident by the comparable duration spent in the two plant volatile sectors.

Figure 3: Mean (μ_{mean}) cumulative duration spent by (left) *Eupoecilia ambiguella* and (right) *Lobesia botrana* in the volatile sector of inflorescences of (orange) alternative plants (A) *Taraxacum sect. Ruderalia,* (B) *Crataegus monogyna,* (C) *Ligustrum vulgare,* (D) *Ribes fructicosus* and (E) *Cornus sanguinea* compared to (green) grapevine *Vitis vinifera* cv. Regent. Statistically significant differences between alternative plants and *V. vinifera* were estimated by GLM and post-hoc comparisons by emmeans (*p<0.05, **p<0.01; in boxplots: black dots = means, colored dots = individual observations).

3.3. Oviposition behaviour on alternative hosts

Oviposition of both species on alternative plants was quantified in a competing setup to grapevine (Figure 4). In general, both species preferred to oviposit on grapevine if compared to an empty water source (Figure 4, control).
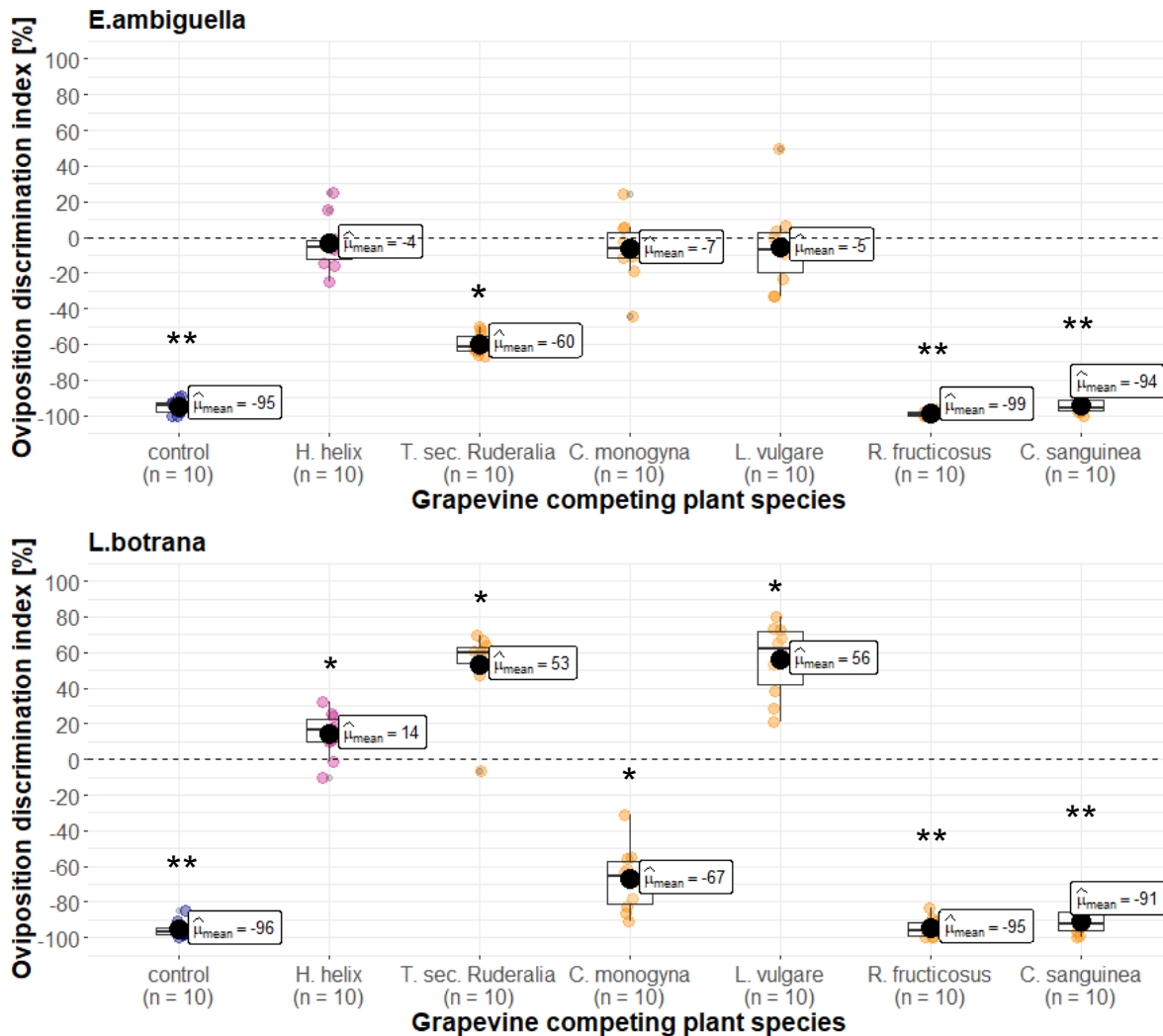
Figure 4: Mean (μ_{mean}) oviposition discrimination index (ODI) of (top) *Eupoecilia ambiguella* and (bottom) *Lobesia botrana* while comparing (blue) water source as control (violet) berries of *Hedera helix* and (orange) inflorescences of *Taraxacum sect. Ruderalia, Crataegus monogyna, Ligustrum vulgare, Ribes fructicosus* and *Cornus sanguinea* against inflorescences of grapevine *Vitis vinifera* cv. Regent. Statistically significant differences between single plant species and *V. vinifera* were estimated by non-parametric Wilcoxon signed rank test (*p<0.05, **p<0.01; in boxplots: black dots = means, colored dots = individual observations). Negative ODIs imply a preference for grapevine and positive ODIs a preference for the competing plant species in case of oviposition.

