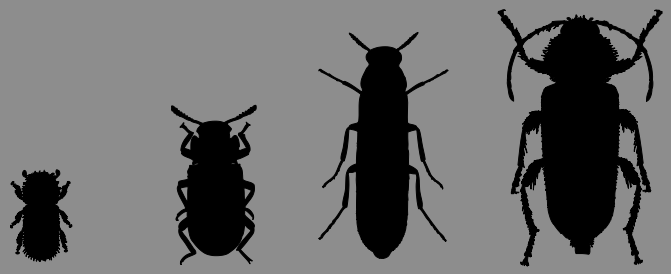

Odour signals relevant to beetles in deadwood habitats

Gerrit Holighaus

“Aber so geht's, wenn man Leuten durchs Auge deutlich machen will, was eigentlich, um vollkommen gefasst zu werden, gerochen werden muss.”

"But this is what happens when you try to make someone understand things by showing them to his eyes, while they must really be smelled in order to be grasped completely."

Georg Christoph Lichtenberg (1742 – 1799)

(Professor of physics, mathematics & astronomy in Göttingen, german satirist)

ODOUR SIGNALS RELEVANT TO BEETLES IN DEADWOOD HABITATS

- ODORANTS, OLFACTION AND BEHAVIOUR -

Dissertation

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ABSTRACT

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The overall objective of this work was to evaluate and determine volatile chemical cues (infochemicals) that determine insect-wood interactions. The deadwood habitat comprises species that reproduce and spend obligatory most of their lifespan in any kind of wood and in any kind of decay stage, including fungi that depend on wood. A gas chromatograph with mass spectrometric-electroantennographic detection (GC-MS/EAD) has been introduced to define sources of volatiles and identify infochemicals, in particular host produced kairomones, but also allomones and pheromones relevant to selected beetle species of an either xylophagous, mycophagous or xylomycetophagous feeding type respectively.

Complex chemical profiles of wood, deadwood and fungal samples have been analysed. Half of thirty compounds identified in the headspace of dry pine timber, the most artificial kind of deadwood, elicited responses in antennae of the Old House Borer *Hylotrupes bajulus*, a major pest of structural softwood. Aliphatic aldehydes, which are however not specific to the host tree species, are proposed as potential infochemicals and complement the number of terpenoid host kairomones previously known for this xylophagous species.

Experiments with felled beech trunks demonstrated that volatile chemistry changes over time and that decay over the length of trunks is quite variable. Potential host kairomones, such as branched alcohols, aldehydes and phenolics have been determined for the fungus farming ambrosia beetle *Trypodendron domesticum*. They provide a good basis for applications in integrated pest management and risk assessment of this secondary scolytine bark beetle. It attacks occasionally apparently healthy trees and is indicative for a recent emergence of pathogenicity in insect-fungus symbioses.

Antennae of the lymexylid *Elateroides dermestoides*, also xylomycetophagous, were tested for response to volatile compounds emitted from a diverse set of infested host trees including hard- and softwoods. Tree species differed substantially in emitted compounds, but beetles perceived those compounds prevalent from all species, explaining the broad host tree range of the beetle. Most of these compounds emanate from basal fungal metabolism, as it could be

demonstrated by analysing isolated strains of beetle-associated fungi. Perception of yeastlike and filamentous fungi by the beetle, and even more its capability to recognise the major fungal cultivar *A. hylecoeti*, which is distinguished by a species specific secondary metabolite, have been clearly demonstrated. However, the importance of predominant ubiquitous fungal volatiles for host recognition has been emphasized. Accordingly, future research should pay more attention to the function of volatile infochemicals within symbiotic relationships and insect-fungus interactions.

Fruiting bodies of polypores are common in the deadwood habitat and their colonisers typically decrease in specialisation degree with advancing maturity. Volatiles of fruiting bodies of the bracket fungus *Fomes fomentarius* were analysed and shown to quantitatively change with maturity in emission of ubiquitous eight-carbon volatiles. They act differentially as infochemicals and control behaviour of the mycophagous specialist *Bolitophagus reticulatus*. The available literature on eight carbon volatiles from mushrooms and their impact on insects is discussed. They are fungal oxylipins and possibly of comparable importance to mycophagous and saproxylic insects as plant oxylipins like the green leaf volatiles (GLV) are to herbivores. Beside the aforementioned, this is the most apparent case of host recognition with ubiquitous volatiles rather than species specific volatiles. This has been demonstrated multitudinously for herbivores and the studies presented here give reason to apply this idea to the deadwood habitat, and to conclude that host recognition in deadwood habitats occurs by using host (species) specific compounds, but even more by the use of ubiquitous host volatiles pertinent to many wood or fungal species.

Beyond such chemical parsimony of host kairomones, further multifunctionality of infochemicals has been demonstrated by attributing a pheromonal function to the defensive secretion of *B. reticulatus*. Species specific phenolic compounds released by both sexes have been defined that attract only male beetles, which are also more sensitive. They represent the first pheromone demonstrated in Bolitophagini. Findings are placed within the context of insect chemoecology, deadwood ecology and symbiosis research, but are also applicable to integrated pest management, wood technology and wood assessment. Modern analytical instruments and examination of comparably well studied insect species revealed that applying Chemical Ecology in basic research of deadwood is a promising task, giving valuable insights in general principles, efficient across the plant, fungal and insect kingdom.

CHAPTER 1

ODOUR SIGNALS RELEVANT TO BEETLES IN DEADWOOD HABITATS: GENERAL INTRODUCTION

Introduction

Among creatures on earth beetles come to the fore in many ways. Speciose among the prevalent insects, especially plant-feeders but also those feeding on death organic substrate or predacious insects account for an exceptionally high diversity (Strong et al., 1984). Ranking systematic taxons, the species number within the insect order Coleoptera exceeds that of any other animal or plant group (Farrell, 1998). Combining both top-ranks, the phytophagous beetles are numerous (Farrell & Sequeira, 2004) but many belong to other feeding guilds. Morphological adaptations make beetles long-living and robust, favouring their success in disparate ecological niches and environments such as water, all plant compartments as stems, roots or wood, dead organic substrates as soil, carcasses or dung, to name only few. The origin of such biodiversity is the crucial question of evolutionary biology. Long persistence of once evolved lineages or diversification within ecological niches is held responsible for having stimulated and sustaining diversity. Adaptive radiation and evolutionary arms races such as the success of herbivory coincident with the rise of angiosperms in geological times are an evident example (Farrell, 1998; Farrell & Sequeira, 2004), but still under debate (Hunt et al., 2007). Quite different disciplines of entomology and ecology contribute to descry the drivers of diversity, its origins, mechanisms - convergent or divergent - across systematic groups and methods (Schoonhoven et al., 2005). The field of Chemical Ecology, which views ecological interactions from a chemical perspective, also made substantial contributions. The well respected Ehrlich and Raven's theory has highlighted the coevolutionary arms race between insects and plants based on chemical interactions as a major source of diversification and is still a major underlying hypothesis of many studies in that field (Janz, 2011). Habitats of high biodiversity have been spotted, and wood or deadwood has been reckoned widely among these (Lonsdale et al., 2008; Rondeux, 2010). Deadwood hosts a plethora of organisms as bacteria, fungi and insects. Among these are bark- and wood-feeders, feeding on wood decomposing or symbiotic fungi, detritivores but also predators and parasitoids representing

the higher trophic levels (Lonsdale et al., 2008). The term *saproxylic* has been used to refer to this entire functional group within the deadwood environment (for a review see Grove, 2002). It is of course impaired by human activities and interests and became an important focus of nature conservation studies and practice (Grove, 2002; Lonsdale et al., 2008; Paillet et al., 2010). In these studies it is emphasised, that this long lasting but scattered habitat is susceptible to landscape fragmentation and forest management. Saproxylic insects dominate forests and include species from all insect orders, particularly numerous beetles and flies (Strong et al., 1984; Paillet et al., 2010). Deadwood arises from dying trees. At first, wooden tissue is often made accessible to colonising organisms by boring activity of beetles, which are favoured by their unique features and adaptations to enter and utilise this impenetrable and undigestible resource. To all intents and purposes, such beetles are ecosystem engineers and pioneer or initiate the transition from living plant tissue to dead organic material (Jones et al., 1997). Although many species colonise living or still living trees, regardless of whether they cause or just attend the transition to tree death, they can hardly be assigned to a phytophagous feeding habit which is defined as *feeding* on *living* plant material. However, they have a lot in common with those species on green plants (Byers et al., 2004). They are confronted with plant defences with the same consequences as phytophagous species are. Hence, as a principle, early colonisers are often specialists on certain tree species and not until defences are depleted, generalists prevail (Klimetzek et al., 1986; Schoonhoven et al., 2005). The assignment of a host plant is straightforward in phytophagous insects. Beetles in the deadwood environment are difficult to classify. A xylophagous assignment might miss out the fact, that many species boring and living in wooden substrate - if it is known at all - feed essentially on, or at least with the help of yeastlike fungi, filamentous fungi or bacteria. The first appearing in deadwood are sugar fungi, consuming building blocks and energy sources that trees use to construct and maintain woody cells. Subsequently staining fungi, structural- and residual wood decayers follow (Stokland et al., 2012). The latter enzymatically digest the wood, which is a complex chemical in form of a very stable lignin/cellulose polymer (Grove, 2002). Apparently, correct classification is dependent on available categories or knowledge. Actually, certain life stages of beetles or species easily cross the border to both sides, combining both habits: on living *and* dead wood-material, actually mostly simultaneously present on an old tree. The division line between living and dead on an organismic level as a tree is less than clear. Now, compared to feeding guilds as phytophages or parasitoids, where host specialisations are manifold (Schoonhoven et al., 2005), the key source of saproxylic

insect diversity has to be quested in the wide range of occurring decay stages and types occurring, each with different species assemblages (Grove, 2002). Ehrlich and Raven's hypothesis highlights the escape from toxic secondary plant metabolites as a source of diversity in phytophagous species. In dying trees, plant defences cease to exist. Thus, in case of saproxylics, this driver of diversity might be displaced by other factors, for example fungal defence, interspecific competition or a mutualism that benefits all involved organisms. Species succession from living to dead wood, is at first mainly affected by tree specific factors such as defence. Subsequently, development paths fan out and decay types are defined by fungal species and involved organisms, and increasingly abiotic factors exert their influence (Klimetzek et al., 1986; Grove, 2002; Rondeaux, 2010). It becomes clear that contrasting the different deadwood habitats, and the originating phase of deadwood, might be particularly suitable to study essential strategies of deadwood colonising beetles. Comparison of differences or similarities to those beetles populating the „animate world" of plants are promising. The impact and information content of chemicals, their unavoidable or purposeful release and also the ability to perceive and render meaningful behaviour has been an endlessly approved result of co-existence in evolutionary times (Schoonhoven et al., 2005). Chemical Ecology is a revealing research field, which uncovers releasers and receivers and in particular triggers or also explain interactions among both. In this study the chemical signals released by different deadwood habitats have been identified and compared. They are essentially emitted by plants, wood or fungi and not least the insects themselves. The signals perceivable by insects are basically odours, mixtures of volatile organic compounds (VOCs). The aim of this work was to identify those perceived by saproxylic beetles and which thereof trigger their behaviour. Across systematic groups and deadwood types, questions have been addressed asking which chemical compounds or classes are released and what the insect olfactoric sense reveals about host associations, or feeding habits such as phytophagy, xylophagy, mycophagy or hybrid forms. General principles or pathways have been of special interest that might exist for the countless beetle species to gain information out of the mass of chemicals released by so many originators as wood, fungi, bacteria, but also insects. Such principles and pathways are well known for phytophagous insects (Bruce et al., 2005; Schoonhoven et al., 2005; Matsui, 2006; Howe & Jander, 2008), but are unknown to a great extent in the diverse deadwood environment. Detailed knowledge of infochemicals in the deadwood context is basically acquired for forest pest species to use it to their control. It is unquestioned that phytophagous insects, much more conflicting human interests as health or resources, are the

best studied. Human crop production exceeds forestry and dominates the applied entomological research. However, Chemical Ecology has contributed much to the so called *integrated pest management*, which has also successfully been applied in forest pest control (Cook et al., 2007). This is a further incentive. Technically limited in earlier days, fantastic discovers now are made in plant-insect-communication and -research, including all trophic levels and model organisms with representatives of moths, flies, bees and more recently also beetles. These are good reasons to study the relevance of odour signals in deadwood habitats.

Main questions of this study

What are the chemical classes of volatile organic compounds which compose the odour of wood and where is their origin? Which compounds of alive or dead trees, wood or xylotrophic fungi qualify as infochemicals in insects? (introductive literature review, Chapter 2)

What are the technical requirements to identify insect infochemicals of wood? (methods paper, Chapter 3)

Which odours determine the type of wood or its suitability in a *xylophagous* beetle attacking timber constructions? (Chapter 3) - *Hylotrupes bajulus*, Cerambycidae (Fig. 1a)

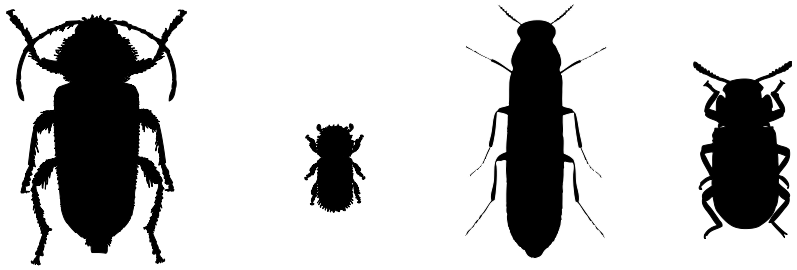
Is the attack on apparently healthy trees by a bark beetle, which otherwise breeds in dead trees, attributable to odorants? (Chapter 4) - *Trypodendron domesticum*, Scolytinae (Fig. 1b)

How do wood odours of a trunk change spatially and temporally? (Chapter 2 & 3)

Which odours allow host recognition in a *xylomycetophagous* beetle that is a tree generalist but also a fungus specialist? (Chapter 5) - *Elateroides dermestoides**, Lymexylidae (Fig. 1c)

Does a *mycophagous* beetle colonising deadwood, rely on ubiquitous fungal volatiles? What is their informational value? (Chapter 6) - *Bolitophagus reticulatus*, Tenebrionidae (Fig. 1d)

Beyond host odours, do other VOCs influence behaviour in deadwood beetles? What might mediate group living, a common habit within fragmented deadwood habitats? (Chapter 7) - *B. reticulatus*, Tenebrionidae (Fig. 1d)



a b c d

Fig.1: Silhouettes of the beetles of this study, classification, feeding type, chapters (drawings not to scale)

a) *Hylotrupes bajulus*, Cerambycidae ——— xylophagy ——— Chapter 3
b) *Trypodendron domesticum*, Scolytinae ——— xylomycetophagy, monophagy ——— Chapter 4
c) *Elateroides dermestoides**, Lymexylidae ——— xylomycetophagy, polyphagy ——— Chapter 5
d) *Bolitophagus reticulatus*, Tenebrionidae ——— mycophagy ——— Chapter 6/7

**Elateroides dermestoides* has been recently recognised as a senior synonym of *Hylecoetus dermestoides* (see Cuccodoro, 2002; Chapter 5), thus, both names appear in this thesis.

Deadwood habitats and Organisms

In this study, beetles from both contrasting feeding strategies and contrasting systematic taxa in different deadwood habitats have been investigated (Fig.1). The selected deadwood habitats are of natural or anthropogenic origin and the studied organisms are always representative beetle species, whose ecology is well studied (for detailed references to literature see denoted Chapters). The order in which the experiments are presented follows feeding habits from a xylophagous over hybrid ones such as xylomycetophagy, to mycophagy (Fig.1). It also represents the natural sequence of tree or wood decay to a certain degree. The term deadwood is generally understood as in the deadwood ecology context, concerning the mature timber habitat and its natural processes (Grove, 2002). As a start another less intuitive „quality“ of deadwood (Chapter 3) is considered. Felled trees, but even more all kinds of timber and subsequent wooden products are *dead* wood. It is made available by humans, and is a secondary habitat to insects - ready to be colonised. Even though only few species make a living in wooden products, which is the most unsuitable deadwood of anthropogenic origin, those coping with it, cause serious damages in timberwork and furniture or carvings for instance. The prime example is the Old House Borer *Hylotrupes bajulus* (Cerambycidae) a longhorn beetle of an anthropogenic worldwide distribution and a major pest of softwood timber used for roof constructions. It is a strictly xylophagous beetle in dry wood hostile to fungal life, and has a long history as a test species in insecticide and integrated pest management research. Raw timber production probably faces the highest losses in the value creation chain of forestry and wood industry and unsurprisingly the most serious forest pest species are bark beetles. A few bark beetles, mainly species of the Curculionid family Scolytinae, can undergo mass outbreaks and eventually kill healthy trees. Such a habit characterises *primary* bark beetles, and those lacking it and instead infest felled or dead trees, are considered as *secondary* bark beetles. Many of the latter are unable to overcome tree defences. It is assumed that in primary bark beetles, associated fungi facilitate overcoming such defences. It has also recently been hypothesised, that once balanced symbiotic relationships are involved in observed novel pathogenicity of secondary bark beetles, and emerge as a new and currently uncontrollable threat to forest ecosystems worldwide (Hulcr & Dunn, 2011). The opportunity to investigate such a case occurred in southern Germany (Chapter 4), where a secondary bark beetle, *Trypodendron domesticum* (Curculionidae, Scolytinae), which is monophagous on beech (*Fagus sylvatica*), has been found to attack apparently healthy trees since the year 2000. Its original habitat is seemingly dead beech trees

or those recently died off. Expectedly, stacks of logs are also attacked by *T. domesticum* (Curculionidae, Scolytinae), which makes it a noticeable forest pest species. Beside Scolytinae, secondary bark beetles also include Cerambycidae, Buprestidae, Platypodinae or Lymexylidae and are bark-, or actually more often wood-breeding species which feature obligate associations with beneficial fungi to facilitate nutritional supply. In some taxa these appear as most sophisticated symbioses, with intriguing morphological and behavioural adaptations as in the so-called *ambrosia beetles* within Curculionidae (Scolytinae and Platypodinae) and Lymexylidae, elsewhere in the insect kingdom only known for ants and termites. Such an illustrious exemplar along with its fungal associates has been examined: The large timber worm *Elateroides dermestoides* (senior synonym of *Hylecoetus dermestoides*), a representative of the enigmatic ship timber beetles (Lymexylidae) (Fig.1). It is unrelated to *T. domesticum*, but they share their habitat at least in hardwood species. However, *E. dermestoides* is polyphagous and attacks also many coniferous tree species (Chapter 5). Pest species of wood, of course, are those related to a relatively early stage of decay, as long as resilience and toughness - the properties essential to constructional wood - are untouched and at risk of insect and fungal disintegration. A more advanced state of wood decay has been examined in the mature timber habitat of beech, bearing typically many fruiting bodies of the Tinder Fungus, *Fomes fomentarius*. It is the sole host of the mycophagous beetle *Bolitophagus reticulatus* (Tenebrionidae), a monophagous specialist spending all life stages in the perennial fruiting bodies. *B. reticulatus* has been intensively studied in joined studies of deadwood ecology together with the field of nature conservation, investigating the human impact of forest fragmentation and forest management on these beetles. The studies suggested volatile chemicals of trunks, fruiting bodies and also beetles to play a role in utilisation patterns of hosts, both, on the spatial scale of sporophore-, and trunk colonisation. Perennial sporophores themselves follow a gradual decay correlated with occurrence and abundance of *B. reticulatus* and further commensal beetles, suggesting fungal host cues to be important (Chapter 6). Pronounced group living in metapopulations on single trunks and „trunk groves“, is known for both *B. reticulatus* and the close relative *Bolitotherus cornutus*. Their aggregation or subsocial group living is common in mycophagous beetles, in particular on perennial sporophores of bracket fungi and highlight once again the intraspecific communication and importance of infochemicals (Chapter 7).

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CHAPTER 2

VOLATILE ORGANIC COMPOUNDS FOR WOOD ASSESSMENT

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* I contributed the parts related to volatile release of trees, trunk & deadwood, wood- & wooden products, as well those dealing with insects on trees, trunk & deadwood, wood- & wooden products

Introduction

Utilisation of **volatile organic compounds (VOCs)** for the quality assessment of wood is basically a bionic concept which is inspired by the impressive achievements of insects in performing this task just by olfaction in order to exploit wood as a resource. The word **"bionics"** is made up of the two words "biology" and "electronics". In German, however, the second part comes from "Technik", which means engineering in this context. In English, this approach of combining biology and engineering is often also called **"biomimetics"**. The two expressions are used more or less synonymously. The interdisciplinary field of bionics is about scrutinising and transferring "natural inventions" into technical applications. In the course of evolution, nature has developed, improved and tested these inventions over millions of years. For technical exploitation, the optimised solutions to a specific set of problems have firstly to be thoroughly analysed. Subsequently, the newly described solutions can be implemented in technical applications with corresponding boundary conditions.

In order to highlight possibilities and limits of an assessment of wood by detecting VOCs released by wood, the first part of this contribution deals with the genesis of VOCs in wood, in fungi and in wood infested by fungi. In the second part, the interaction with insects is used as example how nature exploits the content of information encoded in patterns of VOCs released by wood. By examination of the recognition processes of insects and a parallel trace analysis of related VOC patterns released by fungus infested wood, we might learn how to assess wood quality as well as the kind and state of fungal infestation by detecting wood-released VOCs in a quick and nondestructive manner. In view of that, current techniques are displayed enabling the detection of specific VOCs or of patterns of VOCs released by wood, in order to suggest possible lines of development for devices assessing wood quality.

Volatiles released by living trees

In the discussion of greenhouse gases and their impact on global climate changes (see Chapters 5 and 6 of this book), there is an increasing interest in the complex chemistry of the troposphere. The dynamics of the global atmospheric chemistry through climate forcing is triggered by VOCs (Holopainen 2004, Dindorf et al. 2005). Beside VOCs of anthropogenic origin, especially VOCs emissions from forests which are covering ca. 30% of landmass (FAO 2006) are sources affecting the system. The quantities of **volatiles of natural origin (NVOC)** released above the main landmasses as arable land and forests exceed by far the quantities from anthropogenic sources. Due to their dominance, reactivity and physical properties, they are classified as **VVOCs (very volatile organic compounds** like methane), **reactive VOCs** (isoprene and terpenes) and **non-reactive VOCs** (Guenther et al. 1995).

VOC-emissions by plants are unavoidable due to their metabolic activities (Peñuelas & Llusià 2004). A dominant reactive VOC released by forests for example is isoprene, which is widespread but not generally present throughout the plant kingdom (Harley et al. 1999, Owen & Peñuelas 2005). Isoprene is discussed to play an important role in tropospheric chemistry (Fehsenfeld et al. 1992, Lerdau et al. 1997). Similar to terpenes, its reactivity influences the atmospheric dynamics of ozone, formation and deposition of organic nitrates and organic acids (Harley et al. 1999). Due to this importance in atmospheric processes, algorithms were developed describing the dependence of isoprene and terpene emissions of plants on light and temperature (Dindorf et al. 2005). Further factors as drought, diurnal and seasonal variation or growth conditions were discussed as parameters influencing the VOC emissions of plants (Dudt & Shure 1994, Staudt et al. 2001, 2003). However, there are undisputable many

additional internal (e.g. genetic, biochemical) and external (e.g. interaction with fungi and insects) factors that affect the presence (Litvak & Monson 1998) and emission of different VOCs by trees and other plants (Apel et al. 1999, Peñuelas & Llusià 2001, Schütz et al. 2004) which are not yet covered by known algorithms.