The species *E. ambiguella* (Figure 4, top), significantly preferred grapevine inflorescences over those of dandelion (Wilcoxon signed rank test; p<0.05), blackberry (Wilcoxon signed rank test; p<0.01) and dogwood (Wilcoxon signed rank test; p<0.01) for oviposition. In case of berry

clusters of ivy as well as inflorescences of hawthorn and privet, *E. ambiguella* was not able to distinguish between alternative plant species and the main host plant grapevine.

The situation is different for *L. botrana* (Figure 4, bottom). In all comparative studies on oviposition, this species clearly differentiated between alternative plant species and grapevine. In addition, this species deposited statistically significant more eggs on berry clusters of ivy (Wilcoxon signed rank test; $p<0.05$) and inflorescences of dandelion (Wilcoxon signed rank test; $p<0.05$) and privet (Wilcoxon signed rank test; $p<0.05$) than on inflorescences of grapevine. The number of eggs laid by *L. botrana* on hawthorn (Wilcoxon signed rank test; $p<0.05$), blackberry (Wilcoxon signed rank test; $p<0.01$) and dogwood (Wilcoxon signed rank test; $p<0.01$) was significantly lower than on grapevine inflorescences.

The mean number of eggs laid per female in this bioassay was 38 ± 21 (standard deviation) in case of *L. botrana* and 31 ± 14 in case of *E. ambiguella*. Within the inflorescences, both the flower buds and pedicles were covered with eggs. In case of dandelion buds, *L. botrana* laid significantly more eggs (Wilcoxon signed rank test; $p< 0.05$) on the petals than on the sepals. Within the privet inflorescences, both *E. ambiguella* and *L. botrana* significantly preferred (Wilcoxon signed rank test; $p<0.05$) oviposition on buds over pedicles.

4. Discussion

As a result of climate change, insect pests such as the European grapevine moth *L. botrana* and the European grape berry moth *E. ambiguella* are capable to develop more generations per year than usual and appear earlier in the season (Blümel et al., 2020; Comsa et al., 2022). This may cause an increased asynchrony between moths of the first generation of *L. botrana* and *E. ambiguella* and the inflorescences of grapevine (Caffarra et al., 2012; Ioriatti et al., 2023). As a consequence, moths are dependent on seeking out alternative plant species in case of their actual host plant not being available in order to reproduce and survive. Thereby, the host plant choice depends on the location, with adaptations to the local climate and fauna (Ioriatti et al., 2011). This approach may contribute to maintain population levels, so the vineyard is still at risk of being infected by the following generations of these insect pests.

It was already proven that landscape elements in the surrounding vegetation of vineyards (e.g. hedgerows) host populations of *L. botrana* (Sciarretta et al., 2008). Furthermore, larvae of *L. botrana* were found on inflorescences of *Olea europaea* (olive) trees near vineyards in Greece (Savopoulou-Soultani et al., 1990), Italy (Sciarretta et al., 2008) and Bulgaria (Stoeva, 1982), while up to 45% of olive flowers were infested by first generation larvae in the latter

case. Sciarretta et al. (2008) observed, that the abundance of *L. botrana* of the first generation was higher in olive groves than in vineyards, while moths of the second and third generation rather moved into the vineyard. This could be a result of a different development status of the host plants, as olive inflorescences can emerge 4-6 weeks earlier than those of grapevine (Stavridis & Savopoulou-Soultani, 1998). In addition, Maher & Thiéry (2006) observed, that wild hosts such as berries of *D. gnidium* were preferred over grapes by *L. botrana* as oviposition substrate, while this plant also provided greater nutritional value for the larvae (Thiéry & Moreau, 2005). The authors concluded that the use of alternative plants could be maintained in the host range *L. botrana* because they offer a better fitness than Vitaceae. This concept falls under the preference performance hypothesis of Gripenberg et al. (2010), i.e. female insects oviposit on plants on which the offspring will perform best.

However, in several cases this hypothesis could not be verified, with females choosing plants with a suboptimal fitness for their offspring. This was proven in experiments of Savopoulou-Soultani et al. (1990), who observed higher egg laying rates on grapevine compared to olive inflorescences, but larvae of *L. botrana* developed on olive faster and had a higher weight than those on grapevine. In addition, Gabel (1992) observed that tansy flowers were rather sought out by females in order to take nectar or pollen as food source, while oviposition never occurred on this plant. We suspect a similar background in case of the Common privet and *L. botrana*, as mated females were attracted by its plant odour, but oviposition was not increased compared to grapevine. Whether or not plant volatiles encode for plant quality to gravid female insects is an issue under debate (Cattaneo, 2014; Maher, 2002; Markheiser et al., 2020; Schmidt-Büsser et al., 2009; Tasin, Betta, et al., 2011). Additional evidences are necessary to confirm such an assumption.

Up to date, there seems to be poor information supporting volatiles as an "honest" signal for females in search of oviposition sites, especially in *E. ambiguella* (Tasin, Lucchi et al 2011; Rid et al. 2019). But our results are in line with the prediction that gravid females can disentangle the olfactory stimuli released by the alternative plant to those of their main host, grapevine *V. vinifera* (Cattaneo, 2014; Silva et al., 2019; Tasin, Betta, et al., 2011). However, it is also obvious that the interaction of visual, gustatory and tactile stimuli plays an important role in the final egg deposition process (Maher & Thiéry, 2004; Maher et al., 2006; Markheiser et al., 2018; Rid et al., 2018; Tasin, Lucchi, et al., 2011), finally lead to oviposition for reproduction. Thereby, gustatory stimuli may play a greater role than olfactory stimuli as supposed by Rid et al. (2019). This is evident by the fact that we observed a preference of *L. botrana* for berries of ivy (and inflorescences of dandelion and privet) over those of

grapevine inflorescences after contact to the respective plant organs, which did not emphasize under the exclusive influence of the volatile stimuli.

Nevertheless, it should be highlighted, that a preference of an alternative host plant over grapevine for egg deposition as observed in our study may also be an effect of variations in the size of the surface provided by the different composition of the inflorescences. In case of grapevine leaves, Marshall (1912) assumed that they would be preferred because they are available earlier than inflorescences and represent a larger surface area. In our experimental set up, we tried to reduce this effect by adjusting the two host plants to an identical weight level. In this way, we ensured that the quantity of volatile compounds was comparable.

It is also important to point out, that alternative host plants can play a role not only for the first generation of grapevine moths. In Greece, a fourth generation of *L. botrana* was reported to move to *Rosmarinus officinalis* (rosemary) and *Rubus idaeus* (raspberry) in the fall (Katerinopoulos et al., 2005; Roditakis, 1989). None of the two moth species was attracted by *Rubus fructicosus* in our experiments. However, under field conditions it is unclear whether they are able to complete their entire life cycle on these plants due to falling air temperatures. We therefore consider fall hosts to play a rather subordinate role in the final spread of the two insect pests and it remains an open question if they might be suitable for overwintering of *L. botrana* and *E. ambiguella*. Both species hibernate on vines as pupae under the bark (Bovey, 1966; Stellwaag, 1928), a frost-protected place only provided by woody plants.

Although Caffarra et al. (2012) assumed that the infestation risk posted by an increased number of generations would be limited due to a shorter grapevine cycle, our results together with the cited literature highlight that insects are able to develop strategies in order to ensure their survival. Nonetheless, variation in the plant preference may be feasible at least in *L. botrana*, as this species was preferably attracted to some alternative hosts for oviposition compared to grapevine. The acceptance of dandelion as feeding host was already confirmed by Stavridis & Savopoulou-Soultani (1998), whereas larval performance on this plant was lower than on berries of grapevine. Thus it can be assumed that *L. botrana* will seek out alternative host plants such as privet, dandelion and ivy to reproduce in the absence of the grapevine. However, it cannot be assumed that they disperse long distances to reach these alternative plants. Although the moths are able to fly, they do not migrate more than 80-100 m from their origin (Roehrich & Carles 1981).

For future studies it is mandatory to determine if (i) hatching larvae of these insect species can feed up-on these plants, thus considered "real hosts", (ii) *L. botrana* and *E. ambiguella* are able

to complete a full life cycle on these plants and (iii) offer an adequate fitness for the subsequent generations of the moths. This should enable to finally evaluate the risk of a possible spread with alternative host plants and to adapt improved integrated strategies for the control of these pests.

5. Conclusion

The grapevine moth *L. botrana* and the grape berry moth *E. ambiguella* were attracted to volatiles emitted by inflorescences of grapevine in our experimental setup. The presented results demonstrated that *E. ambiguella* seems to be less triggered by plant-specific stimuli than *L. botrana*, as *E. ambiguella* did not always clearly discriminate between the offered alternative plant species and grapevine with respect to olfactory stimuli, provided on short-range, as well as oviposition. This suggests that *E. ambiguella* is far less dependent on grapevines as host plants than *L. botrana*. Consequently, this species is probably more likely to accept a wide range of alternative plants for oviposition than *L. botrana*. The facts that dandelion was more attractive for *L. botrana* for oviposition than grapevine and larvae are able feed on this plant highlights that they may act as reservoir host for this species. Nevertheless, economic damage posed by single plant species is difficult to estimate, because vineyards are not completely surrounded by these plants. However, they can be a source for initial infestations.

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Chapter VII

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General discussion

Anna Markheiser

Efforts to reduce reliance on chemical insecticides and promote more environmentally friendly and economically viable pest control measures are important for the long-term sustainability of viticulture (Pertot et al., 2017; Sciarretta et al., 2011). Thereby, monitoring of insect pest can help to assess the need to apply insecticides by estimating critical thresholds and, if necessary, to schedule phytosanitary measures (Ammoniaci et al., 2021; Benelli et al., 2023; Pertot et al., 2017). In order to develop a decision support system (DSS) for an egg monitoring of the European grapevine moth *Lobesia botrana* and the European grape berry moth *Eupoecilia ambiguella*, the thesis aimed in deciphering essential factors provided by host plants that may trigger short-range attraction and oviposition of these pest and can be implemented in a DSS, such as the visual appearance of the plant (**chapter II**), the volatiles released by the plants (**chapters IV, V and VI**), the physical properties of the oviposition site (**chapter II**) and the chemical composition of the plant surface (**chapters III and VI**).

Visual factors play a crucial role in seeking host plants by diurnal insects (Prokoby & Owens, 1983) and contribute also to the orientation of nocturnal moths (Warrant & Somanathan, 2022). *Lobesia botrana* and *E. ambiguella* are generally described as crepuscular due to male's flight activity (Lucchi et al., 2018; Stellwaag, 1928). Nevertheless, oviposition activity by *E. ambiguella* was, in contrast to *L. botrana*, also observed at noon (Stellwaag, 1928). Thus, *E. ambiguella* is probably more affected by visual factors than *L. botrana*, although there is a lack of information in these species targeting these aspects (Tasin, Lucchi, et al., 2011a). In general, *E. ambiguella* selected light-protected areas for oviposition (Markheiser et al., 2018). This was also observed in the field in relation to *L. botrana* (Vogel, 1907). In this case, we suspect females were driven to protect their eggs from weather conditions (i.e. solar radiation) during the day as discussed by Zahavi et al. (2017). However, by evaluating oviposition sites in various colours (Markheiser et al., 2018), it was highlighted that females of both species preferably lay eggs on green surfaces, which corresponds to the visual appearance of unripe berries and inflorescences. Furthermore, colours may help the insects to distinguish between healthy and physiologically stressed plants, i.e. by nutrient deficiencies or virus infections, as already observed for Hemipterans (Zhang et al., 2022). Since *E. ambiguella* prefers green over yellow surfaces for oviposition (Markheiser et al., 2018), a similar strategy could lay behind. It may allow to identify hosts that provide sufficient fitness for their offspring.