Most trees are grouped, due to their affinity, in coniferous and broadleaved species. This is also reflected in their VOCs composition: VOCs differ highly from coniferous to broadleaved woodlands. Regarding coniferous trees, VOC-research is almost exclusively done in the family of Pinaceae, e.g. *Pinus, Picea, Larix, Abies, Tsuga*, and *Cedrus* (Hayward et al. 2004, Lee et al. 2005). Broadleaved species were examined on a somewhat broader scale comprising Fagales (*Betula*, *Fagus*, *Quercus*), Sapindales (*Acer*, *Castanea*) and e.g. Salicaceae (*Salix, Populus*) (Pasteels & Rowellrahier 1992, Tollsten & Müller 1996, Hakola et al. 2001, Paczkovska et al. 2006). Further genera such as *Eucalyptus* (Guenther et al. 1993, Zini et al. 2002) are characterised and several comparative studies screened plant species for single VOCs only (Owen et al. 1997). Plant VOCs are mostly alkanes/alkenes, aromatic hydrocarbons, alcohols, phenolics, terpenes, esters, aldehydes and ketones (Kesselmeier & Staudt 1999). However, due to technical restrictions, the analytical window covers currently only compounds with boiling points between 60 °C to 250 °C at atmospheric pressure, and of intermediate to high thermal stability (Schütz 2001).

General processes in plant cells, as the lipoxygenase (LOX)-pathway (Feussner & Wasternack 2002) are responsible for the release of generalistic VOCs as the so called **"green leaf volatiles" (GLV)**. Mainly alcohols, aldehydes of linear six carbon chains and their derivatives such as (Z)-3-hexen-1-ol, (Z)-3-hexen-1-yl-acetate, hexan-1-ol, and (E)-2-hexenal belong to this group (Visser 1979). Whereas the name GLV implies the paradigm that only leaves (not needles) are releasing these compounds, it was proven that coniferous trees release these compounds, too, but only in minute amounts (Schütz et al. 2004). GLV are released in low rates from nearly every plant species (Hatanaka 1993) and show a typical increase on mechanical wounding (de Bruxelles & Roberts 2001, Mithöfer et al. 2005) of any type of plant tissue, be it leaves, needles, stems or roots (Matsui 2006). Especially young developing leaves and damaged leaves - and leaves are damaged by wind or insects in a forest all the time - release increased rates of GLV. With regard to the function of trace compounds with low emission rates as **carrier of information ("infochemicals")**, these minor components must however not be neglected (Schütz 2001, Schütz et al. 2004). GLV are known to play an

important role in insect attraction and aggregation (Visser 1979, Schütz et al. 1997, 2004, Ruther 2000) or insect repellence (Huber & Borden 2001, Zhang & Schlyter 2004) and even in signalling between plant individuals, known as the phenomenon of "talking trees" (Tscharntke et al. 2001, Arimura et al. 2002, Engelberth et al. 2004, Farag et al. 2005). All this points out a complex interactive defence system in plants in which the VOCs play the role of a language. VOCs carry information about the constitutive or induced defence status of the plant, whether it is mechanically wounded, attacked by insects or micro-organisms (Schütz et al. 1997, Schütz 2001, Holopainen 2004, Weissbecker et al. 2004, Holighaus & Schütz 2006, Johne et al. 2006a,b, Paczkovska et al. 2006).

Isoprenoids are characteristic defence chemicals of conifers and are produced through the mevalonate (MEV) or methyl-erythritol-diphosphate (MEP) pathways (Keeling & Bohlmann 2006). They are highly variable in structure (>30,000 terpenes are known) and occur in trees as isoprene (C5), monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20) (Sharkey & Singsaas 1995, Phillips & Croteau 1999, Trapp & Croteau 2001). Following just the name of a compound, for instance, α-pinene should not be mistaken in that it is exclusively released by coniferous trees like *Pinus* spp.. For example, European beech (*Fagus sylvatica*, Fagaceae) seems to be a much stronger monoterpene emitter than expected. The monoterpenes of this species, studied by Dindorf et al. (2005) and Moukhtar et al. (2005), are dominated by sabinene with more than 90% of the daily terpene emission, but the typical coniferous volatiles α-pinene and β-pinene were also found in the VOC pattern of beech trees. This holds also true for Quercus suber, the cork oak (Pio et al. 2005). α-pinene, sabinene, β-pinene and limonene were the main compounds (80%) among the released terpene fraction from the oak.

Within taxonomic groups of lower plants, the VOC patterns are more alike, based on a more similar biochemistry of secondary plant compounds (Asakawa 2004). This relationship is treated in the scientific field of chemotaxonomy (Harborne & Turner 1984). However, variability of VOC patterns can be high, notwithstanding the degree of relationship. The Southern beech *Nothofagus dombeyi* releases α-pinene in considerable amounts, whereas five other species of *Nothofagus* do not at all (Quiroz et al. 1999). A similar variability was shown by Harley et al. (1999) for isoprene emission of several woody and herbaceous plant species of Northern America.

Volatiles released by trunks and deadwood

Trees provide a huge variety of plant tissues and plant surfaces. Compared to herbaceous plants, their surface is much bigger and more sculptured resulting in a higher variety of local VOC pattern and subsequent niches for interacting organisms. For example, 80% of VOCs stored in and released by needles of *Pinus* sp. and *Picea* sp. are identical with those released by the trunk of the trees, but they display significantly different quantitative patterns of VOCs (Sjödin et al. 2000, Schütz et al. 2004). In contrast to leaves and needles, there are only few systematic examinations about the influence of internal or external parameters on VOC patterns or released VOC quantities of wood or bark of trees (Schütz et al. 2004, Holighaus & Schütz 2006). However, various commercial interests lead to the analysis of chemical bark contents. Bark (root or trunk) as well as wood (root or trunk) are outstanding sources for commercial products since rich in essential oils (Wang et al. 2005) which are often VOCs. These defence chemicals against attacking organisms often display antibiotic activity and are used for various aspects in human life, e.g. in medicine (Kalemba & Kunicka 2003), food (Burt 2004) and personal care products (Priest 2002). Besides, VOCs are examined for applications in biotechnical plant protection and biotechnical stored product protection (Manker 2005).

At the beginning of the dieing process of a tree, a remarkable differentiation of the ecological system "tree" takes place resulting in a tremendous diversity of species of insects and micro-organisms (Harmon 1986, Moore et al. 2004). The exact point of initiation of the dieing process, whether caused by storm, insects, fungi or other circumstances, is often hard to define. Although felling or breaking down is often stated as the borderline between living tree and deadwood, when looking closely to physiological and chemical processes, a clear separation is hardly possible. Continuously during life, cells of healthy trees die and are rebuilt. Programmed cell death (PCD) is an integral part of plant development and also of defence. It occurs at all stages of the life cycle, from fertilisation of the ovule up to death of the whole plant. Indeed, without it, tall trees would probably not exist (van Doorn & Woltering 2005). Permanent stress of the environment like oxidative stress, heat or draught, infestation by micro-organisms, etc. causes the loss of protective compounds which have to be renewed (Sharkey & Singsaas 1995, Blokhina et al. 2003, Loreto et al. 2006 and citations therein). The oxidation of unsaturated fatty acids as constituents of lipid membranes or storage compounds of cells leads to the production of aliphatic aldehydes, alcohols, alkanes

and other VOCs (Feussner & Wasternack 2002). These kinds of compounds can all the time be found produced in bark and wood (Weissbecker et al. 2004, Holighaus & Schütz 2006). Attacks of fungi and insects increase oxidative stress on the plant tissue and, in the course, the emission rates of these VOCs (de Bruxelles & Roberts 2001, Schütz 2001). Such biotic stress occurs very often in living plants and in many instances it can be overcome or healed. If the plant is however not able to cope with the related damage, the dieing process is initiated. Regardless of whether biologically initiated or caused by felling, the end of a tree does not result in "chemical silence" since not all the cells of a tree are at the time dead. The defence system and other cell functions are still working, until the storage pools are empty and dieing is completed. In case of such deadwood, the decay process of a tree and wooden substrate results in an extensive release of VOCs, changing considerably due to abiotic environmental factors (humidity, temperature etc.) and biotic interactions (fungi, micro-organisms and insects) (Paiva 2000). The complexity of decay is demonstrated by the VOCs released from bark of a *F. sylvatica* trunk subsequently to felling (Fig. 1, 2). Felled beech trunks release more than 140 volatile compounds in detectable amounts during the first phase of decay (0-2 years after felling), up to 70 of them simultaneously. Differences between small bark samples hint at a high spatial variability of chemical processes of decay and related volatiles within one trunk (Fig. 1; Holighaus & Schütz 2006).

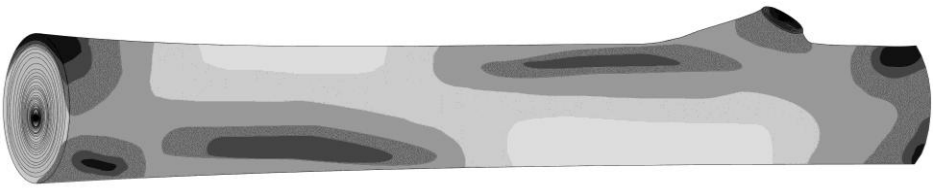

Fig. 1 Distribution of physiological decay states on beech trunk; grey to black patches: fresh to seriously decayed (modified from Holighaus & Schütz 2006)

Starting with felling, the number of volatiles and the emission rates of aldehydes increase (Holighaus & Schütz 2006). Following the decay progress, exemplary chromatograms yielding from a gas-chromatographic separation and subsequent mass-spectrometric detection of VOCs released by the bark of a beech trunk are displayed in Fig. 2 with the main compounds named. Several simple and branched alcohols occur at the beginning of the fermentation process in the headspace of bark tissue (Fig. 2B). Beside terpenes, phenolic compounds as 2-methoxy-phenol, 4-methoxy-phenol and 1,2-dimethoxy-benzene emanate

during the phase of oxidising bark tissue. They vanish fast and the branched alcohols change to longer straight-chained alcohols (Fig. 2B, C). At initial infestation with white rot fungi, up to 30 sesquiterpenes are additionally detected in the bark samples (Fig. 2C). After predominant degradation of lignin and cell structures of the bark by fungi, only sesquiterpenes are left to release (Fig. 2D).

VOCs emitted by wood and wood products

Wood is one of the most widespread building materials. For usage in constructions, the fading of natural metabolic processes in wood is enhanced by drying. The dried "deadwood" does not any more release VOCs on the basis of metabolic processes of the wood cells and, also, a part of the constitutive defence VOCs evaporated during the drying processes. VOC release rates differ between different drying and modification processes (Otwell et al. 2000). Air dried wood releases 8 times more VOCs than thermally modified wood (Manninen et al. 2002). The thermal modification has a high impact on wood chemistry and constructive properties. Thermally modified wood is dominated by aldehydes (hexanal), carboxylic acids and -esters, air dried coniferous wood by terpenes (Tjeerdsma et al. 1998).

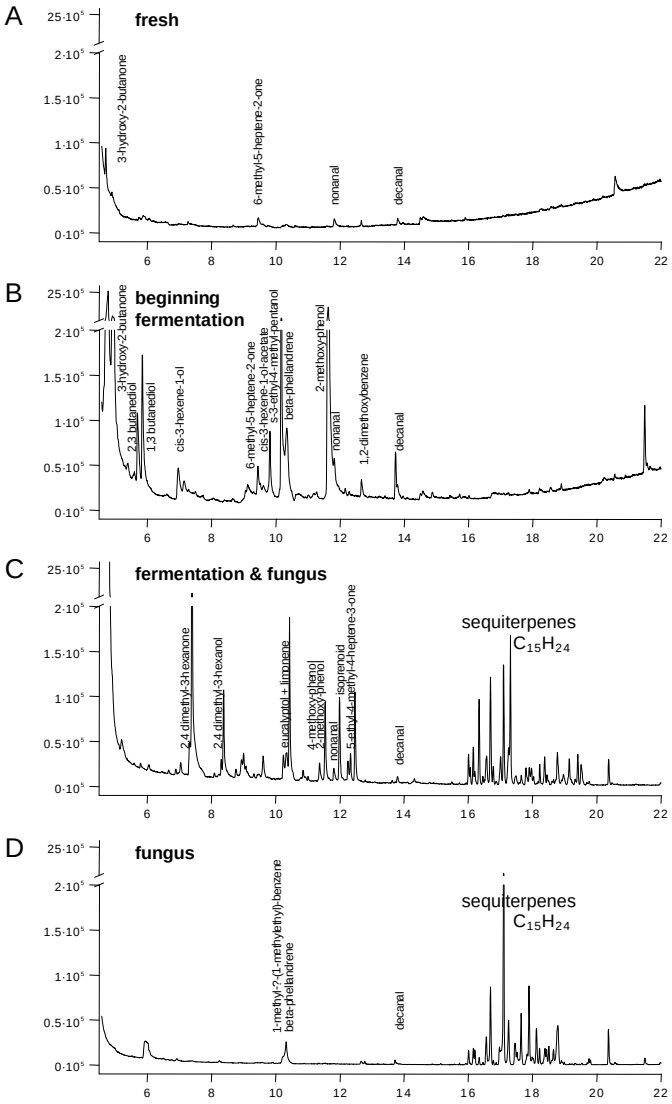

Fig. 2 VOC patterns released by differently decayed bark patches on a trunk of European beech (data from Holighaus & Schütz 2006)

Analytical research on VOCs released by wood and wood products is performed by two reasons. Firstly, several VOCs released from wood are suspected to be toxic or cancerogenic to human beings. The main focus of examinations is therefore on toxic VOCs as well as on unpleasant odours (Bleich et al. 1998). Compared to solid wood, the release rate of VOCs of several derived timber products is significantly reduced (e.g. OSB=0.25x, MDF=0.05x), whereas the rate of aldehyde emission is much higher (Barry & Corneau 1999, Risholm-Sundman et al. 1998, Risholm-Sundman 2002). Of high concern are toxic formaldehyde emissions of processed wooden products (Sundin & Roffael 1992, Bleich et al. 1998, Schäfer & Roffael 2000; see Chapters 15 and 16 of this book). Glues and binding agents are releasers

of this compound (Chapter 15 and 16 of this book). According to Marutzky & Roffael (1977) and own examinations, freshly cut wood itself releases considerable amounts of formaldehyde surpassing sometimes even legal thresholds. However, the quantity of formaldehyde emissions of cut wood decreases quickly. Usually, after 6 month of storage, formaldehyde emissions of wood are below detection limits. Other natural compounds from wood discussed in the context of toxicity belong to the group of monoterpenes (Johansson 1999, Jentoft & Stray 2002). However, the positive affection to wooden products is strongly influenced by the perception of a typical wood-odour and needs also to be considered.

The second focus on VOCs of wood material involves several indoor molds and fungi using the wooden substrate for growth, thereby generating additional VOCs being of considerable concern regarding the "sick-building-syndrome" (Mølhave et al. 1997, Johansson 1999, Fischer & Dott, 2003, Wilkins et al. 2003, Portnoy et al. 2005; see below and Chapter 12 of this book).

Volatiles released by fungi

Fungi are organisms that obtain nutrition by out-of-body digestion, releasing a range of extracellular enzymes to digest their substrates. For degrading wood, they produce cellulases (endo- and exo-cellulases), hemicellulases, α-glucosidase and oxidase, phenoloxidases and laccases (Eaton & Hale 1993; see Chapters 17 and 19 of this book). They further utilise the generated decomposition products for processing metabolism, extending mycelium, and in some cases, developing their fruiting bodies (Kües 2000; see also Chapter 23 of this book). Besides obtaining energy and nutrients, metabolic activity yields also volatile by-products including VOCs. This attributes to the typical odour of each fungus. For example, the edible champignon, oyster mushroom, shiitake, puffball, truffle and straw mushroom all have their own individual aromas (Mau et al. 1997, Venkateshwarlu et al. 1999, Mauriello et al. 2004, Zawirska-Wojtasiak 2004, Chiron & Michelot 2005), motivating our appetite. What contributes to these emblematic odours or VOCs? What is their purpose and function? There is still much about the fungal metabolism, especially the secondary metabolism, to uncover.

Classes of VOCs released by fungi

VOCs released from wood and wood-decaying fungi range from low to high molecular weight and can be further subdivided by their chemical structure (Korpi et al. 1998, Gao & Martin 2002, Schleibinger et al. 2005, Chiron & Michelot 2005, Gao et al. 2005, Thakeow et

al. 2006) into the eight broad categories of alcohols, aldehydes, ketones, acids, esters, S- and N-containing compounds and isoprenoids (monoterpenes, oxidised-monoterpenes, sesquiterpenes and oxidised-sesquiterpenes) as listed in Table 1.

Table 1 VOC classes as released by wood, infested wood, and micro-organisms (data taken from Korpi et al. 1998, Gao & Martin 2002, Schleibinger et al. 2005, Chiron & Michelot 2005, Gao et al. 2005, Thakeow et al. 2006)

Chemical categories	Examples
Alcohols	Ethanol, isopropyl alcohol, octan-1-ol, octan-3-ol, 1-octen-3-ol
Adehydes	Acetaldehyde, bezaldehyde, furfural, nonanal
Acids	Acetic acid, methyl butanoic acids, 2-methyl propanoic acid
Ketones	Acetone, pyranones, hexanones, heptanones, octan-3-one
Esters	Ethyl acetate, methyl propanoate
S-containing compounds	Dimethy disulfide, dimethyl trisulfide
N-containing compounds	Methyl pyrimidine, pyrazine, cyclobutyl amine
Isoprenoids:	
Monoterpenes	αα-pinene, β-myrcene, 3-carene, limonene
Oxidised monoterpenes	Borneol
Sesquiterpenes	Farnesenes, barbatenes, protoilludenes
Oxidised sesquiterpenes	Longiverbenone

The un-branched C8 compounds, 1-octen-3-ol, octan-3-one and octan-3-ol, are considered to be typical fungal constituents found in such diverse species as *Aspergillus*, *Fusarium* and *Penicillium* strains (Schnürer et al. 2002), *Tuber borchii*, *Tuber mesentericum*, *Tuber excavatum* (Mauriello et al. 2004, Menotta et al. 2004), *Lentinus* sp., *Agaricus bisporus*, *Agaricus campestris*, *Lactarius* sp., and *Calvatia* sp. (Overton 1994), and wild *Polyporus sulfureus* and *Fistulina hepatica* (Wu et al. 2005a,b). However, not only filamentous fungi emit these C8 compounds, but, to a lesser extent, yeasts and bacteria, too (Bruce et al. 2004, Nilsson et al. 2004, Schleibinger et al. 2005). On beech wood, the wood rotting fungi *Trametes versicolor*, *Poria placenta* and *Gloeophyllum trabeum* all released the isoprenoides α-pinene, 3-carene, longifolene, and cedrene in addition to the alcohols 1-octen-3-ol and octan-3-ol, and the ketone octan-3-one. In addition, each fungus had its own characteristic compounds in the sesquiterpene-class, *T. versicolor* for example α- and β-barbartene, *G. trabeum* high amounts of protoillud-6-ene, and *P. placenta* fair amounts of daucene (Thakeow et al. 2006).

Impact of fungal development on VOCs released by fungi

VOCs released by fungi can change considerably during their life cycles. For instance, the VOCs produced by live and dead mycelium of *Serpula lacrymans* grown on *Pinus sylvestris* shavings were found to be different. Living mycelium released 1-octen-3-ol as a major volatile component, and dead mycelium 3-methylbutanal and 2-methylbutanal, but only trace amounts of 1-octen-3-ol (Ewen et al. 2004).

Table 2 VOCs released from *T. borchii* fruiting bodies during ascus maturation [ascus stage 0: 0-5%, 1: 6-30%, 2: 31-70%, and 3: 71-90% of the sets of spores in the asci are mature, respectively (after Zeppa et al. 2004)]

VOC-Class	Ascus stage			
	0	**1**	**2**	**3**
Isoprenes	• 2-methyl-5-(1,2,2-trimethyl-cyclopentyl)-, (S)-phenol • valencene • α-patchoulene • longiverbenone • cedrene • aromadendrene	• longifolene • β-cedrene • borneol	• isopino-camphone • 3-thujene	• D-limonene • *trans*-ocimene • (R)-α-pinene • α-farnesene • 4-isopropyl-tropolene
N-containing compounds	• 3-(1-piperazinyl) propanamide			
S-containing compounds			• 5-methyl-3H-pyran-1,2-dithiol-3-one • 2-(1,1-dime-thylethoxy)-5-methylthiophene	• 3-methyl-thio-phene • 2,3-dihydro-5-methyl-thiophene • 1-(methylthio)-1,3-butadiene • 2-methyl 4,5-dihydro-thiophene • 5-methyl-3H-pyran-1,2-dithiol-3-one • 5,6-dihydro-2H-thiopyran • ethyl *tert*-butyl sulphoxide

The change of VOCs in sexual development was followed in fruiting bodies of the ascomycete *T. borchii* over four different stages of spore maturation which were defined by the percentage of asci containing mature spores (Zeppa et al. 2004; Table 2). The stages differed in number and type of VOCs. Immature asci and asci at the end of sporulation released sesquiterpenes, whereas S-containing compounds are released only at the later stages of ascus development. Interestingly, the sesquiterpene aromadendrene released by the immature ascus was also found produced by *T. borchii* mycelium grown in the presence of its host plant *Tilia platyphyllos* but not by free-living mycelium (Zeppa et al. 2004). In conclusion, vegetative and reproductive stages of fungal development produce different sets and also quantities of VOCs, likely as a result of the activation of different metabolic pathways. Therefore, VOC patterns can be used as a destruction-free probe system in order to explore biochemical processes underlying developmental processes of the fungi.

Impact of substrate on VOCs released by fungi

Growth and development of fungi are strongly dependent on nutrients and the physical environment (Kües 2000, Chang & Miles 2004), although they can adapt to a broad scale of conditions. Changes in growth conditions influence their metabolisms, resulting in altering VOC patterns (Wheatley et al. 1997, Gao & Martin 2002). For example during spirits production with carbohydrate-rich substrates such as potato or wheat, the yeast *Saccharomyces cerevisiae* produces ethanol as main product, but the individual substrate provides different and characteristic aroma, caused by the minor components of the yeast and also the substrate (Conner et al. 1998, Pinheiro et al. 2001, Kafkas et al. 2006, Porto et al. 2006).

Some investigations have been carried out on the impact of different media on VOC patterns released by micro-organisms (Wheatley et al. 1997, Bruce et al. 2000, Fiedler et al. 2001, Gao & Martin 2002, Gao et al. 2002, Scotter et al. 2005). Using two main groups of amino acid-rich and carbohydrate-rich media for microbial growth, it was put forward that there are VOCs unique to bacteria and fungi which are called **unique microbial volatile organic compounds (UMVOCs)** (Gao & Martin 2002). VOCs released from fungi on carbohydrate-rich media are mainly alcohols, acids, aldehydes and ketones. In case of amino acid-rich media, higher quantities of nitrogen (N)- and sulphur (S)-containing VOCs are encountered, for instance, cyclobutylamine and dimethyl trisulphide, respectively (Bruce et al. 2004). Zygomycetes, ascomycetes, and deuteromycetes are likely to release the S-containing

compound methanethiol when propagating on protein-rich media, in contrast to basidiomycetes (Scotter et al. 2005; Table 3). In comparison, bacteria on protein-rich media release also broad ranges of VOCs, most markedly S-containing VOCs like dimethyl trisulfide and heptan-2-one, the latter one independently of the media (Gao & Martin 2002).

Looking closer at molds, substrates have a strong effect on VOC production by different species. When *Aspergillus* spp. grow on media rich in nutrients, they proceed the normal primary metabolism and release in course alcohols like 3-methyl-1-butanol, 2-methyl-1-propanol, 1-octen-3-ol and ketones like octan-3-one. Once the nutrients are exhausted, the fungi shift to special secondary metabolisms which yields changed VOC patterns. More and other VOCs are released, including terpineol from the terpene group. In case media are amino acid-rich, this leads to production of S-containing VOCs (Fiedler et al. 2001, Gao & Martin 2002). Moreover, some aspergilli can accept sulphur from inorganic substrate and release it in form of dimethyl-disulfide (Gao & Martin 2002). The situation is contrasting in *Stachybotrys chartarum*, which releases about five times higher quantities of VOCs, when exploiting rich media. VOCs released by *S. chartarum* belong to the group of alcohols, ketones and terpenes. Also *Trichoderma* spp. (*Trichoderma pseudoko-ningii*, *Trichoderma viride*, *Trichoderma harzianum*) release different VOCs when grown on rich malt extract and poor minimal media, respectively (Wheatley et al. 1997, Fiedler et al. 2001, Humphris et al. 2001). However, no N-containing VOCs are observed, very low amounts of S-containing VOCs (benzothiazole) are released in *T. viride*, and ethanol is produced in large amounts, independently of the substrate types.

Table 3 Low molecular weight VOCs released from different types of fungi grown on C- and N-rich media, respectively (data from Scotter et al. 2005)

Phylum	**Zygomycete**		**Ascomycete**		**Deuteromycete**		**Basidiomycete**	
Species	***Mucor racemosus***		***Apergillus* spp.**		***Fusarium solani***		***Cryptococcus neoformans***	
Media	**C-rich**	**N-rich**	**C-rich**	**N-rich**	**C-rich**	**N-rich**	**C-rich**	**N-rich**
VOCs								
Ethanol	+	+	+	+	+	+	+	+
Acetaldehyde	-	+	-	+	+	-	+	+
Acetone	-	+	-	+	-	-	+	-
Methanethiol	-	+	-	+	-	-	-	-

Most wood-rotting fungi belonging to the basidiomycetes on artificial nutrient-rich medium as well as on the natural substrate wood typically release linear aliphatic C8 compounds such as 1-octene, octan-1-ol, 1-octen-3-ol, 2-octenal, 2-octen-1-ol, octan-3-one, and octan-3-ol (Rösecke et al. 2000, Ewen et al. 2004).

These examples from the literature document that VOCs released by micro-organisms are certainly useful to distinguish different groups and even species, but environmental and physiological conditions have to be considered. In order to gain a more consistent picture about growth conditions (temperature, humidity and light), kind of media and developmental stages have to be clearly and in depth addressed in research, since these factors strongly affect the metabolism, leading consequently to changes in VOC patterns (Table 4).

Volatiles released by fungus-infested wood

As discussed already above, VOCs released from fungi are strongly dependent on substrates and the stage of the life cycle and development. Wood as a substrate for wood-decaying fungi can be expected to influence the fungal metabolism. Fungi attack and colonise wood in different ways, depending on the properties of the wood. Wood as a substrate can be living or dead - namely in form of a standing tree, a felled tree, storage wood and wood in service, respectively -, engaging more or fewer living cells in the wood with the ability to render the substrate. When standing in the forest, the tree is a suitable nutrition source for fungi since it contains nutrients like sugars, amino acids and minerals. Anyway, when fungi infect a living tree, they have to adapt to or protect themselves against the tree defense system. In dead wood in contrast, most defence systems of the tree are not anymore active. However, dead wood provides less free sugar and amino acids, and more polymerised substrate which is more difficult to digest. Therefore, when fungi are growing on living or dead wood, the fungal metabolism and, thus, the fungal VOC patterns will be differentially affected. Besides nutrient contents, the water content of the wood is also severely affecting fungal growth and development, why in European standard EN 335-1 (1992) the hazard class for fungal decay is related to the water content in wood.

Table 4 VOCs released by micro-organisms (data taken from Wheatley et al. 1997, Bruce et al. 2000, 2004, Fiedler et al. 2001, Gao & Martin 2002, Gao et al. 2002, Scotter et al. 2005)

Micro-organisms	**Bacteria**		**Zygo-mycetes**		**Asco-mycetes**		**Deutero-mycetes**		**Basidio-mycetes**	
Nutrients (C/N rich)	**C**	**N**	**C**	**N**	**C**	**N**	**C**	**N**	**C**	**N**
VOC categories										
Alcohols										
• C1-C5	+	+	+	+	+	+	+	+	+	+
• C6-C10					+	+			+	
Aldehydes										
• C1-C5				+		+		+	+	+
• C6-C10		+				+			+	
Ketones										
• C1-C5	+	+			+	+		+	+	
• C6-C10	+	+				+			+	
• C10+	+	+								
Acids										
• C1-C5	+									
Esters										
• C1-C5	+									
Alkanes/Alkenes										
• C1-C5	+				+	+				
• C6-C10						+	+			
S-containing										
• C1-C5	+			+		+				
N-containing										
• C1-C5		+								
• C6-C10	+									
Terpenes										
• monoterpenes	+				+		+		+	
• sesquiterpenes					+				+	

Most fungi that infest and decay wood belong to the phylum of basidiomycetes, more precisely to the class of homobasidiomycetes. These wood-decaying fungi are divided into two main types, brown- and white-rot fungi, respectively, according to the colour of the wood in an advanced stage of decay. This difference results from their ability to degrade lignin. Brown rot fungi can degrade all components in wood but lignin. The left-over phenolic substrate lignin turns brown in colour. In contrast, white rot fungi can degrade all types of wood components, even lignin. Their decay mechanism resembles the action of a bleaching agent resulting in whitish-stained cellulose as left-over from the wood (see also Chapter 17 of this book). Another type of fungi softens the cell walls of wood by decay reactions. Such species are therefore called soft-rot fungi and they belong mostly to the phylum of ascomycetes. So far however, little is known between the effect of different rotting abilities of fungi and the release of respective VOC patterns (Thakeow et al. 2006).

Sick building syndrome (SBS) as a consequence of VOCs

When micro-organisms infest buildings, they may produce a potentially hazardous environment. Individuals exposed to environments that contain high concentrations of airborne contaminants from microbial organisms report health symptoms including eye and sinus irritation, headache, nausea, fatigue, congestion, sore throat, and even toxic poisoning. The term "sick-building syndrome" (SBS) was first coined in the mid-1980s referring to ill-health symptoms arising from poor indoor air quality, that further on have been frequently correlated with the presence of fungi (Ahearn 1996). Current methods for detecting microbial contamination include air and material sampling with fungal culture analysis, air sampling coupled with gas-chromatography, mass-spectrometry, and visual inspection (Pasanen 1992, Schiffman et al. 2001). Several micro-organisms infest buildings and release **microbial volatile organic compounds (MVOCs)**. Typical fungi are of the genera *Aspergillus/Eurotium, Penicillium, Cladosporium*, *Trichoderma* and *Stachybotris*. MVOCs released are mainly alcohols (pentanoles, hexanoles, octanoles), ketones (hexanones, heptanones, octanones), and a few N- and S-containing compounds (pyrazine and dimethylsulfide, respectively) (Wilkins et al. 2003, Nilsson et al. 2004, Schleibinger et al. 2005).

Besides contaminants released by micro-organisms, wooden buildings themselves also release VOCs which contribute to SBS. VOCs released from several wood species were examined (Risholm-Sundman et al. 1998), i.e., ash, beech, maple, birch, oak, cherry, rubber wood, pine and spruce. Acetic acid, a compound of corrosive nature, was emitted from every wood species, except pine and spruce. In contrast, terpenes are generally released from pine and spruce wood. Especially 3-carene may irritate skin and mucous membranes. Allergy and chronic lung function impairment might be elicited after prolonged exposure (Falk et al. 1991).

What is the role of VOCs for insects?

Wood is the basis of existence for adapted fungi and insects, influencing each other's living conditions. In the context of the trophic interaction between wood, insects, and fungi, we have seen the functions of the participants and the variation within. Wood can be living or dead, actively or passively defensive. It can be infested by specific insects and/or fungi - under indoor and outdoor environments. Insects can follow just wood VOCs (Weissbecker et al.

2004), fungal VOCs (Fäldt et al. 1999, Holighaus & Schütz 2006) or defense signals (Schütz & Weißbecker 2003). On the one hand, this broad and diverse information is a basis for diverse evolutionary development, marking ecological partitioned niches and suitable environments for hosting species such as insects. On the other hand, this requires a high plasticity of appendant organisms as receivers of the available chemical information (Johne et al. 2003, 2006a). Hence, relying on common VOCs keeps flexibility in a changing and dynamic environment whereas relying on a specific VOC as a kind of marker compound for suitable host plants represents the advantage of highly specific adaptation. Thus, research on a multitrophic system using VOCs as information needs advanced techniques in trace analysis and interpretation (Weissbecker et al. 2004). Sometimes, the crucial information is small and silent, maybe hidden behind abundant noises.

VOCs mediating insect interaction with trees, wood and fungi

Insects on living trees

Insects attacking living trees use the typical host VOCs released by the tissue sought after. In stems of conifers, for example, several monoterpenes such as α-pinene, β-myrcene, terpinolenes and β-pinene are attractive to a large number of conifer inhabiting beetles: an overview of the chemical ecology of bark beetles (Scolytidae) in this complex olfactory landscape is given by Byers (2004), of weevils (Curculionidae) by Schlyter (2004), of longhorn beetles (Cerambycidae) by Allison et al. (2004), and of jewel beetles (Buprestidae) by Schütz et al. (1999a, 2004). The influence of VOCs on insect behaviour is well studied in the case of *Picea abies* in the context of infestation with the bark beetle *Ips typographus*. A cascade of VOCs is released during the process of infestation and colonisation by the beetle: primary attractive VOCs from the bark draw beetles to a weakened tree, followed by production and release of aggregation pheromones by the insects. Subsequently, the release of VOCs from the tree indicating exhaustive overuse of the plant resource leads to repulsion and dispersal of the beetles (Byers 2004). The prospect of successful infestation led obviously during evolution to a high sensitivity of tree invading insects to VOC signals related to different stress factors. Defence reactions of the tree become transparent through shifts in VOC abundance and composition (Petterson & Boland 2003, Schütz et al. 2004). Franceschi et al. (2005) reviewed defence aspects by the wood anatomy influencing chemical defences against insects and blue-staining fungi. Both, anatomical and chemical defense turn out to be strongly interlinked (Hudgins et al. 2004, Erbilgin et al. 2006, Zeneli et al. 2006).

Fungus-insect interaction on trees, trunks and deadwood

Fungi often participate in tree-insect-interactions. These interactions with trees and wood are reviewed by various authors in the past (e.g. Buchner 1953, Wilding et al. 1989, Vega & Blackwell 2005). Insects can be a vector of fungi (Paine et al. 1997), feed on the fungi degrading wood (Mueller et al. 2005), or even host endosymbiotic fungi for wood digestion (Buchner 1953). Especially many xylophageous insects feeding on deadwood co-evolved with fungi to complex symbiotic coenosis (Douglas 1989, Klepzig et al. 1996, Dillon & Dillon 2004). Enzymatic detoxification abilities of these endosymbiotic fungi make otherwise protected lignocellulosic resources accessible - not at least hence, these fungi and their enzymes are of commercial interest (Dowd 1992). Conversely, because of competition for the same resource volatiles from wood decaying fungi can be repellent for insects (Johne et al. 2006a) or toxic fungal metabolites (VOCs and non-VOCs) may keep insects away from the wood (Seybold et al. 2006). Overall, even healthy trees are not aseptic. Fungal interactions with living trees are known in forms of latent infections of the xylem or endophytic colonisations of leaves (Hendry et al. 2002), not to mention the symbiotic mycorrhiza (van der Heijden & Sanders 2002). In the xylem of European beech, for example, *Hypoxylon fragiforme* was identified as a latent invader besides other casual inhabitants (Hendry et al. 2002). Chemotaxonomic studies of this species give hints to metabolites released by the fungus (Stadler et al. 2004). However, up to now there are no data about chemical reactions, resultant VOCs, or insect preferences emerging from this type of fungus-tree interaction.

The insects related to tree trunks can be grouped roughly into phloem- and xylem-feeders. Phloem is rich in nutrients but strongly shielded by the active plant defense system and xylem is hard to digest but less protected (Lieutier 2004). Fungi can play a fundamental role for both groups of insects to overcome the respecttive defensive systems (Dowd 1992). Moreover, insects may feed on fungi utilising the ability of fungi to catabolise cellulose (Watanabe & Tokuda 2001). Some xylophagous or deadwood insects are therefore grouped as mycophagous insects, too (Bouget et al. 2005). For example, the family of bark beetles (Scolytidae) with worldwide about 6000 species presents a huge variability of associations with trees and fungi (Jacobs & Wingfield 2001, Kolařík et al. 2005). These beetles differ widely in their ecology and biochemical adaptations to their host trees. Within this taxonomic group are phloem- and xylem feeders, ambrosia beetles with a compulsive association to symbiotic fungi and there are also several facultative connections between bark inhabiting

insects and fungi (Farrell et al. 2001, Aukema et al. 2005, Mueller et al. 2005). The majority of Scolytidae are phloem-feeders with obviously mutualistic relationships to their fungal associates but the strength of interaction is still subject of considerable debate. Several cases are known where insects act as vectors of serious fungal pests, most noticeable when non-indigenous, newly introduced, and thus not adapted to a given environment (Harrington et al. 2001, Allen & Humble 2002). Many fundamental aspects of the degree of dependence in such insect-fungus relationships are however still poorly known (Kirisits 2004). A strong relationship to fungi is known in the scolytid xylophagous ambrosia beetle *Trypodendron domesticum* and the lymexylid *Hylecoetus dermestoides* which both infest the xylem of *F. sylvatica* trees. They follow the first chemical hints of weakness in living and especially freshly cut trees and initiate ongoing decay processes by introducing several associated "ambrosia" fungi (Farrell et al. 2001, Holighaus & Schütz 2006). VOCs are the main signals for these beetles obtaining information about precise decay and defence status of trunk patches (Holighaus & Schütz 2006). Electrophysiological techniques (EAG = electroantennography) use insect antennae, which are often much more sensitive to VOCs than trace analytical methods, to locate within the hundreds of VOCs those, carrying the information of suitability. General and omnipresent VOCs are little informative. Others correlate with general plant physiological processes and are therefore useful for an assessment of suitability of a trunk as breeding substrate. Further VOCs give highly specific information of e.g. certain fungal species colonising the wooden substrate which can be indispensable for insect development and hence lead to attraction (Belmain et al. 2002), or which are even fatal and have to be avoided. Evolution eventually led to highly specific adaptations which turned VOCs into triggers of these complex interactions. By observing these sensitive signals with analytical techniques, we can obtain the state of wood in aging, decaying and the status of interaction or infestation with fungi and insects (Weissbecker et al. 2004, Holighaus & Schütz 2006, Thakeow et al. 2006).

Insects on wood and wooden products

Not all insects feeding on wooden substrate necessarily need fungal associates. There are species without as *Ergates faber* (Cerambycidae), producing own endogenous cellulases. This ability for cellulose degradation is found sometimes in other insect families, too, for example in cockroaches (Blattaria) and termites (Isoptera) (Douglas 1989, Watanabe & Tokuda 2001). Beside "wood worms" which are larvae from the family of Anobiidae, the Old House Borer

Hylotrupes bajulus (Cerambycidae) is a widespread insect pest of coniferous timbers in buildings. Without any fungal support it can cause substantial damage to roof timbering or framework houses even in temperate climate. An understanding of the volatiles relevant for the orientation of *H. bajulus* could help to find new methods for protection of wood and a control of the beetle (Reddy et al. 2005a). *H. bajulus* is very delicate in the choice of sites for mating and oviposition and obviously, it is guided by olfactory cues. Recent behavioural studies assigned importance to monoterpenoid hydrocarbons as attractants (Fettköther et al. 2000, Reddy et al. 2005b). A direct investigation of the olfactory response of *H. bajulus* to original odour samples of its host trees by GC-EAD/MS yielded a more complex mixture of terpenes, aldehydes, alcohols and other hydrocarbons as VOCs being important to *H. bajulus* (Weissbecker et al. 2004). This knowledge will be crucial for the assessment of thermal wood treatments or chemical wood modification techniques for protecting constructional wood without any poisonous chemicals, just by reducing olfactory traceability and attraction for the Old House Borer.

Siricid woodwasps (Siricidae) (Thomsen & Koch 1999) and Anobiidae, like the death watch beetle *Xestobium rufovillosum* (Belmain et al. 2002), are xylophagous insects which have acquired fungal associates and cause substantial damage on wood and wooden products prior and during service. Both are examples for endosysmbiotic relationships to fungi. Ambrosia beetles (Holighaus & Schütz 2006), as described above, are known for ectosymbiotic relationships. In termite-species (Isoptera), (Brune & Friedrich 2000) both types of symbiosis can be found.

Insects on fungi

Other beetles, for instance of the family of Cisidae, do not bother to prepare the wood for symbiotic fungi but just feed in more or less specialised manner on fruiting bodies of bracket fungi (Jonsell & Nordlander 2004). *Fomitopsis pinicola* and *Fomes fomentarius*, bracket fungi growing on tree trunks of *Pinus* and *Fagus species*, respectively, were shown to release C8 compounds, such as 1-octene, octan-1-ol, octan-3-ol, 2-octene-1-ol, and 1-octen-3-ol, and sesquiterpenes such as β-barbatene. The cisid beetles *Cis glabratus* and *Cis quadridens* can discriminate the host odour of fruiting bodies of *F. pinicola* and *F. fomentarius*, respectively (Fäldt et al. 1999). Moreover, predatory Anaspidae feeding on cisid beetles, namely *Anaspis marginicollis*, *Anaspis rufilabris* and *Epinotia tedella*, were significantly attracted to 1 octen-3-ol released predominantly by damaged fruiting bodies of the bracket fungi (Fäldt et al.

1999). These different degrees of specialisation in insects for fungus infested wood might be used in biomimetic sensor systems for the assessment of wood with respect to fungal infestation.

Techniques for assessing wood quality on the basis of VOCs

It is a bionic concept to utilise VOCs as a parameter for wood quality assessment: This concept is inspired by the impressive achievements of insects in performing this task just by olfaction and taste. However, the approaches to copy these "inventions of nature" are so far sparse.

Biosensors

The selectivity and sensitivity of biological recognition processes motivated the development of biosensors. Biosensors are miniature measuring devices con-sisting of a biological recognition component in close spacial and functional contact with a physical transducer unit. The biocomponent utilised can be of different levels of organisation: whole (micro-) organisms in organismic biosensors, whole sensory systems in biosensors on the basis of sensory organs, and enzymes, antibodies, or receptor-proteins as well as nucleic acids in bio-molecular biosensors. The physical transduction unit is needed to transduce analyte caused changes of the biocomponent (heat, mass, light, resistance, capacity, current, potential, ...) into output signals that can be processed by electronic data processing units (Wollenberger et al. 2000, see also Chapter 12 of this book).

Biosensors for detecting volatile compounds principally fight the problem that biocomponents tend to deteriorate when exposed to air. Especially biomolecules have to be protected by membranes which are hindering diffusion of VOCs and thus, compromising sensitivity. Hence, most biosensors utilising biomolecules as a biocomponent are relying on extraction of VOCs by aqueous solvents, as it is done for the amperometric enzyme biosensor for detecting phenolic compounds from wood pulp (Rosatto et al. 2001), and the amperometric enzyme biosensor for the assessment of wood ageing (Campanella et al. 2005).

In contrast, biosensors on the basis of immobilised micro-organisms show considerable working stability in air. One major field for these biosensors is the measurement of complex parameters like toxicity. Biosensors for the toxicity of VOCs using recombinant bioluminiscent *Escherichia coli* bacteria (Gil et al. 2000, Mwinyihija et al. 2005) or different

strains of algae (Podola et al. 2004) were designed, which might be applicable for wood quality assessments with regard to potential health implications of wood materials. Moreover, biosensors on the basis of immobilised micro-organisms were proposed for the analysis of fermentation characteristics of spoilage micro-organisms (Wang & Wang 2002). This proposal was picked up utilising a potentiometric biosensor based on immobilised yeast cells (Rotariu et al. 2004), and for the analysis of microbial communities utilising an array of electrochemical microsensors and microscale biosensors selectively responding to volatile fatty acids based on immobilised bacteria (Meyer et al. 2002, Revsbech 2005). These biosensors might be applicable for wood quality assessments with regard to microbial infestation and degradation.

Biosensors on the basis of insect olfaction provide unrivalled measuring rates, selectivity and sensitivity in the analysis of VOCs in air (Schroth et al. 1999). Integrating ecological and behavioural observations with electrophysiological measurements of antennal responses to VOC mixtures corresponding to specific situations (for instance: forest fires) yielded sets of insect antennae and marker compounds for the detection of these situations. The black jewel beetle (*Melanophila acuminata*) was found to be attracted by forest fires, because burnt wood is the only suitable substrate for bringing up its offspring. The antennae of the black jewel beetle were proved to be highly sensitive and selective to guaiacol derivatives, compounds which are generated by the pyrolysis of wood (Schütz et al. 1999a). The antennae and with them the set of detected compounds can thus be appointted by the biosensor-designer to assess wood species and fire parameters (temperature, oxygen access) involved in the forest fire in distances of kilometres (Schütz 2004). A few of the compounds recorded by the beetle's antennae are recognised to be of special interest to coopers´ thermally modified oak wood used to produce barrels for wine storage (Chatonnet et al. 1999, Campbell et al. 2005), identifying a promising field of application for such a biosensor in process and quality control of thermally modified wood. Thermal and chemical wood modification is recently discussed to replace treatments of construction wood with poisonous biocides (see Chapter 13 of this book). The house borer *H. bajulus* is one of the major reasons for this problematic biocide treatment (see above). Besides terpenes, there are aldehydes involved in detection and classification of wood by the beetle (Weissbecker et al. 2004). A biosensor on the basis of the antennae of *H. bajulus* can be useful to assess the VOC patterns generated by different wood modification techniques with regard to the detectability and acceptability of treated wood by this dangerous beetle.

The detection and characterisation of fungal infestation and decay in construction wood prior to and during service is another important task by the fact that fungal infestation can have deleterious impacts on the mechanical stability of construction wood. According to their ecology and behaviour, different insect antennae respond differentially to microbial VOCs. However, virtually every insect examined responds to branched- and linear C-8 compounds as markers for microbial activity (Fäldt et al. 1999, Schütz & Weißbecker 2003, Holighaus & Schütz 2006). With this knowledge, biosensors on the basis of insect antennae for the infestation by micro-organisms were established (Schütz et al. 1999b). Despite the high performance of this kind of biosensors, their field of application is limited to on-site-measurements because of the limited life-time of the biocomponent antennae, ranging from hours to days. Further development of biomimetic sensors employing key principles of stablisation, pre-filtering and recognition of insect olfaction will be necessary in order to extend life time and availability of sensor devices on the basis of insect olfaction (Schütz et al. 2001).

Electronic noses

The biological olfactory system inspired furthermore the development of electronic nose technology. An electronic nose is a machine that is designed to detect and discriminate complex mixtures of VOCs (odours) using a sensor array (Eberheim et al. 2004). The sensor array consists of broadly tuned (non-specific) sensors that are treated with a variety of odour-sensitive biological or chemical materials. An odour stimulus generates a characteristic fingerprint from the sensor array. Patterns or fingerprints from known odours are used to construct a database and train a pattern recognition system so that odours within the trained range can subsequently be classified and identified. Thus, electronic nose instruments are comprised of hardware components to collect and transport odours to the sensor array as well as electronic devices to digitise and store the sensor responses for signal processing (Pearce et al. 2003).