Volatile organic compounds cause an olfactory perception in herbivorous insects in order to identify hosts in a complex landscape over a long range (Bruce & Pickett, 2011; Schoonhoven et al., 2005). The oriented flight of *L. botrana* (Anfora et al., 2009; Tasin et al., 2010; Tasin et al., 2006; Tasin, Betta, et al., 2011; von Arx et al., 2011) as well as *E. ambiguella* (Cha et al.,

2008; Schmidt-Büsser et al., 2009) to grapevine is well studied. In *L. botrana* for example, volatile organic compounds (VOCs) enable the insects to distinguish between healthy grapevine plants and grapevine plants infested with *Botrytis cinerea* (Tasin et al., 2012) or other microorganisms (Tasin, Betta, et al., 2011).

Furthermore, insects use VOCs to evaluate the suitability of a plant for oviposition after settlement on a plant (Schoonhoven et al., 2005). Oviposition response to VOCs by *L. botrana* has been barely studied (Tasin, Lucchi, et al., 2011a), while there is also a lack of information addressing *E. ambiguella*. Nevertheless, VOCs were found to increase egg deposition of *L. botrana* even in the absence of gustatory stimuli (Anfora et al., 2009; Tasin, Betta, et al., 2011). Hence, we aimed for a characterization of VOCs emitted by different grapevine cultivars (Rid et al., 2019), as they may explain their variable susceptibility to *L. botrana* egg depositions as observed by Birgücü et al. (2015), Moreau et al. (2008) and Sharon et al. (2009). Thereby, we identified a total of 104 compounds in the volatile samples of the berries of four different cultivars (Regent, Pinot Noir, Müller-Thurgau and Riesling), while their composition play a rather subordinate role in distinguishing the cultivars (Rid et al., 2019). However, we observed quantitative differences (ratios) between cultivars, especially during flowering stage, while being less obvious in the ripening process of the berries (Rid et al., 2019). By reducing these compounds to those that were proven to be perceived by the antennae of the females, variabilities between the cultivars were no longer evident, which indicates that a long-range attraction of females to certain cultivars is not regulated by volatiles only (Rid et al., 2019), although they are in *L. botrana* reported to play a major role for oviposition site acceptance in synergism with visual cues (Tasin, Betta, et al., 2011).

Furthermore, herbivorous insects react rather to volatile blends than to single compounds (Bruce & Pickett, 2011). Several studies were investigating on the development of species-specific volatile blends in order to support integrated pest management strategies (IPM) by increasing the efficiency of pheromones during mating disruption (Fang et al., 2018; Sans et al., 2016; Schmidt-Büsser et al., 2009; von Arx et al., 2012) or monitor pest infestation levels of moths (Loeb et al., 2011). However, our results highlight that the development of attractive blends for field-applications is quite complex due to the high number (24) of perceivable compounds (Rid et al., 2019). Furthermore, release ratios of single compounds within a blend are difficult to control as stressed by (Salvagnin et al., 2018), even in case of 3-component blend attracting *L. botrana* females. With reference to the development of the DSS for egg infestations, we assume that the characteristic VOC profile of grapevines is already provided by the foliage in the vineyard, thus we focus only on VOCs affecting short-range attraction and

oviposition (Markheiser et al., 2020; Markheiser et al, 2023, in preparation). Attraction of female moths over "long-range" is usually measured in wind tunnel studies (Salvagnin et al., 2018; Schmidt-Büsser et al., 2009; Tasin et al., 2006; von Arx et al., 2011), while a conclusive method is missing to measure the behaviour of females on "short-range", after settlement on a plant (Rid et al., 2019). In flying insects, as *L. botrana* and *E. ambiguella*, it is characterized by crawling (Renwick, 1989; Schoonhoven et al., 2005), while *L. botrana* was observed to move restless between grape clusters in order to lay eggs widespread (Galet, 1982). To reflect this behaviour, we developed a method, which allowed to quantify the "short-range" attraction of females to VOCs for oviposition (Markheiser et al., 2020). We coupled a 4-chamber-olfactometer to a video-tracking-system, which enabled to quantify essential behavioural parameters which may be involved in host plant recognition, such as ovipositor-movements, which possibly reflect the tactile and gustatory perception of VOCs by the ovipositor (Markheiser et al., 2020). Indeed, we confirmed that a single compound, (S)-(−)-perillaldehyde, a volatile emitted by a non-host plant *Perilla frutescens*, promotes oviposition of *L. botrana* (Cattaneo et al., 2014) and provoked ovipositor-activities within the olfactometer system in our studies (Markheiser et al., 2020). Furthermore, β-caryophyllene, a compound found to increase in concentration during the ripening of the berries (Rid et al., 2019), had a repellent effect on egg deposition of *E. ambiguella* (Markheiser et al., 2020). This may indicate, that the VOCs emitted by earlier developmental stages of the berry are more attractive to this species than those of matured berries. Another important component in our studies was methyl salicylate, an herbivore induced plant volatile, released upon damage of the plant by feeding insects. They may act as defence strategy of the plant to attract natural enemies (predators and parasitoids) (Simpson et al., 2011). According to (Anfora et al., 2009) it stimulates oviposition of *L. botrana*. The fact that methyl salicylate, which decreases during ripening (Rid et al., 2019) supports the hypothesis of a susceptibility of earlier developmental stages for egg infestations. Nevertheless, in our experiment it had a rather deterrent effect (Markheiser et al., 2020), stressing that the effect of VOCs on oviposition depends on the dose tested.

In addition to VOCs released by grapevine, *L. botrana* is attracted to VOCs emitted by its wild host, the flax-leaved *Daphne gnidium* (Maher & Thiéry, 2006; Tasin et al., 2010), and non-host plants as *P. frutescens* (Cattaneo et al., 2014; Markheiser et al., 2020) and *Tanacetum vulgare* (Gabel et al., 1992) for oviposition. This was taken into account in our studies (Markheiser et al. 2023, in preparation), while focusing on wild plant species in the surrounding of vineyards. Under climatically favourable condition, i.e. high mean air temperature, flight of the moths is initiated earlier in the season (Blümel et al., 2020; Comsa et al., 2022), so females may be

dependent on alternative plant species in the periphery of vineyards for oviposition. Indeed, populations of the first generation of *L. botrana* were already confirmed to move to *Olea europaea* (olive) inflorescences in Greece (Savopoulou-Soultani et al., 1990), Italy (Sciarretta et al., 2008) and Bulgaria (Stoeva, 1982). Our studies (Markheiser et al. 2023, in preparation) confirmed that alternative plant species are able to host at least populations of *L. botrana*. This is supported by the aspects that e.g. oviposition on inflorescences of *Taraxacum* sect. Ruderalia (Common dandelion) was preferred over those of grapevine (Markheiser et al. 2023, in preparation) and larvae are able to develop on this plant (Stavridis & Savopoulou-Soultani, 1998). We assume this plant provides rather gustatory or tactile stimuli, because *L. botrana* did not distinguish between grapevine and dandelion in case of exclusive olfactory stimuli (Markheiser et al. 2023, in preparation).

By addressing the physical properties of the oviposition site (texture and shape), which provide tactile stimuli after settlement on a plant, we confirmed in case of both species a preference for spherical surfaces, as naturally provided by the berries, and a discriminory ability between textures (Markheiser et al., 2018). On berries, epicuticular waxes form the outer layer while consisting out of small, individual, upright wax platelets, which increase during maturation of the berry and cover more and more the ridges of the cuticula of the berry (Rosenquist & Morrison, 1988). As consequence, they can provide variable tactile cues, which may explain the discriminatory ability in our results and other studies (Markheiser et al. 2018; Maher & Thiéry, 2004) and be a hint for the susceptibility of certain varieties or developmental stages for egg infestations. Because females differentiated between materials without texture we supposed that the females are able to notice gustatory stimuli after settlement on oviposition site (Markheiser et al., 2018).

Maher & Thiéry (2004) described several chemo-mechanoreceptor sensilla that are distributed over the tarsi, proboscis, ovipositor and antennae which may allow the selection of suitable oviposition site based on tactile and gustatory stimuli. Indeed, *L. botrana* females offer sensilla on the labial palps (Amat et al., 2022) and the ovipositor (Maher et al., 2006) that perceive i.e. sugars after contact to a plant. In addition, epicuticular waxes are reported to affect host plant selection in Lepidoptera (Brooks et al., 1996; Juma et al., 2016), while its composition changes during ripening and among grape cultivars (Pensec et al., 2014). Hence, we analysed the chemical composition of the epicuticular waxes of different cultivars and phenological stages and combined them with behavioural experiments to address the susceptibility to egg infestations (Rid et al., 2018). To exclude visual and olfactory stimuli, we offered wax extracts to the insects. A total of 59 compounds could be detected in wax samples, while oleanolic acid

and ursolic acid were the predominant substances identified (63%-83% of the total wax amount, depending on cultivar and developmental stage). As the substance attracted females of both species for oviposition and its content was higher in unripe if compared to mature berries (Rid et al., 2018) we assume that earlier developmental stages are more susceptible to egg infestations based on gustatory stimuli only. In addition, females of *E. ambiguella* preferred, in contrast to *L. botrana*, berries with wax layer over dewaxed berries for oviposition (Rid et al., 2018), confirming that gustatory stimuli have a synergistic effect with tactile and/ or olfactory stimuli in *L. botrana* (Tasin, Lucchi, et al., 2011b).

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General conclusion

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The process finally leading to attraction and oviposition in Lepidopterans, such as *Lobesia botrana* and *Eupoecilia ambiguella* is quite complex as the factors initiating this process rely on several interacting stimuli and species differ in the demands on their host plant.

Addressing the physical factors, the species show comparable habits for oviposition while in case of *E. ambiguella*, the stimulus of the spherical shape was suppressed by the instinct to lay eggs on shaded sites. This could be implemented into IPM measures to increase the attractivity of female traps. On the other hand, this could be an indicator for the monitoring of eggs, i.e. *E. ambiguella* lays eggs in less light-exposed areas of the vineyard or berry clusters.

In addition, epicuticular waxes, i.e. the main components oleanolic acid and ursolic acid, were found to decrease during ripening while promoting the oviposition of the two species. Hence late-maturing cultivars could be more affected by infections of the first generation larvae than early-maturing cultivars, as females prefer early developmental stages of the grape for oviposition based of wax extracts. Thereby, *E. ambiguella* is more dependent on a physical barrier attached to the berry cuticula. Furthermore, these two main components could contribute to the targeted DSS as coating of an artificial surfaces, which actually consists out of a green and textured plastic card. The release of ß-caryophyllene (increases during ripening) and methyl-salicylate (decreases during ripening) could be indicators for the susceptibility of certain cultivars or plant species to egg infestations of *L. botrana*. This could be implemented in breeding programs of tolerant cultivars. As methyl-salicylate promotes oviposition in *L. botrana*, it may be one of the key compounds which can be considered in the development of the DSS. Another promising single compound in our study was (S)-(-)-peryllaldehyde for *L. botrana*, whereas no single compound was proven to attract *E. ambiguella*. According to our results, *E. ambiguella* may be less effected by VOCs than *L. botrana*. Furthermore, addressing the susceptibility of other plant species may contribute to the risk assessment i.e. the possible need to expand the mating-disruption techniques to alternative hosts in order to prevent an immigration into the vineyard. It may also enable to identify further key components for the DSS as proven for Common Dandelion.