The pulp and paper industries in eastern Canada need to differentiate black spruce, balsam fir, and jack pine because their proportions in wood chips affect the quality of the pulp and paper produced. A prerequisite to determine their proportions is to be able to rapidly identify the wood of the three conifers. Using a combination of marker compounds and GC profiles of hexane extracts made a distinction even of the sapwood of these tree species possible (Pichette et al. 1998). However, this initial method is too slow and expensive to be used by

paper mills. A more advanced electronic nose consisting of 32 conducting polymer sensors (CyranoseTM 320) was able to rapidly discriminate and identify black spruce, balsam fir and jack pine wood chips, utilising principal component analysis as a data analysis tool (Hobbs et al. 2000, Garneau et al., 2004). In another application, thermally modified oak wood for wine barrels was assessed regarding its toasting level by an electronic nose consisting of 6 metal oxide semiconductor sensors utilising principal component analysis, discriminant function analysis and neuronal network techniques for data analysis (Chatonnet 1999).

The growth of bacteria and fungi on organic matter generates a broad range of VOCs (see above). Most studies with electronic noses deal with the detection and classification of bacteria (Holmberg 1997, Gardner et al. 1998), using sensor-arrays consisting of six to nine metal oxide semiconductor gas sensors. Few reports are available for fungal detection: with an accuracy of 93% six spoilage fungi of meat (four *Eurotium* spp., each one *Penicillium* and *Wallemia* species) were classified on blood agar 24 hours after infestation and prior to visible growth, using an electronic nose consisting of 14 polymer sensors (Keshri et al. 1998). With the same electronic nose, seven homobasidiomycetes (*Agaricus arvensis, A. bisporus, A. campestris, Agaricus maleolens, Agaricus nivescens, Pleurotus sajor-caju,* and *Volvariella bombycina*) were differentiated (Keshri et al. 2003). Twenty-four hours after their inoculation on rich potato-dextrose-agar (PDA) and a minimal medium (Czapek-Dox agar), 5 fungi suspected to be involved in SBS (*Aspergillus flavus, Aspergillus niger, Cladosporium cladosporioides, Penicillium chrysogenum,* and *S. chartarum*) were detected and classified by an electronic nose designed at North Carolina State University (NC State E-Nose) consisting of 15 metal oxide sensors. The classification was independent on type of the growth medium. The raw data of this analysis were transferred to an electronic data processing system. They were first compressed using windowing functions which provided a set of four features for each sensor. Linear-discriminant analysis was then applied to the compressed data to maximise class separability. Sixty percent of the compressed data were randomly selected to form a training set for the classification algorithms. K-nearest-neighbours (KNN) and least-squares (LS) techniques were both employed to classify the remaining 40% of the compressed data. The KNN technique resulted in 90% accuracy of species identification after the first day of inoculation (Schiffman et al. 2000).

Outlook

Bionic noses integrate two different approaches by copying algorithms used by nature in odour perception on different level of organisation. Biosensors selectively tuned to marker compounds are amended by algorithms of electronic noses operating with an array of broadly tuned chemical sensors in order to discriminate complex situations based on a set of marker compounds.

As a complex sensing device, an insect antenna can serve as a blueprint for technical sensor optimisation in a "constructive bionics" approach, using for instance the principles of the porous cuticle for sample enrichment, as shelter against air and dust, and as a chemical pre-filter. Algorithms used by insects for contrast enhancement in odour mixture recognition can be exploited as a source of inspiration in an "informational bionics" approach. The identification of marker compounds or pattern recognition algorithms from sensory ecology of insects interacting with wood and degrading micro-organisms might serve as a guideline in a "process bionics" approach in the development of new bionic sensors for wood assessment. Thus, in the near future the possibilities in wood assessment can be considerably extended by thorough application of a bionics/biomimetic approach.

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CHAPTER 3

GAS CHROMATOGRAPHY WITH MASS SPECTROMETRIC AND ELECTROANTENNOGRAPHIC DETECTION: ANALYSIS OF WOOD ODORANTS BY DIRECT COUPLING OF INSECT OLFACTION AND MASS SPECTROMETRY

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Abstract

A gas chromatography-mass spectrometry-electroantennographic detection (GC-MS/EAD) setup has been designed by adapting a commercially available "Olfactory Detector Port" to the use with an insect antenna. Measurements were performed with antennae of the old house borer *Hylotrupes bajulus*, a widespread insect pest of coniferous timbers. Headspace volatiles from timber of *Pinus sylvestris* were collected and analysed by GC-MS. About 30 compounds were identified in the Kovacs range from 500 to 1200, especially terpenoids and aliphatic alcohols and aldehydes. The antennae of *H. bajulus* responded to nearly half of the detected volatiles with a peculiar sensitivity for α-pinene among the terpenes and for hexanal among the aldehydes.

Keywords

Electroantennographic detection; Detection, GC; *Hylotrupes bajulus*; Wood volatiles; Alcohols; Aldehydes; Terpenes

Introduction

The ability of insect antennae to produce electrical potentials when stimulated with volatiles was demonstrated by Schneider (Schneider, 1957). The so-called electroantennogram (EAG) can be recorded with electrodes positioned in the hemolymph at the base and the tip of an antenna. The EAG represents a superposition of receptor potentials of sensory cells with different specifications. When the antenna is stimulated with isolated compounds the amplitude of an EAG signal correlates with the concentration of the applied substance. The high sensitivity and selectivity of insect olfactory receptors combined with a gas chromatographic separation provides a powerful analytical technique (Moorhouse et al., 1969, Weissbecker et al., 1997) referred to as GC-EAG or GC-electroantennographic detection (EAD). This technique is predominantly used by chemical ecologists in order to investigate the interrelation between insects and their environment. The main apparative challenges of this method are the development of an "interface" for the adaptation of the hot and dry GC effluent to the requirements of the insect (i.e. humid and at a biocompatible temperature) and the use of a high-impedance amplifier which allows the recording of the EADsignal with a plotter or a data processor (Struble & Arn, 1984). Examples of recent publications show the usefulness of this method, e.g. in pheromone research (Cossé et al., 2002; Kalinova et al., 2003; Tolasch et al., 2003), host finding of parasitoids (Pettersson et al., 2001; Aldrich & Zhang, 2002), and food location of phytophagous insects (Bédard et al., 2002; Nojima et al., 2003; Syed et al., 2003). Other examples are interactions like chemical mimicry (Haynes et al., 2002; Schiestl & Ayasse, 2002) or multitrophic interactions (Smid et al., 2002).

Often EAD is used in parallel to flame ionisation detection (FID) as a GC-FID/EAD technique. When samples of unknown composition are analysed, in addition to the GC-FID/EAD technique an analysis by GC-MS is essential. However, the comparison of chromatograms recorded with two different setups can be very difficult and time consuming. Moreover, modern sampling techniques like solid-phase microextraction (SPME) or thermo-desorption produce samples that are exhausted in the moment of injection and cannot be used for a second injection in another analytical instrument. This raises the demand for a combination of both methods to a coupled GC-MS/EAD technique. The main difficulty of this method is the controlled splitting of the GC effluent between atmosphere (EAD) and vacuum (MS).

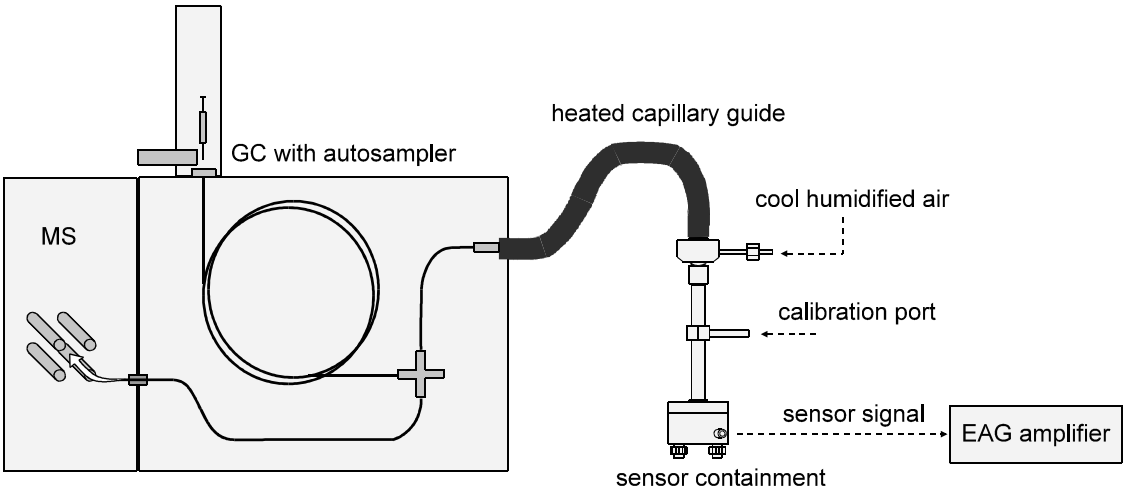

Fig. 1: Schematic drawing of the GC-MS/EAD setup.

A stimulus for solving this problem came from another closely related field of application. In parallel to GC-EAD, the technique of combining gas chromatography with a "sniffing detector" has been developed. This technique, sometimes called gas chromatography-olfactometry (GC-O) is quite similar to GC-EAD, but a human nose is used as detector and the operator is able to record character and intensity of the odours. The main field of application of this technique is found in the production of perfumes, nourishments and stimulants (Jordan et al., 2002; Boscaini et al., 2003; le Fur et al., 2003).

However, also other branches of manufacturing industry (e.g. cloths, furniture, cars) realised the importance of the sense of smell for purchase decisions of the consumers. Maybe this demand from economically important industries finally led to the emergence of GC-MS/O as an ultimative method in combining analytical power with the sensory impression of a human operator. Today, devices for the coupling of MS with an olfactory detection are available from several suppliers of analytical equipment. Applications are found for example in the production process of synthetics (Villberg & Veijanen, 2001) but also in the monitoring of malodours from agricultural facilities (Rabaud et al., 2002).We now performed the next step by adapting a commercially available GC-MS/O interface to a GC-MS/EAD setup (Fig. 1).

First results with the new method have already been obtained with *Cameraria ohridella*, an invasive pest of horse chestnut trees (Johne et al., 2003). As a new challenge we chose the old house borer (also called house longhorn beetle) *Hylotrupes bajulus*. This beetle is a widespread insect pest of coniferous timbers and can cause substantial damage to roof

timbering or framework houses.With an origin in Europe the beetle has dispersed into areas of moderate climate all over the world. Adults of *H. bajulus* are found in sizes ranging from 8 to 20 mm. Female beetles lay eggs into cracks of timber or of truncated trees after windblows. The damage is done by the larvae which spend from 2 up to 8 years feeding in the wood before they pupate and emerge as adult beetles. The tunnelling of the larvae often results in considerable financial loss since infested wood in buildings has to be replaced. An understanding of the volatiles relevant for the orientation of *H. bajulus* could help to find new methods for protection of wood and a control of the beetle. *H. bajulus* is very delicate in the choice of sites for mating and oviposition and obviously is guided by olfactory cues (Becker, 1943; Higgs & Evans, 1978; Plarre & Hertel, 2003). Recent behavioural studies supported these results and attached importance to monoterpenoid hydrocarbons as attractants (Fettköther et al., 2000). These experiments were performed with hexane extracts of wood or odorant standards of volatiles that were previously identified as constituents of the coniferous aroma. A direct investigation of the olfactory response of *H. bajulus* to an original odorant sample of its host trees by GC-EAD so far has not been made. The volatile emissions of wood are a complex mixture of terpenes, aldehydes, alcohols and other hydrocarbons which make it inevitable to perform an additional analysis by GC-MS. The use of a combined GC-MS/EAD setup offers the advantage that no toilsome comparison between the chromatograms of two different setups is necessary. The obtained results give a new insight into the chemical ecology of *H. bajulus* which might be used for the development of new methods of wood protection.

Experimental

Sampling of volatiles

Samples for GC-MS/EAD analysis were collected using the closed-loop-stripping-analysis (CLSA) method (Boland et al. 1984). Timber of Scots pine (*Pinus sylvestris* L.) was reduced to small cuboids of about 5 cm^3 volume. Of these cuboids 100 g were put into a 500 ml glass flask with a ground neck outlet. The outlet was closed with a PTFE-stopper. Stainless steel capillaries (i.d. 1 mm) were fed through the stoppers. A miniature pump (Fürgut, Tannheim, Germany) circulated air from the flask to an adsorbent trap loaded with 1.5 mg charcoal (CLSA-Filter, Daumazan sur Arize, France). Sampling was performed for 45 min with a flow of 1 l/min. Volatiles were eluted from the charcoal with 75 µl of a mixture consisting of methylene chloride (two parts) and methanol (one part) (both solvents Suprasolv quality, Fa.

Merck/VWR, Darmstadt, Germany). Samples were stored in an ultra low temperature freezer at -80 °C.

GC-MS/EAD system

The system (Fig. 1) is based on aGC-MSsystem produced by Agilent (Palo Alto, USA) and consists of a type 6890N gas chromatograph connected to a type 5973N quadrupole mass spectrometer. The GC is equipped with a type 7163 autosampler and a split/splitless injector. Data acquisition is done with the MS ChemStation software (Agilent). A J&W Scientific HP-5MS column (Agilent) is used (30m × 0.25mm i.d., film thickness 0.25 µm). The effluent from the column is splitted into two pieces of deactivated capillary using a Graphpack 3D/2 flow splitter (Gerstel, Mülheim, Germany). One capillary (1m×0.1mmi.d.) leads to the mass spectrometer, the other (1m × 0.15 mm i.d.) to an "olfactory detector port" (ODP-2, Gerstel). The split and the restriction capillaries were part of the ODP setup and were "factory adjusted" in order to result in an equal split of the gas flow into the two setups.

The ODP incorporates a flexible heating sleeve (length outside GCoven: 35 cm) which guides the capillary out of the GC oven. When the volatiles elute from the end of the capillary they are enveloped by a flow of helium used as a make-up gas in order to prevent contact of the volatiles with the surfaces of the setup. The nose-adapter that normally belongs to the ODP is replaced by a mixing chamber in which the effluent of the capillary is mixed with humidified air [23 ◦C, 80% relative humidity (RH)]. The humidified air is provided by bubbling synthetic air through a 500 mlwashing bottle at a rate of 500 ml/min. The airflow is directed vertically through the flow tube (length: 15 cm, i.d. 6 mm, PTFE) to the insect antenna which is housed in a detector cell made of PTFE. This setup is in the following referred to as EAD interface. For peak identification the National Institute of Standards and Technology mass spectral library (NIST, Gaithersburg, USA) and the MassFinder 2.1 software together with the library "Terpenoids and Related Constituents of Essential Oils" (Hochmuth, König, Joulain, Hamburg, Germany) are used.

Analytical conditions

Samples are injected in a quantitiy of 1 µl into the injector in the pulsed splitless mode (pulse pressure 150 kPa until 1.5 min) at a temperature of 250 °C. The GC is operated in the following temperature program: start: 50 °C, hold for 1.5 min, ramp 6 °C/min to 200 °C, hold for 5 min. The chosen temperature program is a compromise between optimal separation

conditions and a moderate run time which is essential because of the limited lifetime of the employed antenna. Helium (purity 99.999%) is used as carrier gas after passing through a combined adsorbent trap for removal of traces of water, oxygen and hydrocarbons ("Big Universal Trap", Agilent). The carrier gas flow is set to 1 ml/min resulting in a gas vector of 24 cm/s. The GC-MS interface is operated at a temperature of 280 °C. The heating sleeve of the ODP is set to 230 °C. The mass spectrometer uses electron ionisation (EI) at 70 eV and is used in the scan mode with a mass range from 35 to 300 mass units at a scan speed of 2.78 scans per second.

EAD

Excised antennae of *H. bajulus* are placed in an antenna holder (Professor Koch, Kaiserslautern, Germany) modified from a model designed for a portable EAG system (Färbert et al. 1997). Within this holder the ends of the antennae are in touch with an electrolyte solution adapted to the insect's hemolymph (Kaissling & Thorson, 1980) which provides electrical contact to a pair of Ag/AgCl electrodes (Fig. 2). The antenna holder provides a stable support for the antenna in which its surface is freely accessible to the air flow from the EAD interface.

For amplification of the EAD potentials an electronic setup (Professor Koch) is used that consists of a pre-amplifier, a main amplifier, a frequency filter and an adjustment amplifier. Pre-amplifier (input impedance 100M) and main amplifier each provide amplification by a factor of 10, resulting in a total amplification of 100. The amplifier has a built-in low pass filter which is set to a cut-off frequency of 1 Hz in order to suppress the ubiquitous mains frequency of 50 Hz.

The amplified signal is recorded by the Agilent GC Chem- Station software (which is installed on the data acquisition system in addition to the MS ChemStation software) using the type 35900E A/D converter (Agilent). The A/D converter is connected to the acquisition system via LAN and synchronised by the start signal of the GC. The following steps were taken in order to match the amplifier output to the input signal range of the A/D converter (0-1 V):

(1) The additional frequency filter is used as a high pass filter with a cut-off frequency of 0.01 Hz to suppress the slow drift which is often observed in the EAD signal and can cover several volts in the amplified signal.

(2) Since EAG signals may be positive or negative (depending on the mounting of the antenna in the antenna holder) it is convenient to use the adjustment amplifier to add a constant voltage of 0.5V to the amplified, high pass filtered signal. This keeps the signal in the center of the signal input range of the A/D converter (0-1 V) and allows to record positive deflections as well as negative deflections.

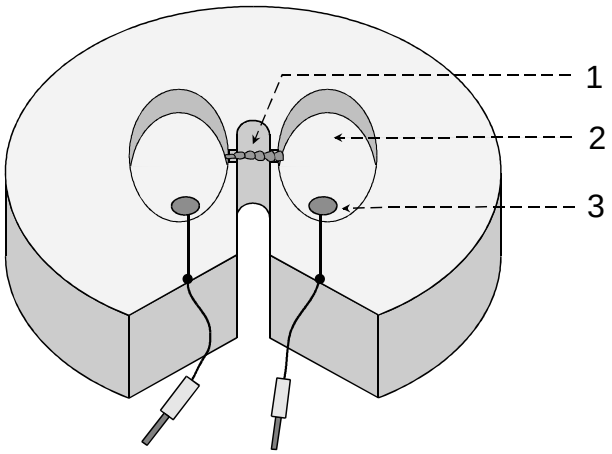

Fig. 2: Antenna holder milled from a perspex disc (∅ 27 mm, height 12 mm). The antenna (1) is stretched over the central hole of the holder while its ends are in contact with the electrolyte solution reservoir (2). The Ag/AgCl-electrodes (3) are connected to micro plugs via silver wires.

Calibration of the EAD

For a quick calibration of the EAD system a calibration port is installed into the flow tube of the EAD interface (Fig. 1). The calibration port is positioned 4.5 cm below the upper end of the flow tube. Odorant standards are produced using dilution series of the respective compounds in paraffin oil (Uvasol quality, Merck/VWR). Small pieces of filter paper (2 cm^2) are drenched with a small amount of the standard dilution (100µl). The filter paper is put into a 10 ml glass syringe. Inside the air volume of the syringe the odorant will accumulate in a concentration that is proportional to the concentration of the substance in the solution and its vapour pressure according to Henry's law (Schütz et al., 1997). By injecting a fixed volume (5 ml) of air onto the antenna a reproducible stimulus can be applied. Pentanal (97% purity, Sigma-Aldrich, Taufkirchen, Germany), hexanal (98% purity, Sigma-Aldrich) and heptanal (95% purity, Acros, Geel, Belgium) are used as calibration standards.

Hylotrupes bajulus

Specimen of the old house borer are obtained from a laboratory rearing of the Federal Institute for Materials Research and Testing (BAM), Berlin. The rearing uses small blocks of pinewood in which individual larvae of the beetle are housed. Under these optimised rearing conditions larval development is shortened to less than 1 year. Adult beetles are used for the experiments at an age of 1-4 weeks after emergence.

Results

Calibration of the setup

Every individual antenna of *H. bajulus* has a unique sensitivity for the volatiles emitted from pine timber. In addition, the sensitivity will change in the course of time. Considering the limited lifetime of an excised antenna a calibration by injection of standard solutions in the GC would consume too much time. However, in order to compare measurements obtained from different antennae it is important to get information on the sensitivity of the employed antenna. This can be done by injection of volatiles into the calibration port of the GC-EAD interface. When a stimulus is delivered to the antenna as a short pulse (e.g. as a manual injection within 0.5 s), the EAD potential will show a steep depolarisation followed by a slow return to the baseline which may last for some seconds. The duration of the quenching of the signal varies with the fitness of the antenna and the concentration of the odorant. This implies that the area of a peak recorded in the EAD signal is not a good measure for the amount of the stimulus. However, the amplitude of the deflection correlates with the strength of the stimulus. Fig. 3 depicts the response of antennae of *H. bajulus* to dilution series of three different aldehydes. The results show that among the aliphatic aldehydes hexanal is detected with the highest sensitivity. Experiments with lower (C1-C4) and higher (C8-C12) aliphatic aldehydes show that the sensitivity for aldehydes decreases all the more as the chain length deviates from C6 (data not shown). A dilution of hexanal in paraffin oil with a concentration of 10−6 results in a concentration of ca. 2.8 ppbv.

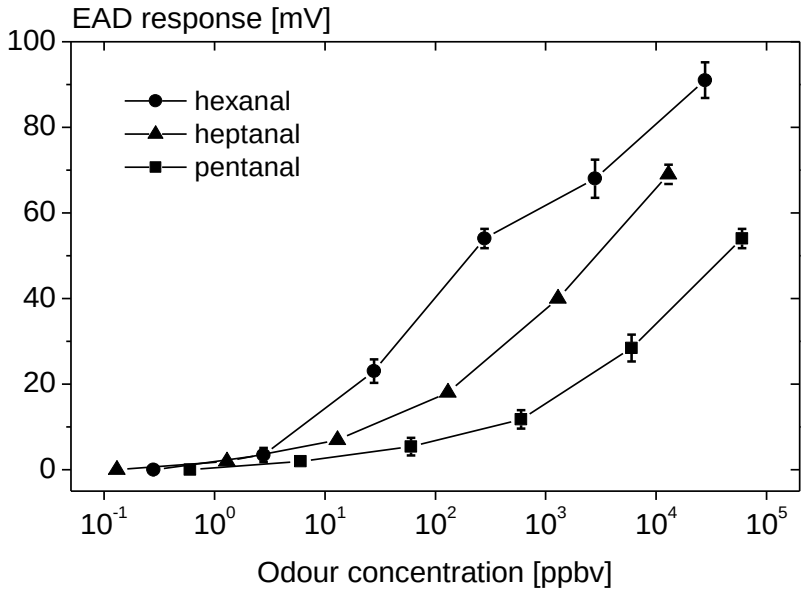

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Fig. 3: Calibration of antennae of *H. bajulus* with dilution series of aldehydes. Depicted are EAG amplitudes in response to short (0.5 s) stimulations. Error bars represent standard deviations (n=5).

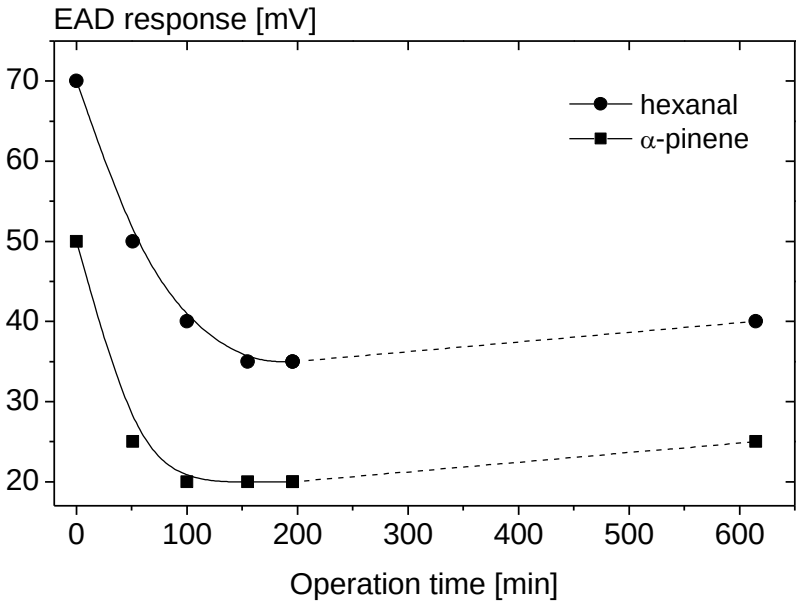

Fig. 4. Response of an antenna of *H. bajulus* to calibration standards (2.8 ppmv hexanal, 5.3 ppmv α-pinene). The dotted line represents a time in which no GC runs were performed.