Overall, the response to VOCs and the plant surface composition are probably the most important factors affecting the attraction to suitable host plants for reproduction. Therefore, future studies should address on the interaction of single oviposition promoting stimuli.

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The greatest thanks go to my husband, who is my rock and positive pole, who has always motivated me and kept me going with the vital measures during the writing process. I would also like to thank my little son, who always puts a smile on my face (even if he also contributed to the sleep deficit during the writing process). Of course, none of this would have been possible without my parents, who support me unconditionally in all situations.

Finally, I would like to thank all my colleagues at JKI, both in the office and in the lab, who have always been pleasant companions in suffering.

Appendix

Appendix A: Status and author contributions of publications included in the thesis

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Chapter II

Published as peer-reviewed article in Journal of Applied Entomology:

Markheiser A, Rid M, Biancu S, Gross J, Hoffmann C (2018) Physical factors influencing the oviposition behaviour of European grapevine moths *Lobesia botrana* and *Eupoecilia ambiguella*. *Journal of Applied Entomology* 142: 201-210. https://doi.org/10.1111/jen.12423

CH and AM designed the study. AM collected data with technical support of SB. AM performed statistical analyses. AM wrote the first draft of the manuscript, which was approved, discussed and edited by MR, JG and CH. JG and CH provided funding. AM was responsible for correspondence.

Chapter III

Published as peer-reviewed article in Journal Pest Science:

Rid M, Markheiser A, Hoffmann C and Gross J (2018) Waxy bloom on grape berry surface is one important factor for oviposition of European grapevine moths. *Journal of Pest Science.* 91:1225-1239. https://doi.org/10.1007/s10340-018-0988-7

CH initiated the idea of decision support system. MR, AM, JG and CH designed the study. MR conducted the chemical analysis and MR and AM the behavioural bioassays. The results were analysed and discussed by all authors. MR and JG wrote the first draft of the manuscript, which was approved by all authors. JG and MR were responsible for correspondence.

Chapter IV

Published as peer-reviewed article in Journal of Plant Diseases and Protection:

Rid M, Markheiser A, Stein S, Hoffmann C, Gross J (2019). Volatiles of several grapevine cultivars emitted at different phenological stages linked to discriminatory ability of grapevine moths. Journal of Plant Diseases and Protection 126: 115-127. https://doi.org/10.1007/s41348-019-00214-y

MR, CH and AM designed the study. MR collected and analysed data. MR wrote the first draft of the manuscript. AM, JG and CH critically revised and contributed to the manuscript. JG and CH provided funding. JG and MR were responsible for correspondence.

Chapter V

Published as peer-reviewed article in Insects

Markheiser A, Rid M, Biancu S, Gross, Hoffmann C (2020): Tracking Short-Range Attraction and Oviposition of European Grapevine Moths Affected by Volatile Organic Compounds in a Four-Chamber Olfactometer. *Insects* 11: 45. https://doi.org/10.3390/insects11010045

AM, CH, MR and JG designed the study. AM investigated on olfactometer assays and video-tracking and collected data with technical support of SB. AM performed statistical analyses. MR collected data on EAG experiments and analysed these data. AM wrote the first draft of the manuscript, which was approved, discussed and edited by MR, JG and CH. JG and CH provided funding. AM was responsible for correspondence.

Chapter VI

Manuscript in preparation

Markheiser A, Kayser R, Biancu S, Hoffmann C (in prep): May alternative plants contribute to the spread of the European grapevine moth *Lobesia botrana* and European grape berry moth *Eupoecilia amb*iguella in vineyards?

AM, CH and RK designed the study. AM developed methology; AM and RK collected data with technical support of SB. AM performed statistical analyses. AM wrote the first draft of the manuscript. JG and CH provided funding. AM is responsible for correspondence.

Appendix B: Curriculum Vitae

Anna Markheiser

Education

Since 04/2020	**PhD student** in Environmental Science, iES Landau, RPTU Kaiserslautern-Landau, Landau, Germany *Thesis: Plant-specific factors affecting the short-range attraction and oviposition of European grapevine moths*
03/2012 - 07/2013	**Master of Science** in Horticultural Science Hochschule Geisenheim, Geisenheim, Germany *Thesis: Einfluss der Wirtspflanze sowie Herkunft von Botrytis cinerea-Isolaten auf die Wirkung von Triazol-Fungiziden gegen Botrytis cinerea im Gewächshaus und Halbfreiland*
08/2008 - 02/2012	**Bachelor of Science** in Horticulture Hochschule RheinMain, Geisenheim, Germany *Thesis: Einsatz entomopathogener Pilze zur Kontrolle schädlicher Käfer (Coleoptera) im Gartenbau – Infektionsmöglichkeiten von Otiorhynchus crataegi und Harmonia axyridis*
10/2006 - 05/2008	**Diplom** in Chemistry (no graduation) Technische Univerität Kaiserslautern, Kaiserslautern, Germany
09/2003 - 06/2006	**Abitur** Berufsbildende Schule Technik I, Ludwigshafen, Germany

Professional Experience

03/2021 – now	**Research assistant** Julius Kühn-Institut (JKI), Institute for Plant Protection in Fruit Crops and Viticulture, Siebeldingen, Germany Working group: Epidemiology *Project: Vectoscreen (Vektormonitoring zu Flavecence dorée, Xylella fastidiosa und geregelten Nicht-Quarantäneschad-organismen im Obst- und Weinbau)*
03/2017 - 03/2020	**Research assistant** Julius Kühn-Institut (JKI), Institute for Plant Protection in Fruit Crops and Viticulture, Siebeldingen, Germany Working group: Epidemiology *EU-Project: Xf-actors (Xylella fastidiosa active containment through a multidisciplinary-oriented research strategy)*