Antennae of *H. bajulus* show a good response over a period of a few hours after abscission from the insect. The sensitivity decreases in the course of time, especially in the first hour of the measurements. After this decline the sensitivity remains constant for a considerable time. Often even an increase of sensitivity can be observed, especially if the antenna is not charged with further measurements (Fig. 4). Using the temperature program described above it was possible to record up to 10 chromatograms with one antenna (depending on the performance of the antenna).

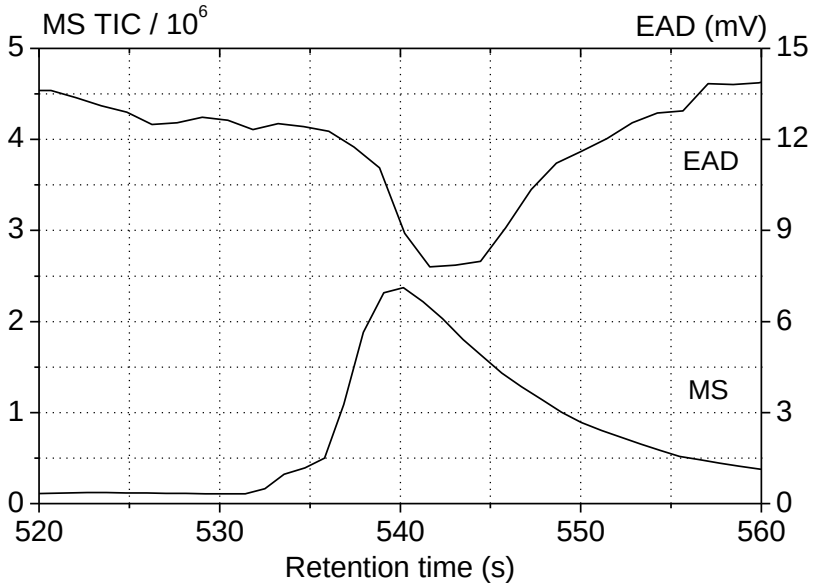

Fig. 5. Detail of a GC-MS/EAD chromatogram recorded with an antenna of a male *H. bajulus* and a sample of volatiles from scotch pine. The EAD peak is delayed about 3 s in comparison to the TIC peak of α-pinene at 9.0 min (540 s).

GC-MS/EAD results

The potential measured between tip and base of an antenna normally has a value of several mV and is subject to a slow drift in the course of time. EAD peaks are small deflections in this baseline. The information about total height and drift of the antennal potential is lost in the high pass filter of the EAD amplifier. Thus, the scale of the EAD axis is valid only as a measure for the amplitude of the deflections whereas the absolute value of the potential is irrelevant. For the data presented in Figs. 5 and 6 the position of the baseline has been freely

chosen in a way that allows a favourable presentation. All EAD potentials displayed in the following figures represent the amplified (100×) signals of the antenna.

A precondition for the comparability of the chromatograms from MS and EAD is the simultaneousness of the peaks in both signals or at least a constant shift in both signals. The induction of an electrical signal in the antenna is a dynamic physiological process followed by a quenching of the stimulus and a return to the baseline. Thus, the maximum of an EAD peak is not necessarily in coincidence with the maximum of the stimulus. In addition, the peak shape of the EAD signal is modified by the frequency filter. However, the onsets of the peaks obtained by the mass selective detector should match the EAD signals.

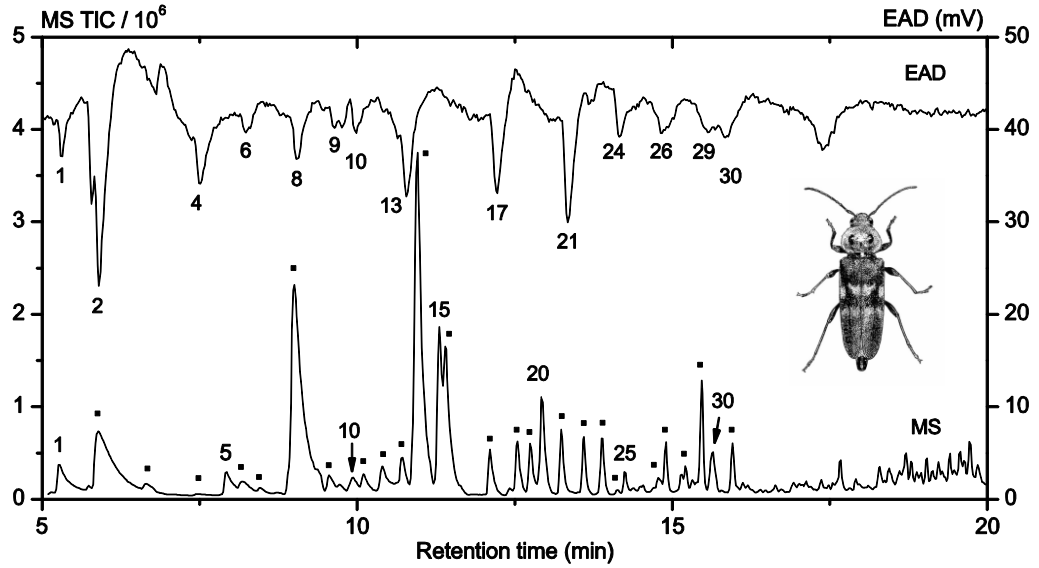

Fig. 6. GC-MS/EAD chromatogram of volatiles sampled from sawed timber of scotch pine (*Pinus sylvestris* L.). The upper line represents the EAD signal of a male old house borer, the lower line depicts the MS-TIC (total ion current). Numbers assign the peak numbers as listed in Table 1. In the MS chromatogram peak numbers are partly replaced by dots. The absolut height of the EAD signal is shifted in comparison to Fig. 5. Insert: Drawing of *Hylotrupes bajulus*.

In order to demonstrate the simultaneousness of the peaks in EAD and MS signal a detail from a GC-MS/EAD chromatogram is depicted in Fig. 5. The restriction capillaries leading to MS (i.d. 0.1 mm) and EAD (i.d. 0.15 mm) cause a delay of the EAD signal of about 1 s. The EAD signal is further delayed by the passing of the gas through the flowtube of the EAD interface which lasts about 1.5 s. So, the total delay of the EAD signal should amount to 2.5 s

compared to the MS signal. Fig. 5 depicts peaks corresponding to α-pinene in both signals. The onset of the peaks as well as the peak maxima are delayed about 3 s in the EAD signal.

With the knowledge of the delay between the signals of EAD and MS an interpretation of complex chromatograms is possible. Fig. 6 depicts a chromatogram of volatiles collected from timber of Scots pine. The peaks with the highest abundances in the MS signal are monoterpenes which are typical coniferous wood volatiles, especially α-pinene (peak 8, tR = 9.00 min) and 3-carene (peak 14, tR = 10.95 min). However, the highest deflection in the EAD signal is observed at tR = 5.85 min and corresponds to hexanal. Further aliphatic aldehydes and alcohols were also detected by the insect antennae. Table 1 shows a summary of the peak assignments obtained from the NIST database and the MassFinder software. Volatiles which elicit a response from the insect antenna are marked (+).

Discussion

The presented data demonstrate that the employed setup is capable of recording GC-MS/EAD chromatograms even from highly complex samples. Odorant samples collected from pine wood using the CLSA method comprise a multitude of volatiles, especially terpenoids, aliphatic aldehydes and alcohols.

The quantitative calibration of the EAD signal is performed by a single-point calibration by injection of stimuli into the calibration port of the EAD interface before and after each GC run. Thus, it is possible to keep track of changes in the sensitivity of an antenna from run to run and to compare chromatograms that were recorded with different antennae. A multi-point calibration with a dilution series is not applied because of the limited lifetime of the antenna. A more time consuming calibration via injection of defined quantities of odorant substances into the injector is possible if a precise quantitative interpretation of the electroantennographic response is necessary.

As a rule, a quantification should be performed using the peak areas of the TIC chromatogram from the mass spectrometer. However, in some cases the insect antenna shows remarkable responses whereas the MS is close to the detection limit, e.g. peak 4 (hexanol) in the shown chromatogram (Fig. 6). The use of the EAD data for a quantification can also be essential when bioactive compounds coelute with other substances, e.g. peak 30 in Fig. 6. In this case,

a quantification is possible if the dose-response relationship of the antenna for all involved compounds is known.

Table 1: Volatiles identified in the headspace of pine wood (*Pinus silvestris*). Peak numbers correspond with assignments in Fig. 6. A "+" in the last column indicates that the respective compound elicits a response in the EAD signal. Retention indices are calculated from the chromatograms obtained with a J&W Scientific HP-5MS column (Agilent).

Peak-#	RI	Substance	EAD
1	779	1-Pentanol	+
2	806	Hexanal	+
3	839	Furfural	+
4	874	1-Hexanol	+
5	894	2-Heptanone	
6	904	Heptanal	+
7	916	Acetic acid, pentyl ester	
8	938	α-Pinene	+
9	960	2-Heptenal	+
10	975	1-Heptanol	+
11	981	1-Hepten-3-ol	
12	993	2-Pentyl-Furan	
13	1006	Octanal	+
14	1015	3-Carene	
15	1029	p-Cymene	
16	1033	Limonene	
17	1061/63	2-Octenal & p-Mentha-1,4-diene	+
18	1078	Unknown terpenoid	
19	1086	Benzene, 4-ethenyl, 1,2-dimethyl-	
20	1094	Benzene, 1-methyl-4-(1-methylethenyl)-	
21	1106	Nonanal	+
22	1121	Fenchol	

23	1133	α-Campholenal	
24	1143	cis-p-Menth-2,8-dienal & unknown ketone	+
25	1148	trans-Verbenol	
26	1169	Pinocamphone	+
27	1174	Borneol	
28	1187	Thymol	
29	1198	(+)-α-Terpineol	+
30	1203/05/07	Benzene, 1-methoxy-4-(2-propenyl)- & Myrtenal & Decanal	+
31	1219	Verbenone	

An interpretation of the results for the chemical ecology of *H. bajulus* suggests a significance of hexanal, pinene and other aldehydes and terpenes for the host location of the beetle. Previous studies by Fettköther et al. (2000) demonstrate that (among other volatiles) α-pinene, α-terpineol, and verbenone induce a behavioural response from *H. bajulus*. This fact is supported by the finding that α-pinene and α-terpineol both elicit a response in the antenna of the beetle (Table 1). The behavioural response to verbenone was observed only for female beetles which is consistent with our results that the male beetle does not perceive this terpenoid. Verbenone is produced by two subsequent oxidation processes from α-pinene and thus might be a measure for the age of naturally degrading wood. It is also a major monoterpenoid emitted from larval frass of *H. bajulus* (Fettköther et al., 2000). Lindgren and Miller (2002) stated that beetles associated with fresh wood are rather repelled by verbenone whereas beetles associated with aged wood are not repelled or even attracted by this terpenoid. *H. bajulus* also has a preference for aged wood (Adelsberger, 1975) which might be a strategy to avoid competition by adapting to a less nutrient diet with a low content of water and soluble sugars (Höll et al., 2002). The sexual dimorphism in the response to verbenone might be a hint that females of *H. bajulus* use the scent of larval frass as a clue for an adequate site for oviposition as stated by Fettköther et al. (2000).

The EAD chromatograms did not show a reaction of *H. bajulus* to limonene. The behavioural response of the beetle to limonene was only weak (Fettköther et al., 2000) which suggests that

it perceives limonene only in high concentrations and possibly uses it for a short range orientation.

The finding that aldehydes like hexanal are perceptible to the beetle with high sensitivity is noteworthy. Aldehydes ranging from C6 to C10 were identified in the sample and all of them elicited responses from *H. bajulus*.

The high sensitivity of *H. bajulus* for hexanal is interesting. The relative intensity of hexanal can diverge from the normal pattern when lipoxygenase activity is induced. Hexanal is considered a major indicator of lipid oxidation and is ubiquitous in the biosphere. It is a part of the green leaf odour (GLO) of plants, it is also found in the bark of nonconiferous trees (Vrkočová et al., 2000) and it contributes to the emissions of meat (Ahn et al., 2001) or flowers (Schade et al., 2001). The role of hexanal for other tree-associated beetles is controversial. Some bark beetles show an EAD response to hexanal (Huber et al., 2000) while others do not (Zhang et al., 1999; Zhang et al., 2001). A possible deterrent effect is discussed (Poland et al., 1998; de Groot & MacDonald, 1999).

It is not clear if volatiles like hexanal are used by the beetle for its orientation since they may occur in much higher abundances from sources not related to the habitat of the beetle, e.g. green leaves or flowers. However, the fact that an insect is able to detect a volatile does not give an information if the volatile is an attractant, a deterrent, or does not elicit any behavioural response at all. This can only be clarified in behavioural essays. The presented results suggest that in particular the aldehydes ranging from hexanal to decanal should be subject to further studies in order to investigate their influence on behaviour of *H. bajulus*.

The use of the demonstrated technique is not restricted to the investigation of interrelations between insects and plants. Compounds with high significance to an insect are often detected with remarkable sensitivity (sub-picogram range) and/or a wide dynamic range (e.g. 10^6). Phytophagous insects, for example, often use olfactoric cues to get information about the physical condition of a host plant. This implies that the insect (or its antenna) can be used as a sensor for the health of plants (Schütz et al., 1996; Schütz et al., 1999a). Another application is the use of insect antennae to measure pheromone concentrations in the field in the context of the pheromone disruption method (Sauer et al., 1992).

Possible applications for insect antennae as sensors are also found in other disciplines. An example is *Melanophila acuminata*, a beetle that uses burnt trees for oviposition and thus is

able to detect the volatiles from burnt wood which makes it a perfect sensor for forest fires (Schütz et al., 1999b). It has also been observed that insects are able to detect anthropogenic compounds which have no direct significance for their normal life. An example is the ability ofwasps to detect some kinds of explosives (Park et al., 2001). These findings open up new vistas in solving analytical problems with the help of insect antennae even if the application is not at all associated with the chemical ecology of insects.

Conclusions

Recapitulating the presented results it can be stated that the combination of GC-MS with an electroantennographic detection to a GC-MS/EAD setup results in a most powerful analytical tool that combines high sensitivity and specificity of an insect antenna with the analytical power of a mass spectrometer. Experiments with *H. bajulus* revealed that this beetle does not only perceive typical coniferous volatiles like α-pinene and α-terpineol but also has a high sensitivity for aldehydes like hexanal. The role of these compounds and their possible significance for control measures against the beetle have to be clarified in behavioural studies.

Implementations of the presented method are not restricted to questions of chemical ecology but comprise a multitude of applications, e.g. in quality control or plant protection.

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CHAPTER 4

ODOURS OF WOOD DECAY AS SEMIOCHEMICALS FOR *Trypodendron domesticum* L. (COL., SCOLYTIDAE)

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* I performed all experiments and both authors prepared the manuscript for which I am the corresponding author

Abstract

Volatile chemicals of decaying beech wood were collected by enclosure of bark including phloem and outer xylem, and enclosure of infested trunks and infested but apparently healthy trees. GC-MS/EAD was employed to identify among those, volatile samples putative infochemicals for *Trypodendron domesticum*. A first list of compounds, the insect reacts with is given. Numerous volatiles of decaying trunks occur and vary patchwise over the length of the trunk, suggesting also a chronological succession defining states of decay and susceptibility for secondary bark beetles. Healthy trees probably fully recovered after an initial beetle attack, lacking any of the putative infochemicals. However, potential infochemicals for development of IPM strategies are established.

Keywords

Xyloterus domesticus, *Trypodendron*, *Fagus sylvatica*, European beech bark disease, ambrosia beetle, wood decay, deadwood, GC-EAD

Introduction

Beginning in Belgium 1999, low mountain ranges of middle Europe were afflicted with the "European beech bark disease" (EBBD). It was first described by Hartig in 1878 as a complex disease where infestation of beech scale (*Cryptococcus fagisuga*; Hemiptera, Eriococcidae) is followed by fungal affection with *Nectria coccinea* and several white rot fungi. This often causes die back of mature beech trees, they tumble down and are colonised by woodbreeding beetles. Beside Belgium with 1 million cubic meter solid of beech wood (*Fagus sylvatica*); Luxembourg, France, and Southern Germany were affected in the last 6 years. In addition to known symptoms of EBBD, in all regions beech trees of an healthy appearance were surprisingly infested by the wood-breeding beetle *Trypodendron domesticum*. In order to understand mechanisms of this disease a chemo-ecological study was carried out, comparing the new phenomenon with the classical situation. A number of investigations of the involved beetles of the family of Scolytidae and Lymexylidae (Kerck, 1976; Klimetzek, 1984; Byers, 1992) suggest that the mechanisms of host-selection consist in the chemosensory differentiation of states of wood decay. The presence at the "border" between living and dying trees, *T. domesticum* turns out to be an interesting research object on xylobiont insects and physiological dying- and decaying-processes in trees.

The underlying hypotheses of this work are: 1) Volatile organic compounds change successively during aging and decay of wood and characterise the most susceptible phase and breeding site for *T. domesticum*. 2) Volatiles released by trees afflicted by the new disease phenomenon are similar to volatiles of felled, susceptible deadwood.

Material and Methods

In order to examine hypothesis 1), the volatiles of decaying beech logs were sampled and analysed. The perception of these compounds was scrutinised using a GC-MS/EAD setup (Weissbecker et al., 2004) and additionally tested with pure compounds in an EAG experiment. Volatiles were sampled with a specialised trunk sampling jacket (Schütz et al., 2004) to trap emanated volatiles from dead and living trees in a non-invasive manner. The sampling jacket surrounds the trunk and generates an enclosure of air above a bark surface of $0.5m^2$ in size. In order to trap volatile compounds emitted by this surface, the enclosed air was circulated with a miniature pump through a charcoal filter with a flow of 1 l/min for a sampling time of 1 hour. Enriched volatiles were eluted from the charcoal with a mixture of methylene chloride and methanol. Odour samples were repeatedly taken in march and April during the early flight period of the beetle and separated into non-infested, highly attractive, beetle-infested and fungus-infested samples respectively. The attractive sites selected by females for creating the entrance holes of breeding galleries correspond to small bark patches which can be sub-cortically differentiated by visual cues as colouration, or smell with notes of e.g. ethanol, smoke or fungus (Kerck, 1976). For more detailed information, bark and wood chips of 1cm diameter were punched out, classified visually according to decay states (Fig. 1), and examined for emanating volatiles under lab conditions.

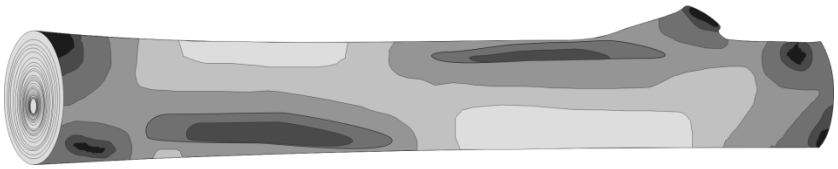

Fig.1: Distribution of physiological decay states on beech trunk (grey to black parts: fresh to seriously decayed).

The investigations on living beech trees concerning hypothesis 2) were done at a monitoring site of the Forest Research Institute Rheinland-Pfalz (FAWF), placed in the “Hunsrück” low mountain range in the forest district “Saar-Hochwald”, southern on a mountain top of 800m altitude. During the flight season between march and may, infested and non-infested living

trees, all of a healthy appearance, were sampled with 7 replications each, using the sampling jacket as described.

Odour samples were analysed by combined gas chromatography-mass spectrometry and electroantennographic detection GC-MS/EAD as described by Weissbecker et al. (2004). For the analyses by GC (model 6890N, Agilent, Palo Alto, USA) we used the following temperature program: start: 50 °C, hold for 1.5 min, ramp 6 °C/min to 200 °C, hold for 5 min. It was equipped with a split/splitless-injector operated at 250 °C in the pulsed-splitless-mode and a HP-5MS column (length 30m, ID 0.25 mm, film thickness 0.25 µm, Agilent). Helium was used as carrier gas at a constant flow of 1 ml/min. The effluent of the column was splitted, leading to a quadrupole mass spectrometer, and carried by a humidified air flow to a sensor containment including the insect antenna. Signals from the antenna were amplified by a factor of 100 and recorded using an A/D-converter and the HP- Chemstation software. For peak identification the National Institute of Standards and Technology mass spectral library (NIST, Gaithersburg, USA) was used.

Results

Analysis with GC-MS showed that felled beech trunks release over all more than 140 volatile compounds in noticeable amounts during the first phase of decay (0-2 years after felling), up to 70 contemporary in one bark sample. Differences between the small bark samples showed a high spatial variability of chemical processes and related volatiles within one trunk. Drawing an overview of the physiological aging processes starting with felling, the number of volatiles and emission rates increased beginning with aldehydes (exemplary GC-runs with named main compounds shown in Fig. 2 following decay progress from (a) to (d)). Several single and branched alcohols occured with beginning fermentation processes in the headspace of bark tissue (b). Beside terpenes, phenolic compounds, as 2-methoxy-phenol, 4-methoxy-phenol and 1.2-dimethoxy-benzene emanated during the most attractive phase for oviposition of *T. domesticum* (b) & (c).

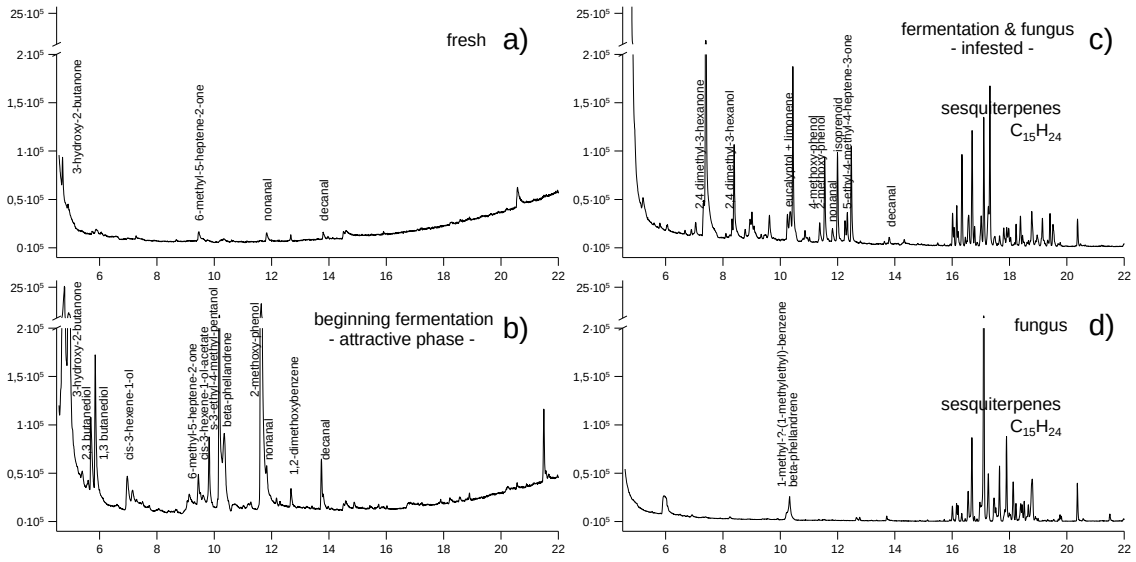

Fig. 2: Total Ion Chromatogram of physiological decay states from fresh to seriously decayed (a-d). Attractive tunnelling sites (see Fig 1): b) and c). Bark patches in state a) not yet attractive. d) unsuitable.

They vanished fast and the branched alcohols changed to longer carbon-chains. With the entrance of white rotting fungi (c) up to 30 sesquiterpenes were additionally detected in the bark samples. After degradation of lignin and cell structures, only these were found (d).

GC-EAD: Recordings from antennae of female *T. domesticum* with GC-MS/EAD showed reproducible EAD signals to thirteen compounds. Nine of them were also perceived by *Hylecoetus dermestoides* L. (Coleoptera: Lymexylidae), a syntopic beetle, often found at the

same host-trunks. (Fig. 3). The sensitivity of the two species is similar, but differs for single compounds (e.g. nonanal & 1,2-dimethoxybenzene).

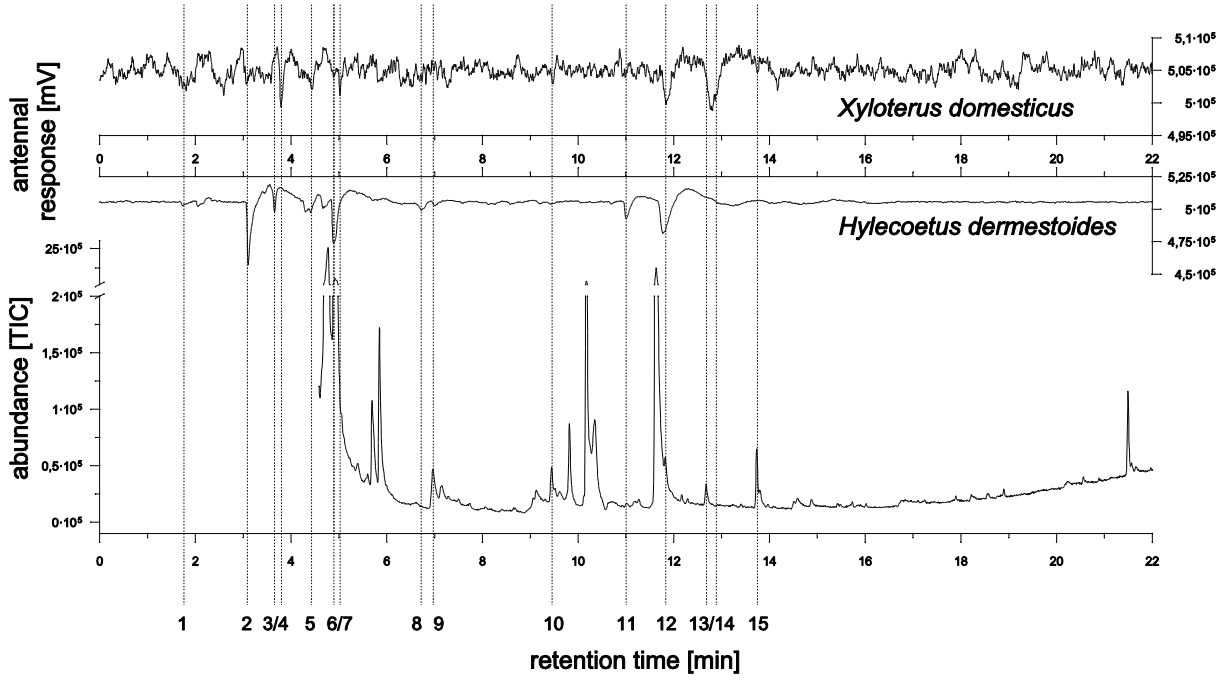

Fig. 3: Comparison of two GC-MS/EAD recordings of the same odour sample of highly attractive decaying beech bark with antenna of *T. domesticum* (upper, top) and antenna of *H. dermestoides* (upper, down). Corresponding ion-chromatogram (lower line). Identifcation of volatiles 1: solvent ($MeOH/CH_2Cl_2$) - 2-5, 8, 11, 14: not identified - 6: 3-methyl-1-butanol -7: 2-methyl-1-butanol - 9: cis-3-hexene-1-ol - 10: 6-methyl-5-heptene-2-one - 12: 2-methoxy-phenol + nonanal - 13: 1,2-dimethoxybenzene - 15: decanal

Discovered compounds which may play a role as semiochemicals in *T. domesticum* are shown in Fig 4. Four were tested as pure compounds and are perceived highly sensitive. Identification of both butanols and 6-methyl-5-heptene-2-one is not fully assured and has to be proven with synthetic compounds.

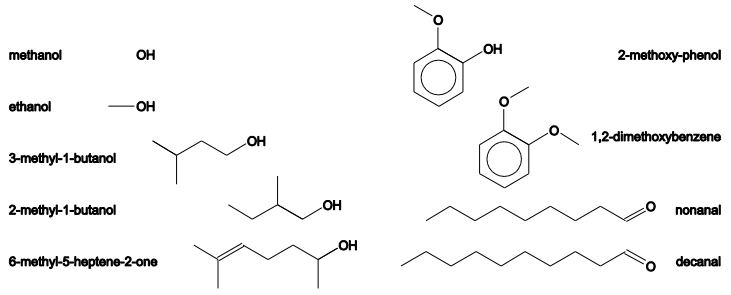

Fig. 4: Perceived substances discovered in GC-MS/EAD experiments. Ethanol, 2-methoxy-phenol, nonanal and additionally 4-methoxy-phenol were tested as pure compounds down to concentrations of 0.1 ppb in air.

The volatile-analysis of living trees in the context of EBBD showed that proposed semiochemicals (Fig. 5) could not be found in any living trees, except of nonanal. Trees infested with *T. domesticum* did not differ significantly from uninfested trees of the same stand. Therefore, control trees were measured again one year later, and they differed clearly from the others (Fig. 5).

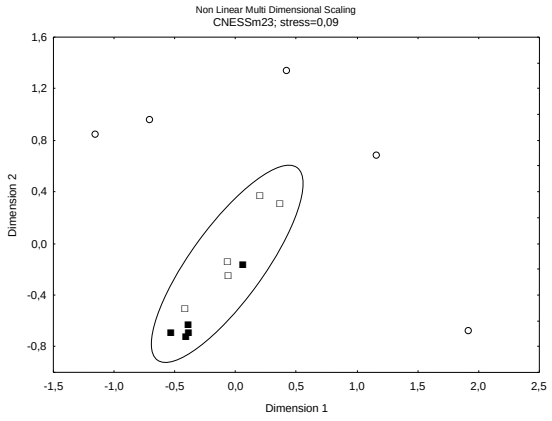

Fig. 5: Non linear multidimensional scaling: Volatile composition of living trees. Infested trees (■), non-infested (□), controls one year later (○).

Discussion

The variability of decay processes is very high. Decay processes at a trunk occur discontinuously in time-course and patch-wise in space. Depending on abiotic factors, chemical processes, and involved organisms decay related volatiles vary in presence and quantity. They are used by deadwood colonising insects in host finding. The suitability of wood for deadwood-insects is determined by its state of decay. *T. domesticum* is highly selective in choosing breeding sites at spots of a specific decay state. Our investigations showed several candidates as marking semiochemicals eliciting behavioural response. Catches of *T. domesticum* in a trap experiment showed that beside ethanol, 2-methoxy-phenol and methyl-butanol isomers may act as attractants to *T. domesticum*. Further studies should complete the list of compounds that are perceived. Specific micro-organisms and fungi are known to be distributed by *T. domesticum*. Their impact on volatile production and wood decay chemistry may reveal trophic interactions and mechanisms of host finding. The

investigations on infested living trees with EBBD symptoms showed huge differences to infested deadwood. Unknown plant defense signals, which might have caused the beetle attack, or an already expired infection can be an explanation for the lack of expected semiochemicals. The homogeneity between infested living trees and uninfested controls may refer back to a stress situation in 2003 similar to all trees. The uninfested control trees measured in 2004 differ clearly in volatile composition as shown in Fig. 5. An effect of a recovery within this climatically intermediate year might be the reason for this result.

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CHAPTER 5

ELECTROPHYSIOLOGICAL RESPONSES OF A DEADWOOD BEETLE TO TRUNK VOLATILES: NO MATTER OF TREE SPECIES.

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this is a manuscript prepared for submission

* I performed all experiments and prepared the manuscript for which I will be the corresponding author

Abstract

The feeding habit of agriculture in the enigmatic ship timber beetles (Lymexyloidea) is predicated on studies of the xylomycetophagous *Elateroides dermestoides*. Its larvae cultivate the yeastlike fungus *Ascoidea hylecoeti* provided by maternal transmission in galleries of many arboreal hosts including dying soft- and hardwoods. We studied host recognition in this generalist, by analyzing volatile chemistry of infested host trees: beech (*Fagus sylvatica*), oak (*Quercus petraea*), larch (*Larix decidua*) and spruce (*Picea abies*). Electroantennographic recordings of antennae of *E. dermestoides* female with a GC/MS-EAD setup identified among the host trees disparate and species specific chemical profiles. Surprisingly, major antennal responses were recorded in equal measure to compounds present in all four host trees: isoamyl alcohols, ethyl- and propylacetate - volatiles of general fungal metabolism. We could demonstrate that perceived compounds present in all hosts, were also released by the fungal cultivar *A. hylecoeti* on artificial medium. EAG dose-response experiments confirmed strong antennal responses and high sensitivity for (Z)- and (E)-roseoxide isomers, species specific to the fungal cultivar *A. hylecoeti*. This corroborates the assignment as a fungal specialist. The alcohols and acetates, citronellol and nerol/geraniol-derived terpenoids are also products of yeasts and ophiostomatoid fungi, occasionally found as "weed" fungi in galleries of *E.*

dermestoides. We conclude that host recognition of female *E. dermestoides* barely includes host-tree specific volatiles and is primarily dependent on fungal activity in dying trees or presence of conspecifics that reveal themselves by release of volatile metabolites of the fungal cultivar. This probably accounts for the indiscriminative host tree selection. However, the outstanding sensitivity of *E. dermestoides* to the fungal cultivar *A. hylecoeti* addresses new questions on the symbiotic nature of the association between both species.

Keywords

Hylecoetus dermestoides, Lymexylidae, ambrosia beetles, fungus-growing, ants, termites, symbiosis, *Ophiostomatales*, *Ceratocystis*, yeast, volatiles, EAG, dose-response, EC50, ethanol, bark beetle, host specificity, non-host volatiles, saproxylic

Introduction

The large timber worm *Elateroides dermestoides* (senior synonym of *Hylecoetus dermestoides*: Cuccodoro, 2002), is a mycophagous wood-boring beetle in the small superfamily of Lymexyloidea. Its ecology and fungal farming behaviour is similar in many respects to true ambrosia beetles (Scolytinae: Curculionoidea) in the sense of females providing their offspring with symbiotic fungi which the larvae feed on (Neger, 1909; Buchner, 1930, 1953; Francke-Grosmann, 1952; Batra & Francke-Grosmann, 1961). Most ambrosia beetles are related to deadwood and therefore called secondary bark beetles. Considering their host tree species, they are by the majority generalists. Primary bark beetles attacking healthy trees encounter effective plant defences, and are often specialists. (Klimetzek et al., 1986; Farell et al., 2001; Gibson & Hunter, 2010). *E. dermestoides* infests numerous soft- and hardwood species (Online Resource Suppl. Tab. 1). Logs, stumps or diseased trees are the ideal substrate for larval development. Abundant occurrence in wood piles turns *E. dermestoides* into a serious forest pest. For pest control or application in integrated pest management, unlike for many other bark beetles, effective attractants are unknown for *E. dermestoides* (Francke & Brümmer, 1978a,1978b; Redlich et al., 1981) and the presence of an aggregation pheromone has been even put into question (Klimetzek et al., 1986).

In search for infochemicals, bark volatiles of host and non-host tree species were shown to effectively enhance or inhibit response to ambrosia beetle pheromones. Host-specificity and reproductive isolation may also be maintained by responses to host volatiles (Lindgren et al., 2000; Kühnholz et al., 2001). Among those, an ubiquitous host signal is ethanol (Byers et al., 2004; Bouget et al., 2009) which is generated naturally by anaerobic fermentation in moist decaying wood tissue or stressed trees (Moeck, 1970; Klimetzek et al., 1986; Kimmerer & Stringer, 1988). First of all, it has been identified as a host cue for *Trypodendron* species (Moeck, 1970; Kerck, 1972), and it turned out that the co-occurring *E. dermestoides* is also attracted (Klimetzek et al., 1986). Comparative studies of secondary bark beetles (Klimetzek et al., 1986) suggested that generally the importance of ethanol as a host cue coincides with host specialisation: ethanol was proposed as the major host signal for those having the broadest host range as *E. dermestoides* (Klimetzek et al., 1986). However, as in this case, many attractive host volatiles are deduced from analogies to related beetle species or by surmising that compounds present in the host would be attractive (Byers et al., 2004). In this study, we investigated which host volatiles are perceived by *E. dermestoides* by inspecting its major host trees beech, oak, larch and spruce and applying present-day gas chromatographic and electrophysiological methods. This would allow testing the proposed hypothesis, that polyphagous species perceive odours which are common in many plant species whereas monophagous species may primarily rely on odours typical for their host plant (Klimetzek et al., 1986; Bernays, 1996,2001; Mustaparta, 2002; Schoonhoven et al., 2005). *E. dermestoides* is most unrelated to scolytid ambrosia beetles, and convergent evolution might has borne similar strategies in host selection and perception. However, very little is generally known about host infochemicals (kairomones) in deadwood beetles.

Material and Methods

Sampling of Bark Volatiles and Structure Elucidation

In mixed and deciduous forests in the surroundings of Göttingen, Lower Saxony, we prospected for *E. dermestoides* infested trunks of representative host species in early June 2003. In July and August, larval growth and boring activity reach their maximum and infestations become most apparent (Richter, 1933). Complete bark samples of infested areas were cut out, brought to the lab in a cool bag and sampling preparation was accomplished within 60 min at room temperature. Cambium and phloem were dissected from the outer bark and 20 g were enclosed in a round bottom flask of 500 ml with a PTFE stopper. An odour sample was collected by dynamic headspace sampling applying the CLSA method. A rotary vane pump (type DC12/16NK; Fürgut, Tannheim, Germany) circulated the headspace volume over an adsorbent trap (glass tube, 6 cm long, 0.3 mm ID, loaded with 1.5 mg charcoal) for 15 min at 1.0 l/min. Volatiles were eluted with 50 µl solvent (CH_2CL_2/MeOH, 2:1, Suprasolv-quality; Merck/VWR, Darmstadt, Germany) and an aliquot of 1 µl of the extract was directly analysed by means of coupled gas chromatography - mass spectrometry - electroantennography as described by Weissbecker et al. (2004). The GC-MS consisted of a 6890N gas chromatograph connected to a 5973N quadrupole mass spectrometer with electronic ionisation at 70 eV, both Agilent (Palo Alto, USA). A HP-5ms fused silica column (Agilent, 30 m, 0.25 mm ID, 0.25 µm coating thickness, phenyl/dimethylpolysiloxane) was used with a constant Helium flow of 1 ml/min. The temperature program was 50 °C (1.5 min), followed by an increase of 7.5 °C/min to 200 °C. A GRAPHPACK 3D/2 flow splitter (Gerstel, Mülheim, Germany) was used to split the effluent from the column into two pieces of deactivated capillary leading to the mass spectrometer (length 1 m, ID 0.1 mm) and to the EAD setup (length 1 m, ID 0.2 mm). The restriction of these capillaries resulted in a 1:1 split of the gas flow into the two setups. Compounds were identified by comparison of full scan (m/z 35-300) mass spectra and GC retention values with those of reference compounds, mass spectral databases and published parameters (Tab. 1). The used databases were Wiley 9 combined with NIST ´08, (McLafferty, 2009) and "Terpenoids and Related Constituents of Essential Oils", a database available from MassFinder 3.07 software (Hochmuth Scientific Consulting, Hamburg, Germany).

Identification of low molecular weight compounds

A typical sample of beech bark was prepared as described above. An odour sample was collected by direct headspace sampling using solid phase micro extraction, 85 µm Carboxen™/Polydimethylsiloxane (PDMS) StableFlex™ fibre type (Supelco, USA). Before sampling, the fibre was conditioned in a programmed temperature vapouriser (PTV) at 250 °C for 5 min. The septum-piercing needle was inserted through the PTFE stopper and the fibre exposed for 30 min. The above described GC-MS/EAD setup was equipped with an HP-INNOWax fused silica column (Agilent, 30 m, 0.25 mm ID, 0.25 µm coating thickness, polyethylene glycol) connected to the EAD setup and the mass spectrometer scanning a mass range from 15 to 300. The needle was inserted into the PTV injector held at 250 °C, exposed, and then the programmed GC resting at 50 °C was started manually: temperature hold for 1.5 min, increase of 6.0 °C/min to 200 °C.

Tab. 1 Compounds in bark samples of beech - *F. sylvatica*, oak - *Q. petraea*, larch - *L. decidua* and spruce - *P. abies*. Identification method, relative amounts (+++++ >20%, ++++ <20%, +++ <5%, ++ <1%, + trace, - not detected), electroantennographic activity of *E. dermestoides* antennae and occurrence in fungal cultures of its symbiont *A. hylecoeti*. Order according to elution on a non-polar column, numbers correspond to Fig. 1.

No	Compound	ID[a)]	LRI[b)]	*Fagus sylvatica*	*Quercus petraea*	*Larix decidua*	*Picea abies*	EAD	*Ascoidea hylecoeti* [d)]
1	unknown		-	○	○	○	○	●	
2	Ethyl acetate	A	617[c)]	○	○	○	○	●	a,b
3	unknown		659[c)]	○	○	○	○	●	
4	n-Propyl acetate	A	717[c)]	○	○	-	-	●	a,b
5	3-Methyl-1-butanol	A	756[c)]	○	○	○	○	●	a,b
6	2-Methyl-1-butanol	A	758[c)]	○	○	○	○	●	b
7	unknown		850	-	○	○	-	●	
8	1-Hexanol	A	876	+++	++	+	+	●	a
9	Methoxy-benzene	B	925	-	+	++	-	●	
10	α-Pinene	A	938	-	-	+++++	+++++	●	
11	unknown		945	○	○	○	○	●	
12	Camphene	A	957	-	-	+++	+++[e)]		
13	1-Heptanol	B	974	+	++++	-	-	●	
14	β-Pinene	A	984	-	-	++++	+++++	●	
15	6-Methylhept-5-en-2-one	A	990	-	++	-	-		b
16	β-Myrcene	A	992	-	-	+++	++++		a,b
17	3-Carene	A	1015	-	-	-	+++		
18	Hexyl acetate	A	1016	+++++	++	+++	++[e)]		a,b
19	p-Cymene	A	1026	-	-	++	++++		
20	Limonene	A	1036	-	-	++++	++++		a,b
21	unknown terpenoid		1040	-	-	+++	+++		
22	unknown		1062	○	○	○	○	●	
23	γ-Terpinene	A	1063	-	-	++	++		a
24	(*E*)-linalool oxide (furanoid)	A	1079	-	++	++	++[e)]		a,b
25	(*Z*)-linalool oxide (furanoid)	A	1094	-	++	-	-		a,b
26	2-Methoxy-phenol	A	1096	+++	++	++	++	●	
27	Fenchone	B	1097	-	-	-	++		
28	Heptyl acetate	B	1112	++	++++	++	+[e)]		
29	(*Z*)-roseoxide	A	1115	++	++++	+++	++	●	b
30	α-Fenchol	B	1123	-	-	+++	+++		
31	(*E*)-roseoxide	A	1132	++	+++	++	++	●	a,b
32	Camphor	A	1154	-	-	++	++		
33	Citronellal	B	1157	++	+	++	++[e)]		a,b
34	Borneol	B	1176	-	-	+++	+++[e)]		
35	Octyl acetate	A	1210	++++	++++	+++	++[e)]		a
36	Citronellol	B	1232	+++++	+++++	+++	++[e)]		a,b
37	Geraniol	B	1257	++++	-	-	-		a,b
38	Citronellyl acetate	B	1353	++++	++++	++	++[e)]		a,b
39	Geranyl acetate	A	1387	+++	-	-	-		
40	Decyl acetate	B	1408	+++	+++	++	+[e)]		

[a)] Identification is based upon: A, mass spectrum and linear retention index (*LRI*) agreed with standards; B, mass spectrum and *LRI* agreed with literature data. [b)] Experimental *LRI* on HP-5ms column. [c)] *LRI* extrapolated; for tentative identification see text and Fig. 2. [d)] a, Volatile compounds found in *A. hylecoeti* extracts (Francke & Brümmer, 1978a,1978b); b, compounds found in headspace of *A. hylecoeti* cultures (Holighaus unpublished). [e)] Compounds were not found in whole trunk sample shown in Fig. 1. EAD-activity is marked with a filled circle (●), open circles (○) indicate an EAD response, where no eliciting compound was identified.

EAD / EAG recording and analyses

The electroantennographic detector (EAD) connected to the GC-MS used a modified version of an 'olfactory detector port' (ODP, Gerstel, Mülheim, Germany). This incorporated a flexible heating sleeve held at 230 °C guiding the capillary out of the GC oven where the effluent of the capillary is mixed with humidified air. The airflow was directed to the insect antenna housed in a detector cell made of Perspex and PTFE (Weissbecker et al., 2004). EAD measures, often flawed with a slow drift, passed through a high-pass filter with a cut-off frequency of 0.01 Hz. For dose-response experiments, the same setup was used to record EAG signals. Solvent controls (paraffin - Uvasol®, spectrosc. qual., high visc., Merck, Darmstadt, Germany) and a standard stimulus of 1-octen-3-ol at a 10^{-3} dilution in paraffin were puffed over the antenna first. Test odours were prepared by soaking a piece of filter paper (1 cm^2) with 50 µl of the dilution of a pure test compound prepared in six concentrations from 0.0000001 to 0.01 (w/w) in paraffin and immediately put into a gas tight 10-ml glass syringe (Poulten & Graf GmbH, Wertheim, Germany). The syringe was flushed with synthetic air and after a short equilibration time a stimulus was applied. Reproducibility was achieved by always puffing 3 ml of syringe headspace over the antenna, after a standard resting time of 0.5 min. Each set of experiments started with the lowest concentration, every puff was repeated three times. Every compound was tested in the six given concentrations for 2-5 beetles. Only female *E. dermestoides* were used for electroantennographic recordings.

In order to compensate for the interindividual variation in absolute responses (mV), the signals were normalised (corrected) to the standard stimulus (1-octen-3-ol, 10^{-3}) response, set as 1. Therefore, data for six doses (blank, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} (w/w) in paraffin) were obtained to describe a dose-response relationship for each tested substance. EAG responses were characterised by nonlinear curve fitting using the software package "drc" generally designed for analysis of multiple dose response curves within the software environment "R" (Ritz & Streibig, 2008; Knezevic et al., 2007). Logistic non-linear models were applied to calculate dose-response-curves (Getz & Lánsky, 2001). An Akaike's information criterion (AIC) was calculated to select the minimal adequate model of a three-, four- or five-parameter logistic model (details see Knezevic et al., 2007). Parameters relating to particular characteristics of the sigmoid curve were calculated: The ED_{50} is the effective dose which provokes a response halfway (50%) between zero and maximum response and is the inflection point of the curve. ED_{10} (10% response), we took in our sole discretion as an

odour detection threshold. Accordingly, ED_{90} - ED_{10} dimensions a detection range (compare van Giessen et al., 1994; Cometto-Muñiz & Abraham, 2010).