03/2014 - 02/2017	**Graduate research Assistant** Julius Kühn-Institut (JKI), Institute for Plant Protection in Fruit Crops and Viticulture, Siebeldingen, Germany Working Group: Zoology and Integrated Plant Protection *Project: M-ovicard (Entwicklung eines Entscheidungshilfewerkzeuges beim Einsatz von Insektiziden gegen Traubenwickler im Weinbau im Rahmen des integrierten Pflanzenschutzes)*

Internship

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04/2018 – 05/2018	**Guest Researcher** Institute of Agricultural Sciences (ICA) Spanish National Research Council (CSIC), Madrid, Spain *Project: Establishing the Electrical Penetration Graph (EPG) technique in order to assess the feeding behaviour of Philaenus spumarius, vector of Xylella fastidiosa*
03/2013 – 08/2013	**Research Assistant** BASF SE, Agrarzentrum Limburgerhof, Germany *Biological Research Fungicides*
03/2011 – 08/2011	**Practical study semester** BASF SE, Agrarzentrum Limburgerhof, Germany *Global Product Safety/ Registration – Terrestrial Organisms*
06/2008 – 08/2008	**Assistant** Gartenbaumschule Schwarz, Worms, Germany

Scientific membership

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Since 2015	International organisation of biological control (IOBC) Working Group of "Integrated Protection in Viticulture"; **Convenor of the sub-group insects** (since 10/2023)
Since 2015	Deutsche Gesellschaft für allgemeine und angewandte Entomlogie (DGaaE)

Academic publications (peer-reviewed)

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Jarausch B, **Markheiser A**, Jarausch W, Biancu S, Kugler S, Runne M and Maixner M (**2023**): Risk assessment for the spread of Flavescence dorée-related phytoplasmas from alder to grapevine by alternative insect vectors in Germany. *Microorganisms* 11(11), 2766. https://doi.org/10.1111/jen.12423

Markheiser A, Santoiemma G, Fereres A, Kugler S, Maixner M, Cornara D (**2022**): DC-EPG assisted comparison of European spittlebugs and sharpshooters feeding behaviour on grapevine. *Journal of Applied Entomology* 147 (3), 192-204. https://doi.org/10.1111/jen.13098

Cornara D, Morente M, Lago C, **Markheiser A**, Garzo E, Moreno A, Fereres A (**2021**): Probing behavior of Neophilaenus campestris on various plant species. *Entomologia Experimentalis et Applicata* 169 (12), 1126-1136. https://doi.org/10.1111/eea.13109

Markheiser A, Rid M, Biancu S, Gross J, Hoffmann C (**2020**): Tracking short-range attraction and oviposition of European grapevine moths affected by volatile organic compounds in a four-chamber olfactometer. *Insects*, 11(1), 45. https://doi.org/10.3390/insects11010045

Markheiser A, Cornara D, Fereres A, Maixner M (**2020**): Analysis of vector behavior as a tool to predict *Xylella fastidiosa* patterns of spread. *Entomologia Generalis* 40 (1), 1-13. https://doi.org/10.1127/entomologia/2019/0841

Cornara D, Morente M, **Markheiser A**, Bodino N, Tsai CW, Fereres A, Redak RA, Perring T, Lopes JRS (**2019**): An overview on the worldwide vectors of *Xylella fastidiosa*. *Entomologia Generalis* 39(3-4), 157-181 https://doi.org/10.1127/entomologia/2019/0811

Rid M, **Markheiser A,** Stein S, Hoffmann C, Gross J (**2019**): Volatiles of several grapevine cultivars emitted at different phenological stages linked to discriminatory ability of grapevine moths. *J Plant Dis Protect* 126, 115-127. https://doi.org/10.1007/s41348-019-00214-y

Rid M, **Markheiser A**, Hoffmann C, Gross J. (**2018**): Waxy bloom on grape berry surface is one important factor for oviposition of European grapevine moths. *J Pest Sci*, *91*, 1225-1239. https://doi.org/10.1007/s10340-018-0988-7

Markheiser A, Rid M, Biancu S, Gross J, Hoffmann C (**2018**): Physical factors influencing the oviposition behaviour of European grapevine moths *Lobesia botrana* and *Eupoecilia ambiguella*. *J Appl Entomol,* 142, 201-210. https://doi.org/10.1111/jen.12423

Conference contributions (first author only)

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Markheiser A, Biancu S, Minges N, Seinsche S, Zikeli K, Maixner M, Jelkmann W, Hoffmann C (**2023**): High-throughput monitoring as an approach for an early detection of quarantine pests in viticulture. IOBC-WPRS Meeting of the Working Group „Integrated Protection in Viticulture"; 3.-5. Oct 23, Logroño, Spain

Markheiser A, Biancu S, Minges N, Seinsche S, Zikeli K, Maixner M, Jelkmann W, Hoffmann C (**2023**)**:** Effiziente Monitoringstrategie zum Nachweis der Vektoren von Quarantäneschädlingen im Obst- und Weinbau. In: 63. Deutsche Pflanzenschutztagung:

Pflanzenschutz morgen - Transformation durch Wissenschaft; 26. - 29. September 2023, - Kurzfassungen der Vorträge und Poster - 475: 551–552.