Results

Volatiles from infested host plants of E. dermestoides

Twenty-five tree species belonging to ten families are known as hosts for *E. dermestoides*. (Online Resource Suppl. Table 1). The chemical analysis of bark of four of the most frequently cited hardwood and softwood hosts infested with larvae of *E. dermestoides* is shown in Table 1. Complex mixtures of acetates, aliphatic and branched alcohols, phenolics and terpenoids were identified in samples of beech, oak, larch and spruce. The chromatograms of the conifers are clearly dominated by characteristic conifer terpenes, namely α-pinene, β-pinene, camphene, β-myrcene, δ-3-carene, cymene, limonene, β-phellandrene, γ-terpinene, as well as borneol and α-fenchol and their oxidation products camphor and fenchone comprising 83 % and 93 % of the mixtures in larch and spruce respectively. A second group of terpenoids was found in all tree species including the two roseoxide isomers and citronellol, of which the acetate and aldehyde were also present. Together they account for one third to half of the mixture in beech and oak, while due to the dominance of conifer terpenes, only 0.5 % and 5 % in spruce and larch. Further ubiquitous compounds were 1-hexanol and hexyl acetate, as well as heptyl-, octyl- and decyl acetates. 1-Heptanol was, albeit present in low amounts, the only compound exclusively found in the hardwood species. Two phenolics, methoxy-benzene and 2-methoxy-phenol were rather minor components, but present in hard- and softwoods.

Antennal responses of E. dermestoides to host plants

In the electroantennographic study, overall 17 reproducible peaks of the EA-detector were recorded and 8 of them could be at first assigned to a chemical compound. Comparisons of EAD and GC-MS traces are shown in Figure 1. At first sight *E. dermestoides* antenna responded strongest to compounds present in all host species, those were neither major compounds, nor those specific for coniferous or deciduous species or single host species. Strongest responses were recorded to (Z)-roseoxide and (E)-roseoxide, 2-methoxy-phenol and an unknown compound (22), all present in all tree species. Weaker responses were found for 1-hexanol, 1-heptanol, and in particular for α-pinene and β-pinene considering the large

quantities of both terpenes. The antenna of *E. dermestoides* did not respond to any other of the abundant terpenoids. Strong responses were recorded for very volatile compounds (1-6), not detectable under the solvent peak while the MS was operated with a solvent delay.

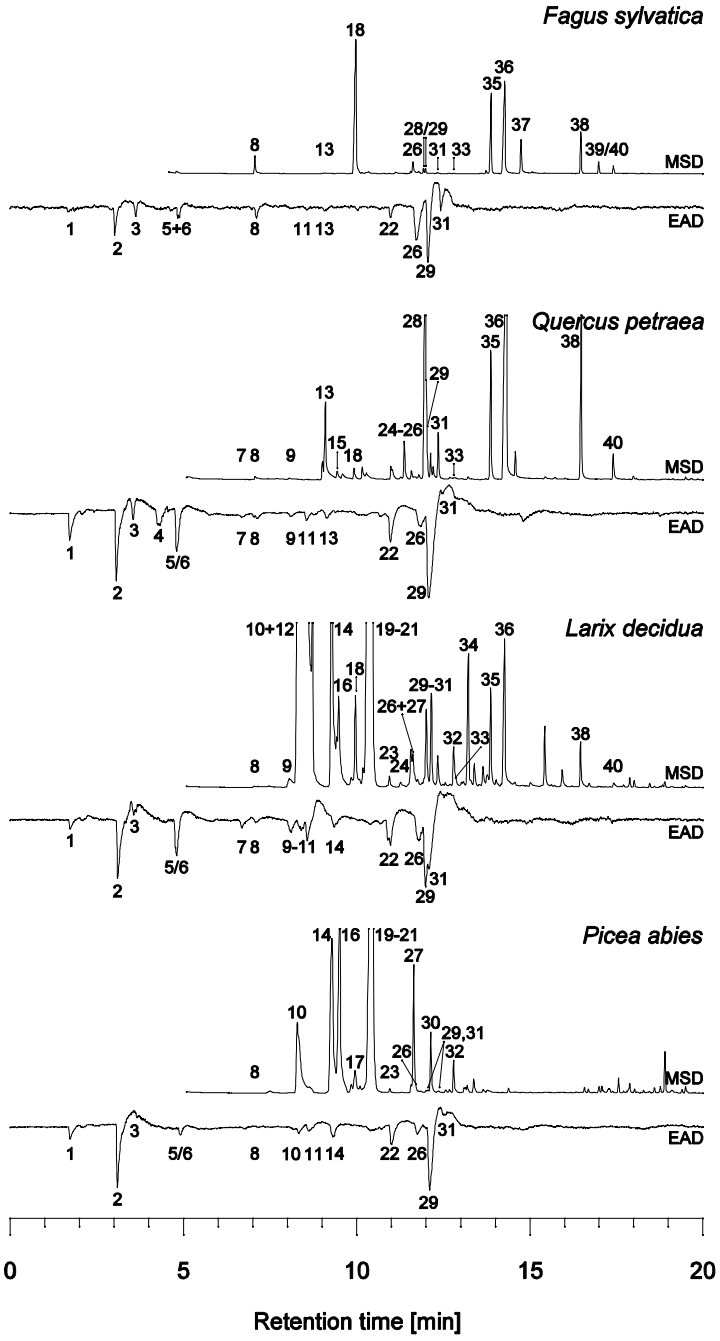

Fig. 1 Coupled gas chromatographic and electroantennographic recordings (GC/MS-EAD) of infested hard- and softwoods with antennae of female *Elateroides dermestoides*. Bark samples of beech - *Fagus sylvatica*, oak - *Quercus petraea*, larch - *Larix decidua* and a whole trunk sample of spruce - *Picea abies*. Numbers correspond to Table 1.

Solvent free SPME samples of beech bark were analysed and are shown in Figure 2. EAD responses to the roseoxides and 1-hexanol were recovered, and single ion traces of the GC/MS-EAD allowed identifying ethyl- and propyl acetate as the elicitors of major EAD signals at Rt 4.4 min and 5.8 min. The third major signal matched the ion traces of 3-methyl-1-butanol and 2-methyl-1-butanol, coeluting at Rt 10.6 min.

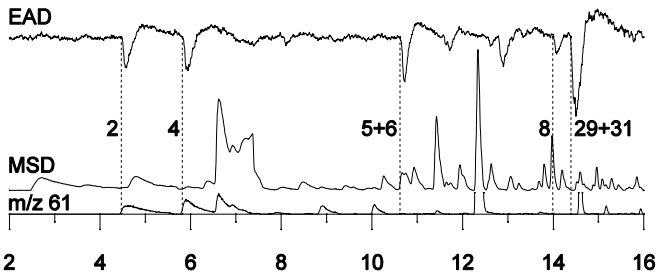

Fig. 2 Coupled gas chromatographic and electroantennographic recording (GC/MS-EAD) of infested beech - *F. sylvatica* - with antenna of female *Elateroides dermestoides*. Solvent free sample acquired with SPME, polar column. Single ion trace of characteristic mass fragment (m/z 61) of ethyl- (2) and propyl acetate (4) illustrate the matching EAD-response. Numbers correspond to Table 1.

Dose-EAG-response experiments with authentic standards revealed both 3-methyl- and 2-methyl-butanol to be perceived with similar sensitivity, reflected in comparable sensitivity parameters calculated from dose-response curves (Table 2). Overall, the thresholds quantified in ED_{10} values are lowest for the available mixture of roseoxide isomers and ethyl acetate. All other compounds resulted in effective doses ED_{10} around 10^{-5} (w/w).

Tab. 2 Sensitivity parameters[a] derived from fitted dose vs. EAG-response curves of female *Elateroides dermestoides*.

	Threshold ED_{10}	Half-response ED_{50}	Detection range $ED_{50} \sim ED_{10}$
Compound	Log_{10}conc. (w/w) ± SE	Log_{10}conc. (w/w) ± SE	Log_{10}conc. (w/w)
Ethanol	-3.5 ± 0.2	-2.8 ± 0.2	0.7
Ethylacetate	-7.0 ± 0.0	-4.5 ± 0.5	2.6
Propylacetate	-4.9 ± 0.9	-1.5 ± 0.8	3.4
3-Methyl-1-butanol	-4.5 ± 0.5	-1.7 ± 0.4	2.8
2-Methyl-1-butanol	-5.2 ± 0.0	-1.8 ± 0.5	3.4
2-Methoxy-Phenol	-5.1 ± 0.0	-3.1 ± 0.7	1.9
(-)-(Z/E)-Roseoxide	-5.8 ± 0.0	-3.3 ± 0.7	2.6

[a]Sensitivity parameters as defined in Material and Methods

However, at this given concentration in paraffin, distinctive vapour pressures would cause different headspace concentrations: 2 ppbV for 2-methoxy-phenol, 27 ppbV for 2-methyl-butanol or 350 ppbV for propyl acetate, calculated with Antoine coefficients (Yaws, 2007) assuming Henry's gas law. This indicates the presence of effective receptors to detect compounds with low vapour pressures such as 2-methoxy-phenol or the roseoxides. Ethanol is a general attractant for ambrosia beetles and *E. dermestoides* (Bouget et al., 2009), and indeed traces of this compound were detected, but did not evoke an EAD response. Sensitivity parameters derived from the EAG dose-response experiments indicate a detection of ethanol only at higher concentrations in the ppm range. For comparison, exemplary dose-response curves are opposed to the response to ethanol (Fig. 3). Regardless of ED values as relative expressions referring to a maximum response, absolute responses shown in Figure 3 were highest for the roseoxide and 2-methoxy-phenol with intermediate responses to methyl-butanols and acetates (not shown) and lowest for ethanol. The antennal response to roseoxide increased almost log-linearly over four orders of magnitude, suggesting that the antenna has a high discriminatory power in low as well as in high concentrations for this odorant.

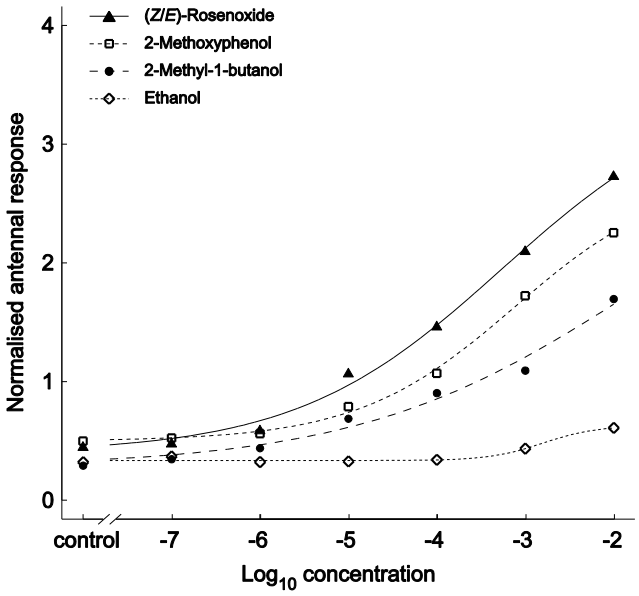

Fig. 3 Fitted dose response curves of ethanol (◊), 2-methyl-1-butanol (▲), 2-methoxy-phenol (□) and (±)-(Z/E)-roseoxide mixture of isomers (●). Marks are means of responses relative to a standard response of 1-octen-3-ol (log_{10}-3). Doses are shown as log_{10} concentrations in paraffin (w/w).

The low volatile compounds ethyl acetate, propyl acetate, 3-methyl-1-butanol and 2-methyl-1-butanol, confirmed with SPME measures to evoke substantial EAD signals (Fig. 2), were tentatively assigned to the chromatograms in Figure 1 and Table 1 by comparing their

literature LRI to experimental LRIs calculated from the EAD signals (1-6) and an extrapolated alkane retention time. 3-Methyl-1-butanol and 2-methyl-1-butanol matched the EAD signals at Rt 4.8 and 4.85 (5 and 6), generally coeluting on both used separating columns, but due to low amounts separating in the beech sample as discrete peaks. Ethyl acetate and propyl acetate where assigned to EAD signals number 2 and 4.

Discussion

In order to corroborate the proposed hypothesis, *E. dermestoides* as a generalist should perceive odours present in all host tree species, or according to the converse argument do not perceive compounds restricted to single host taxa. The tested tree species were preferred hosts of *E. dermestoides* (Online Resource Suppl. Fig.1), and showed characteristical and contrasting chemical profiles of bark volatiles. They clearly separate the hardwood from the softwood species and to some extent the individual species (Stairs, 1968; Persson et al., 1996; Vrkočová et al., 2000; Holighaus & Schütz, 2006). Comparing the antennal responses of *E. dermestoides* to the different host trees, the similarity of the signals is conspicuous and shows that most compounds perceived are present in all tested host tree species. Beyond the equality in occurrence, the signal to signal ratios among compounds are alike - of course the relationship from source strength to signal response is logarithmic (Fig. 3), damping the response amplitude to variability of emission rates in different samples. These results indicate that the compounds might have a common metabolic origin. Anaerobic metabolism as a source of ethanol is documented in stems of trees, and further pathways producing very volatile compounds might exist (Kimmerer & Stringer, 1988), in particular in a dying tree. The electrophysiological response to ethanol is rather weak compared to ethyl- and propyl acetate, 3- and 2-methyl-1-butanol (Fig. 3) and is in agreement with a rather weak attractiveness of ethanol in the field (Graf & Manser, 2000; Bouget et al., 2009; Henin et al., 2003). However, both acetates and the two branched alcohols are general metabolic products of filamentous fungi and yeasts (Hazelwood et al., 2008; Park et al., 2009). No tree is sterile and endophytic fungi, quiescent in a living tree, might be activated in dying tissue (Sieber, 2007). Fungi occurring in the galleries of *E. dermestoides* and spreading through infested trunks can be narrowed down to few known species. Together with the symbiont *Ascoidea hylecoeti*, an ascomycetous yeast-like fungus, further ophiostomatoid „weed“ fungi of the genus *Ceratocystis* and *Graphium* (Francke-Grosmann, 1967; Zimmermann, 1973; Carlier et al., 2006), along with several yeasts and undetermined bacteria (Bibikova et al., 1988) are

regularly found in the beetle galleries. Additionally to ethanol, higher alcohols, esters, aldehydes and aromatic compounds are produced by many unicellular yeasts (Park et al., 2009), and mycelium forming yeasts as *Ceratocystis* emit acyclic terpenes as well (Hanssen, 1993). Citronellol, citronellal and citronellyl acetate have been regularly found in *Ceratocystis* species together with ethanol, ethyl acetate and 3-methyl-1-butanol (Hanssen, 1993) and those we also found in *Ceratocystis torulosa*, one of the „weed fungi“ in *E. dermestoides* galleries (Holighaus unpublished; Zimmermann, 1973). Our attributions to possible sources are deductions, and the fungal community present in the galleries depends to a certain degree on region, tree species or companion beetle species (Batra & Francke-Grosmann, 1961; Zimmermann, 1973, Korolyev & Ucastnova, 1990). Without doubt, *A. hylecoeti* is obligatory in the galleries (Francke-Grossmann, 1961) and metabolites should be present in our samples. Volatile compounds from *A. hylecoeti* are well studied (Francke & Brümmer, 1978a,b) and for better comparison, the compounds identified in cultures on synthetic media are supplemented to our data in Table 1. It becomes evident that the symbiont of *E. dermestoides* at least contributes to several of the very volatile compounds present in the bark samples and is likely to be the major source of citronellol, citronellyl and citronellyl acetate, together with *Ceratocystis* weed fungi. Definitely it is the source of the roseoxides - probably a species specific terpenoid for *A. hylecoeti* (Francke & Brümmer, 1978b; Hanssen, 1993). As a further note, citronellol, several terpenoids and long chained acetates do not appear in the chromatogram of *Picea abies* (Fig. 1), which represents a singular exemplary sample of a whole trunk (for method details see Schütz et al., 2004; Holighaus & Schütz, 2006) instead of bark samples of the remaining tree species and one of the same *Picea* tree shown in Table 1. This indicates that these compounds are not emitted until tissue is disrupted. Disruption also enhances the overall abundance, but the pattern of perceived compounds is not affected compared to bark samples of the other trees, and shows that relevant host infochemicals are readily released from an entire trunk. Bark analysis is in our case a sufficient method and might be appropriate for secondary bark beetles (Tømmerås & Mustaparta, 1989). Methoxy-phenol, also a minor but ubiquitous compound in our samples might not be attributable directly to fungal metabolism, since it is an ingredient of some wood species such as oak (Alañón, 2012). Nevertheless, it is a common product of enzymatic lignin degradation by micro-organisms (Crawford & Crawford, 1980; Leonowicz et al., 2001). Methoxy-phenol is perceived by several bark beetles (Huber et al., 2000) and strongly increases pheromone attraction in the ambrosia beetle *Trypodendron lineatum* (Borden, 2001). The high sensitivity

of *E. dermestoides* to this phenolic compound gives rise to the hope that it has similar behavioural function with regard to IPM strategies. Of course behavioural experiments have to confirm such potential. The set of compounds perceived indicates that *E. dermestoides* orientates in an olfactoric landscape that is caused by stressed and dying trees, by deadwood rather than living hard- and softwoods, a collapsed tree defence, wood decay by fungi or bacteria, conspecifics (enabling kin recognition) and predecessing or competitive fungi or fungal farming beetles, rather than host or non-host tree species. The olfactoric sense seems tuned to high-contrast signals indicating deadwood material appropriate for larval and symbiont development. Such decay-specific "sign signals" should allow efficient decision making for a generalist (Bernays, 2001), when referring to host tree species, which is apparently an olfactoric specialist of primarily fungal signals in the deadwood environment.

This table is intended for submission as an online supplementary to the journal manuscript.

Online Resource Supplementary Tab. 1 Summarised host records of *Elateroides dermestoides*.

Scientific name		Times cited[a)]
CONIFEROPSIDA		
PINACEAE		
Abies	*A. alba*	23
Larix	*L. decidua*	6
Pseudotsuga	*P. menziesii*	5
Picea	*P. abies, P. orientalis, P. pungens*[b)]	23
Pinus	*P. silvestris, P. nigra*	8
MAGNOLIOPSIDA		
SALICACEAE		
Populus	*P. davidiana*[c)]	1
ROSACEAE		
Prunus	*P.* spec., *P. avium*	2
BETULACEAE		
Alnus	*A.* spec.	7
Betula	*B. pendula, B. pubescens, B. tortuosa*	20
Carpinus	*C. betulus*	1
FAGACEAE		
Fagus	*F. sylvatica, F. orientalis*	48
Quercus	*Q.* spec, *Q. petraea, Q. robur, Q. pubescens, Q. cerris*	39
JUGLANDACEAE		
Juglans	*J. regia*	2
ACERACEAE		
Acer	*A.* spec.	5
HIPPOCASTANACEAE		
Aesculus	*A. hippocastanum*[d)]	1
OLEACEAE		
Fraxinus	*F.* spec.	2

[a)] Number of articles referring to the corresponding tree species as a host of *E. dermestoides* reviewed by Kurir, 1972, and further references: [b)] Kula et al., 2011; [c)] Bibikova, 1988; [d)] personal observation.

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CHAPTER 6

EIGHT-CARBON VOLATILES ARE INFOCHEMICALS FOR A SPECIALIST FUNGIVORE AND CHARACTERISE SUCCESSIONAL STAGES OF BASIDIOCARPS DURING BEETLE COLONISATION

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this is a manuscript prepared for submission

* I performed all experiments and prepared the manuscript for which I will be the corresponding author. M. Fragstein assisted in experimental work and statistics, B. Weissbecker facilitated direct headspace measures and assisted in quantification procedures.

Abstract

Volatile emissions of fruiting bodies of the bracket fungus *Fomes fomentarius* were collected by entrainment and analysed by full scan GC-MS. Nine eight-carbon volatiles, which are volatile products of lipid oxidation, and another two related nine-carbon oxylipins, benzaldehyde and two sequiterpenes were detected. Direct headspace sampling together with GC-MS (SIM) was employed to quantify release of selected eight-carbon volatiles among successional stages of infestation of fruiting bodies with a mycophagous specialist, the darkling beetle *Bolitophagus reticulatus*. The release of 1-octen-3-ol, 3-octanone and 3-octanol diverged substantially and correlates with beetle abundance in the field when considering behavioural responses previously ascertained in laboratory experiments. Walking bioassays demonstrated 3-octanone to be attractant and 1-octen-3-ol to be repellent to both sexes of *B. reticulatus*. EAG dose-response experiments corroborate those findings. We propose eight-carbon volatiles to be key infochemicals for *B. reticulatus*, referring to host quality or indicating host exploitation. Due to their ubiquity as volatile oxylipins and signalling capacity in the fungal kingdom and cross-kingdom communication, together with

perception capability and responses by many insect species - in particular specialist and generalist fungivores - we presume, that eight-carbon volatiles are of comparable importance to mycophagous and saproxylic insect communities as plant oxylipins like green-leaf volatiles (GLV) are to herbivores.

Keywords

eight-carbon, C_8, oxylipin, fungivory, fungal grazing, fungal development, fungal defense, octanone, octanol, ethanol, Tenebrionidae, Polyporales, LOX, DOX, saproxylic, EC50, host selection

Introduction

Eight-carbon volatiles are ubiquitous among fungi and characterise the fungal aroma for the human nose. They derive from polyunsaturated fatty acids and are classified as oxylipins which play key roles in numerous processes like development, cell growth, sporulation, pathogenicity or apoptosis (Tsitsigiannis & Keller, 2007). Oxylipins have recently gained attention because of their diverse signaling capacity in and particularly amongst and between plants and fungi (Tsitsigiannis & Keller, 2007; Kachroo & Kachroo, 2009; Christensen & Kolomiets, 2011), but many of these features remain to be investigated. It is also hypothesised that oxylipins are involved in regulation of secondary metabolites and modulate behaviour of antagonistic animals (Kempken & Rohlfs, 2010; Rohlfs & Churchill, 2011). Their regulators or byproducts - oxylipin related compounds - eventually become host cues, attractants or repellents, stimulants or deterrents for fungivores (Hanski, 1989). Odorants as infochemicals presuppose volatility, and mainly the eight-carbon volatiles, but also aldehydes, isoprenoids, alcohols or esters (Chiron & Michelot, 2005; Thakeow, 2008) come into consideration. Eight carbon volatiles have been occasionally investigated to that effects and have shown to attract several insect species, mainly dipterans or beetles (Pierce et al., 1989; Fäldt et al., 1999) and even their predators (Collatz, 2009). They were considered as possible attractants for fungivores in general (Hanski, 1989; Spiteller, 2008), but repellent effects were also demonstrated (Pfeil & Mumma, 1993; Wood et al., 2001; Sawahata et al., 2008). Studies of metabolic origins of eight carbon volatiles, suggest enzymatic activities not necessarily resulting in concurrent release of all C8 representatives (Chen & Wu, 1984; Combet et al., 2006). Since we suppose involvement in basic developmental processes and consider the

widespread perception in the insect kingdom, we hypothesise eight-carbon volatiles to be potent and distinctive host infochemicals for fungivorous insects.