Markheiser A, Reinhard L, Kröhner D, Kappel Y, Maixner M (**2021**): Effizienz unterschiedlicher Fallensysteme zum Monitoring der Wiesenschaumzikade *Philaenus spumarius*, dem Vektor des Bakteriums *Xylella fastidiosa*. In: 62. Deutsche Pflanzenschutztagung: Gesunde Pflanzen in Verantwortung für unsere Welt; 62. Deutsche Pflanzenschutztagung; 21. - 23. September 2021, Kurzfassungen der Vorträge und Poster- 467: 511

Markheiser A, Kugler S, Maixner M (**2021**) Untersuchungen zum Fraßverhalten von Zikaden, Ein Beitrag zur Risikoabschätzung einer Etablierung von *Xylella fastidiosa* in Deutschland. 62. Deutsche Pflanzenschutztagung; 21. - 23. September 2021, Kurzfassungen der Vorträge und Poster- 467:137–138

Markheiser A, Maixner M (**2019**): Host plant affiliation of xylem-feeders in Central Europe. In: Proceedings of the 2nd European Conference on *Xylella fastidiosa*, 29.-30. October 2019, Ajaccio, France, 82. https://doi.org/10.5281/zenodo.3577893

Markheiser A, Maixner M (**2018**): Understanding *Philaenus spumarius* behavior and host preference for predicting *Xylella fastidiosa* spread under Central European conditions. Contributions of the H2020 POnTE and XF-ACTORS projects, 2nd Annual Meeting, 23.-26. October 2018, Valencia, Spain, 75. https://doi.org/0.5281/zenodo.3577762

Markheiser A, Biancu S, Maixner M (**2018**): Potentielle Überträger des Feuerbakteriums *Xylella fastidiosa* im deutschen Obst- und Weinbau. In: JKI (Hrsg.): 61. Deutsche Pflanzenschutztagung: Herausforderung Pflanzenschutz - Wege in die Zukunft, 11.-14. September 2018, Universität Hohenheim -Kurzfassungen der Vorträge und Poster- (Julius-Kühn-Archiv 461), Braunschweig, 329-330. https://doi.org/10.5281/zenodo.3577619

***Markheiser A**, Maixner M. (**2018**): XF-ACTORS – Ein internationales Forschungsprojekt zur Vermeidung der Einschleppung und Ausbreitung von *Xylella fastidiosa*. In: JKI (Hrsg.): 61. Deutsche Pflanzenschutztagung, 11. - 14. September 2018, Universität Hohenheim - Kurzfassungen der Vorträge und Poster- (Julius-Kühn-Archiv 461), Braunschweig, 584-585. doi: 10.5073/20180821-135218

Markheiser A, Maixner M (**2017**): Significance of xylem feeding Auchenorrhyncha in orchards and vineyards in Germany. European Conference on *Xylella fastidiosa* – Finding answers to a global problem, 13.-15. November 2017, Palma de Mallorca, Spain. Book of Abstracts, 21.

Markheiser A, Maixner M (**2017**): Potential vectors of *Xylella fastidiosa* and their host plants found in Germany. European Conference on *Xylella fastidiosa* – Finding answers to a global problem, 13.-15. November 2017, Palma de Mallorca, Spain. Book of Abstracts, 35.

Greif A, Rid M, Gross J, Hoffmann C (**2017**): Oviposition behavior of European grapevine moths induced by different grape varieties. In: Gross, J.; Harari, A.: 40 Years of the IOBC-WPRS Working Group "Pheromones and Other Semiochemicals in Integrated Production": Proceedings of the Meeting "The Good Sense of Scent" at Mishkenot Sha'ananim, 8.-13. November 2015, Jerusalem, Israel, (IOBC WPRS bulletin = Bulletin OILB SROP 126), 115.

Greif A, Rid M, Gross J, Hoffmann C. (**2016**): M-OVICARD-Entscheidungshilfewerkzeug für den Einsatz von Insektiziden gegen Traubenwickler im Weinbau. Innovationstage der BLE 25.-26.Oktober 2016, Bonn, Tagungsband, 94-96.

Greif A, Rid M, Biancu S, Gross J, Hoffmann C (**2016**): Nachweis der eiablageinduzierenden Wirkung synthetischer Duftstoffe für Traubenwickler mittels verschiedener Verhaltensbiotests. In: JKI (Hrsg.): 60. Deutsche Pflanzenschutztagung: 20. - 23. September 2016, Halle-Wittenberg; Kurzfassungen der Beiträge, 177-178.

Greif A, Rid M, Gross J, Hoffmann C (**2015**): Guiding tour: M-Ovicard – A decision support system for integrated pest management of grapevine moths. IOBC Working Group meeting "Integrated Protection and Production in Viticulture", Vienna, Austria.

Greif A, Rid, Gross J, Hoffmann C (**2015**): Oviposition behaviour of grapevine moths induced by different grape varieties: 40 Years of the IOBC Working Group "Pheromones and other semio-chemicals in integrated production”: the good sense of scent; November 8-13, Jerusalem, Israel, Program and abstracts, 62.

Greif A, Rid M, Gross J, Hoffmann C (**2015**): M-Ovicard: Analyzing physical cues for grapevine moth oviposition for the development of a Decision Support System. In: Schmitt, T.; Blank, S.M.; Köhler, A.; Kramp, K.; Weyer, J. (eds.): Entomologentagung: Programm und Abstracts; 02.–05. März 2015, Frankfurt/M, 90-91.

*honored as outstanding poster presentation at the 61st German Plant Protection Conference at the University of Hohenheim, Germany

Appendix C: Declaration according to §8 of Promotionsordnung des Fachbereichs: Natur- und Umweltwissenschaften der RPTU Landau vom 19.08 2014

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I declare that:

- The submitted dissertation was written independently and all aids and sources used in this work were indicated and the contributions of any collaborators or other authors have been clearly highlighted;

- I had no paid assistance of by any sort of PhD service agencies;

- I have not made use of AI (artificial intelligence) tools while writing this work

- The dissertation has neither identically nor in a similar form been submitted for any scientific examination in Germany or in another country;

- The dissertation has neither earlier nor simultaneously been submitted at any other university or faculty;

I am aware that the PhD title can be revoked as a result of failure regarding any of the above mentioned points and that legal ramifications are also possible.

Worms, 20. November 2023

Anna Markheiser