Fungi of the genus *Fomes* are bracket fungi within the Basidiomycota and grow on various wooden substrates. *Fomes fomentarius* - the Tinder Fungus - is common, circumboreally distributed and develops large perennial sporophores in contrast to many ephemeral fruiting bodies of Agaricales. It has not been recognised until recently that latent infections of many deciduous tree species favour its primal and dominant occurrence on dying trees (Baum et al., 2003; Schwarze, 2007; Sieber, 2007; Müller-Using & Bartsch, 2009; Fuentes et al., 2010). Consequentially, it bears a fungivorous insect community richest in species among polypores in boreal old-growth forests (Schigel, 2009), and substantially contributes to occurrence and survival of threatened deadwood insect species (Martikainen & Kaila, 2004; Müller et al., 2008). The beetle fauna of *F. fomentarius* has been investigated to test model theories of fungivore diet breadth and it has been inferred from a disproportionately high frequency of monophagous beetles in young fruiting bodies, that at least those stages are chemically defended (Hanski, 1989; Jonsell et al., 2001; Jonsell & Nordlander, 2004). Volatile chemistry of *F. fomentarius* has been studied *in situ*: Eight-carbon volatiles, terpenoids and aldehydes are influenced by season and sporulation (Fäldt et al., 1999). Here we resume the details of its eight-carbon volatiles. Selected representatives were tracked over sporophore developmental stages of *F. fomentarius* and their impact on behaviour of a fungivorous beetle has been examined. We chose the character species of the Tinder Fungus in boreal Eurasia, the black tinder beetle *Bolitophagus reticulatus.* The monophagous fungivore is prevalent on *F. fomentarius,* pioneers the mycophagous community (Jonsell et al., 2001), and entirely develops in succeeding life stages of the bracket fungus. These are a scattered food source of a certain nested spatial occurrence (Rukke, 2002; Müller-Using & Bartsch, 2003,2009). Many polypore-beetle interaction- and spatial ecology studies included *F. fomentarius* and *B. reticulatus* (Nilsson, 1997a,b; Rukke & Midtgaard, 1998; Sverdrup-Thygeson & Midtgaard, 1998; Schigel, 2009; Knutsen et al., 2000), and pointed out that the discontinuous spatial and temporal availability requires sophisticated abilities of the beetle to locate and evaluate adequate substrate for each life stage (Nadvornaya & Nadvornyy, 1991; Nilsson 1997a,b; Lik, 2005). An influence of volatiles including eight-carbon volatiles in host-finding of *B. reticulatus* has been supposed in earlier studies (Nilsson, 1997a; Fäldt et. al., 1999), but no behavioural activity could be assigned to any of the examined chemicals (Fäldt et. al., 1999). Behavioural observations and an evident correlation of *B. reticulatus* occurrence to

sporophore development (Nadvornaya & Nadvornyy, 1991; Nilsson, 1997a,b; Jonsell et al., 2001 and others), gave rise to re-examine volatile chemistry of *F. fomentarius* and determine quantitative changes during fruiting body development and colonization of *B. reticulatus*. Particular attention was paid to electrophysiological and behavioral experiments to assess whether eight-carbon volatiles serve as differentiating host infochemicals in the comparably well studied fungus-insect interaction of *B. reticulatus* and *F. fomentarius*.

Materials & Methods

Populations

Fruiting bodies: Bracket fungi *F. fomentarius* were collected in a near natural 160-year-old beech forest in the "Solling" low mountain range, central Germany, not forested since 1967. Approaching the climax, the amount of deadwood is currently increasing (3.61 trunks ha^{-1} in 2000, 80% lying, 20% standing) with a volume of 73 m^3 ha^{-1} in 2002 (Müller-Using & Bartsch, 2003). The cause of mortality was brittleness and half of the dying trunks showed sporocarps of *F. fomentarius* (Müller-Using & Bartsch, 2003), bearing a well-established population of *B. reticulatus*. We classified the collected sporocarps into four successional stages to describe development and decay. Groups were as follows: [1] "Living", [2] "partly dead", [3] "with cracks & holes", [4] "falling apart". Here we widely follow the classification of Jonsell et al. (2001, see also Midtgaard et al., 1998). The analysed material consisted of five to seven fruiting bodies per group collected on eight different dead trunks of beech (*Fagus sylvatica*), half upright, half overthrown. The longer radius of the fruiting bodies described as a half spherical cap ranged from 40-200 mm. Living sporocarps were sporulating during sampling on 20th of April 2010.

Insects: *B. reticulatus* specimen have been collected from colonised sporocarps growing on beech trunks before flight period between February and April 2008 at "Egge" and "Solling" mountains and 2010 ibidem. Sex was distinguished using antennal *setae* as well as *setae* and apical thorns on *meso*- and *metatarsi* as a character, distinctly pronounced in males. Beetles were kept in the dark at 8 °C with a piece of sporocarp until one hour before using them for further studies under ambient laboratory conditions.

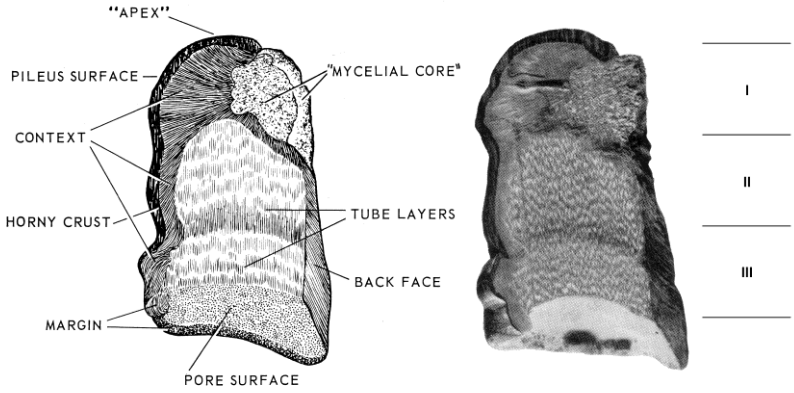

Fig. 1. *Fomes fomentarius* sporophore. Diagrammatical section (after Matthewman & Pielou, 1971; p. 779) on the left. Corresponding transverse section on the right with zones I, II and II, bearing most, average and least numbers of *Bolitophagus reticulatus* living in them (after Nadvornaya & Nadvornyy, 1991).

Methods

Dynamic Headspace Sampling of Fungal Volatiles and Identification with GC-MS: Fruiting bodies were brought to the lab and sampling preparation was accomplished within 30 min at room temperature to minimise storage effects (Wurzenberger & Grosch, 1983). A mixed sample of one fruiting body always included parts of the context or so called *trama*, tube layers, mycelial core and pore surface (Fig. 1). The material was cut to pieces of 5 mm^3 with a scalpel and 80 g were enclosed in a round bottom flask of 250 ml. For a qualitative content analysis of fungal scent, dynamic headspace sampling was applied to collect an odour sample. Filtered clean air was sucked through the flask over an adsorbent trap (glass tube, 6 cm long, 0.3 mm ID, loaded with 1.5 mg charcoal) by means of a rotary vane pump (type DC12/16NK; Fürgut, Tannheim, Germany) for 1.5 h. Volatiles were eluted with 50 µl solvent (CH_2Cl_2/MeOH, 2:1, Suprasolv-quality; Merck/VWR, Darmstadt, Germany), then an aliquot of 1 µl of the extract was directly analysed by means of coupled gaschromatography-mass spectrometry. The GC-MS consisted of a 6890N gas chromatograph connected to a 5973N quadrupole mass spectrometer with electronic ionisation (EI, 70 eV; scan speed 1.58 scans s^{-1}), both Agilent (Palo Alto, USA). A HP-INNOWax fused silica column (Agilent, 30 m, 0.25 mm ID, 0.25 µm coating thickness, polyethyleneglycol) was used with a constant Helium (purity >99.999%) flow of 1 ml/min. The temperature program was 50 °C (1.5 min), followed by an increase of 7.5 °C/min to 200 °C. A permanently installed olfactory detection port

resulted in a split of about 1:1 (see Weissbecker et al., 2004). Compounds were identified by comparison of full scan (m/z 15-300) mass spectra and GC retention values with those of reference compounds, mass spectral databases and published results were used (see Fig. 2). Databases were Wiley 9 combined with NIST ´08, (McLafferty, 2009) and "Terpenoids and Related Constituents of Essential Oils", a database available from MassFinder 3.07 software (Hochmuth Scientific Consulting, Hamburg, Germany).

Quantification of eight-carbon volatiles with Direct Headspace Sampling and GC-MS (SIM): In order to minimze matrix effects when enriching the sample with an adsorbent, we chose direct headspace sampling to acquire more reliable quantitative analysis. We used a GC-MS equipped with a gas syringe and a cooled PTV injector (programmed temperature vapouriser), (Gerstel, Mülheim, Germany). In order to increase sensitivity we programmed a GC-MS setup equal to the one described above in the single ion mode (SIM), measuring only the main and an additional characteristic ion mass for m-cresol (m/z 108), 3-ethyl-phenol (m/z 107, 122) (these are secretions of *B. reticulatus*, Holighaus, unpublished), and the eight carbon volatiles 1-octen-3-ol (m/z 57), 3-octanone (m/z 43) and 3-octanol (m/z 59, 83). This resulted in a scan speed of 1.82 scans s^{-1} with a dwell of 75 msec for each mass. Glass vials of 20 ml content (75,5 mm x 22,5 mm diam.) were filled with chopped pieces of fruiting bodies, prepared as described above, of the different sporophore developmental stages to a mark of 35,0 mm and closed with twisted caps (18,0 mm diam.) fitted with PTFE cladded silicone septae. Vials were maintained in the agitator of a multi purpose sampler (MPS-3, Gerstel, Mülheim, Germany) at a constant temperature of 20 °C. A HP-5ms fused silica column (Agilent, 30 m, 0.25 mm ID, coating thickness 0.25 µm 5% phenylmethylsiloxane) was used. The following temperature program was employed: 50 °C hold for 1.5 min, ramp 3 °C min^{-1} to 125 °C. In order to clean the system, a second ramp followed with 15 °C min^{-1} to 220 °C (3 min). Between each run, the gas tight direct headspace syringe of 2.5 ml was held at 75 °C in a heated cartridge (both Gerstel, Mülheim, Germany) to minimise memory effects. Sampling vials with the fungus were incubated for 10 min at 40 °C and subsequently one stroke of 2 ml was injected with a speed of 0.1 ml/sec into a PTV (CIS 4, Gerstel, Mühlheim, Germany). The PTV was equipped with a Tenax TA® filled liner and held at -40 °C in solvent vent mode during injection, followed by an increase of 12 °C sec^{-1} up to 260 °C. In order to quantify the amount of each volatile in the blend, standard compounds were diluted to concentrations of 10^{-3} to 10^{-7} (w/w) in paraffin (Uvasol®, spectrosc. qual., high visc., Merck, Darmstadt, Germany). An aliquot of this dilution of ~60 µg was filled into the 20 ml sampling

vials and analysed like the fungal samples. Peak area of the major characteristic mass ion was used for quantity calculation.

Authentic standard compounds: Compounds with the highest available purity were purchased from commercial suppliers (3-octanone, octanal, 3-octanol, 2-octenal, 1-octen-3-ol, benzaldehyde, 1-octanol, 2-octen-1-ol).

Electroantennographic Recordings: For 1-octen-3-ol, 3-octanone and 3-octanol dose-response experiments were carried out with an EAG setup as described by Weissbecker et al. (2004). For recording EAG potentials, the amplified signal passed through a high-pass filter with a cut-off frequency of 0.01 Hz to suppress the slow drift often observed in the EAD signal. Afterwards, a constant voltage of 0.5 V was added to the signal. These steps were necessary to match the amplifier output to the input signal range (0-1 V) of a 35900E A/D-converter (Agilent). The signal was recorded using the Agilent Chemstation software. Test compounds were diluted to concentrations of 10^{-2} to 10^{-5} (w/w) in paraffin (Uvasol®, spectrosc. qual., high visc., Merck, Darmstadt, Germany). A reproducible stimulus was applied by puffing 3 ml of air after a standard resting time of 2 min over the antenna. Negative controls (synthetic air; 50 µl paraffin on filter paper) were puffed over the antenna first, then a piece of filter paper (1 cm^2) was soaked with 50 µl of the test dilution and directly put into a 10-ml glass syringe (Poulten & Graf GmbH, Wertheim, Germany). The syringe was flushed then with synthetic air and a stimulus was applied. We always started with the lowest concentration, each puff was repeated twice. Every compound was tested in the five given concentrations for 10 beetles, five of each sex.

Behavioral studies: The behavior towards 1-octen-3-ol, 3-octanone and 3-octanol was tested in dual-choice walking bioassays at room temperature (20 °C) and daylight. The walking arena, placed on a dark table, consisted of an open Petri dish (20 x 140 mm diam., Polystyrene) with two drilled bores (90 mm apart, 20 mm diam.) supported by two 100 ml Erlenmeyer flasks as pitfalls. In each run, a test compound was dissolved in paraffin (Uvasol®, spectrosc. qual., high visc., Merck, Darmstadt, Germany) to a concentration of 10^{-3} w/w. Five droplets (equates to 0.06 gr) of this solution were then filled into a 1.5 ml glass vial and put into one of the Erlenmeyer flasks. As a control, pure paraffin was prepared similarly and placed into the opposite flask. Test compound and control were always positioned randomly. The full arena setup was cleaned in a lab dishwasher at 55 °C, and dried in a ventilated oven for 30 min at 60 °C between experiments. Six beetles of the same sex were

placed in the middle of an arena and were allowed to walk for 60 min at daylight and room temperature. The experiment was repeated until a minimum of thirty beetles made a choice by dropping into pitfalls. Beetles were interchanged from a stock of 130 specimen held at 8 °C to equalise conditions and avoid pseudoreplications.

Data Analyses

Fungal Volatiles: The calibration curves for 1-octen-3-ol and 3-octanone standards in paraffin were visually linear down to 10^{-6}, for 3-octanol to 10^{-7} (w/w). Headspace concentrations for fungal samples were then calculated using experimental Antoine coefficients (Yaws, 2007) and NTP condition values, assuming an ideal gas and thus validity of the gas law of William Henry. For each eight-carbon volatile, we conducted a generalised linear model with its headspace concentration (log_{10}-transformed) as a response variable, and the fungal developmental stage as a categorical explanatory variable. The residuals for normailty and variance homogeneity were graphically examined. We compared the estimates for the different stages using a Tukey post-hoc test. In order to compare the change in concentration from stage 1 to stage 2 between the two volatiles 3-octanone and 1-octen-3-ol, a linear mixed model with the headspace concentration (log_{10}-transformed) as a response variable and developmental stage, volatile and the interaction between stage and volatile as explanatory variables has been fitted. Sample was included as a random effect, to account for the non-independence of volatiles within sample. The significance of the interaction was analysed by conducting a likelihood ratio-test to compare the above model with a model without interactions (both models fitted with maximum likelihood).

Electroantennographic recordings: The mV-response of each initial calibration puff with synthetic air was subtracted from all response values. Thus data was obtained for five doses: 0, 0.00001 to 0.01 in logarithmic increments to describe a dose response relationship. No further normalisation was applied. EAG responses were characterised through nonlinear curve fitting using the software package "drc" generally designed for analysis of multiple dose response curves within the software environment "R" (Ritz & Streibig, 2008). We followed considerations of Getz & Lánsky (2001) and applied logisitic non-linear models to calculate a dose-response-curve, using the "drc" package. An Akaike's information criterion (AIC) was calculated to select the minimal adequate model of a three-, four- or five-parameter logistic

model (see Knezevic et al. 2007, Ritz & Streibig, 2008). Established parameters relating to particular characteristics of the sigmoid curve were calculated: The ED_{50} is the effective dose which provokes a response halfway (50%) between zero and maximum response and is the inflection point of the curve. ED_{10} (10% response) was taken as an odour detection threshold in our sole discretion. Accordingly, the interval between ED_{90} and ED_{10} dimensions a detection range (compare van Giessen et al., 1994; Cometto-Muñiz & Abraham, 2010).

Behavioral studies: In order to test independence of the olfactometer setup from surrounding conditions we tested the reaction to solvent paraffin against itself or an empty pitfall. The proportion of choices was equal to both sides und thus we performed Chi-Square analyses for an expected proportion of 1:1 for all choice experiments using a common spreadsheet program.

Results

FOMES - fungal volatiles

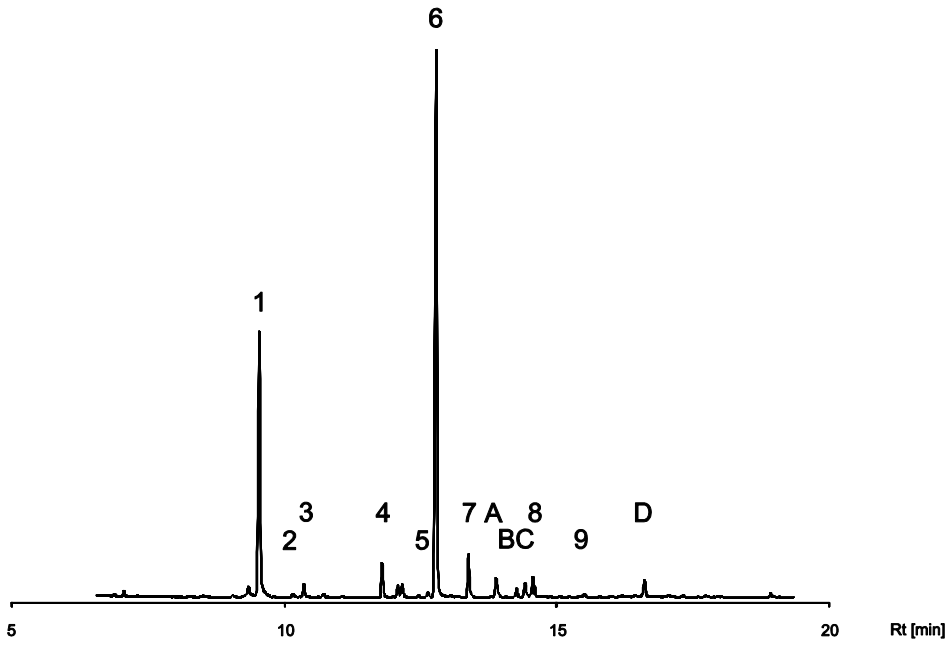

Fig. 2 Qualitative GC-MS total ion chromatogram of a charcoal trap eluate, obtained by CLS-analysis of a chopped young fruiting body of *Fomes fomentarius* grown on *Fagus sylvatica*. All peaks labeled with a number are eight carbon volatiles: 3-octanone[a,b,c] (1), octanal[a,b,c] (2), 1-octen-3-one[c] (3), 3-octanol[a,b] (4), 2-octenal[c] (5), 1-octen-3-ol[a,b,c] (6), (5,Z)-octa-1,5-dien-3-ol[c,d] (7), protuillud-6-ene[b] (A), benzaldehyde[a,c] (B), 2-nonenal[c] (C), 1-octanol[a,b] (8), 2-octen-1-ol[a,c] (9), 3-nonen-1-ol[a] (D), remaining trace compounds are unknown.

superscripts: all compounds identified by MS (Wiley 9 & NIST ´08 combined library) and confirmed by:
a) authentic standard; b) LR_I/MS of MassFinder 3.1 database; c) LR_I in Piveteau et al., 2000; d) MS in Tressl et al., 1982

The aroma of young, actively growing fruiting bodies is fairly dominated by eight carbon volatiles, as it is shown in Figure 2. Most of the typical eight-carbon volatiles of mushrooms (Chiron & Michelot 2005) are present in a *F. fomentarius* chromatogram (number 1-9, Fig. 2), except 1-octene, not detectable due to the used polar column. The eight carbon volatiles contribute with ¾ to the total detected amount of volatiles, from which 90 % fall upon the three most abundant compounds 1-octen-3-ol (6), 3-octanone (1) and 3-octanol (4) (Fig. 3). Another six eight-carbon volatiles (2-3, 5, 7-9) constitute the remaining tenth part, including the tentatively identified (5,Z)-octa-1,5-dien-3-ol (7). Benzaldehyde (B), two nine-carbon volatiles (C & D) and a sesquiterpene (A) constitute most of the remaining traces of the *F. fomentarius* aroma.

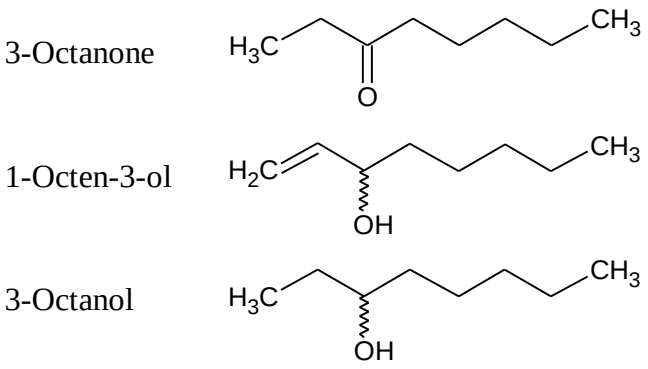

Fig. 3 Chemical structure of main eight-carbon volatiles selected for direct headspace quantification, EAG experiments and behavioural studies. Enantiomeric composition of the two alcohols was not examined.

C8 changes and sporophore development

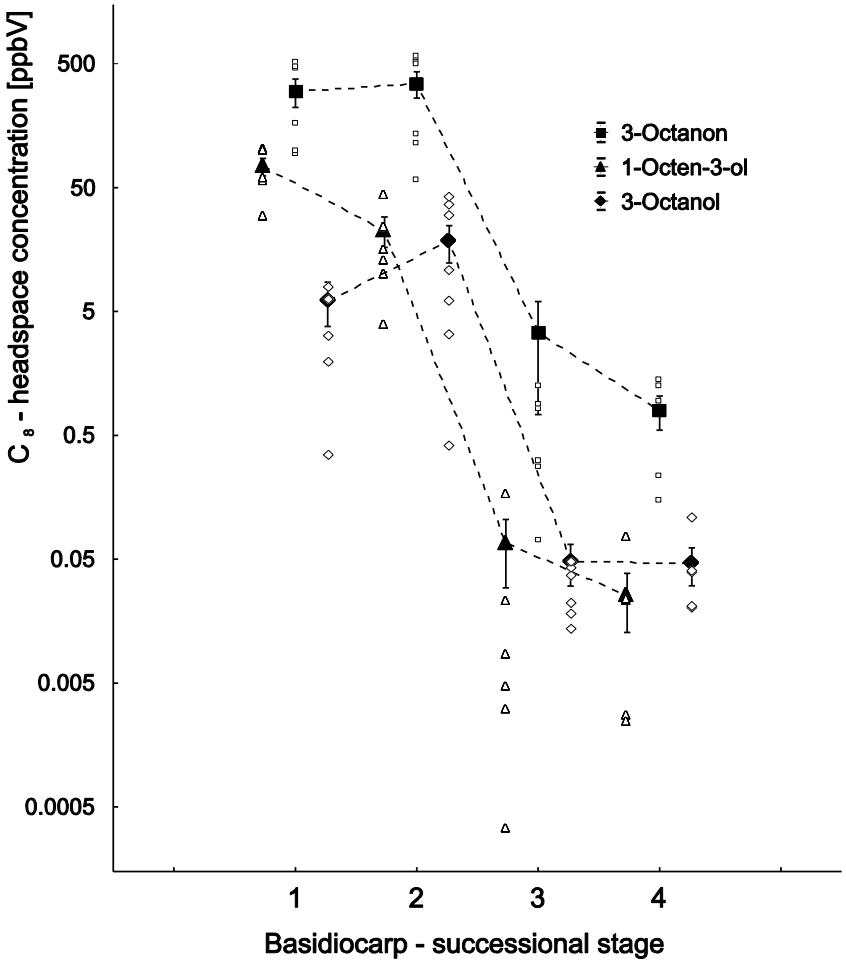

Fig. 4 Artificial time course of the eight carbon volatiles 3-octanone (■), 1-octen-3-ol (▲) and 3-octanol (□) from sliced basidiocarps of *Fomes fomentarius* grown on beech, according to successional stages of basidiocarps "living" (1), "partly dead" (2), "with cracks & holes" (3), "falling apart" (4). Concentrations in headspace are derived from standard dilutions and given on a logarithmic scale as median ± SE in parts per billion by air volume under NTP conditions. (n_1=6, n_2=7, n_3=7, n_4=5; subscripts indicate stage of development).

The absolute concentration of all three monitored eight-carbon volatiles (Fig. 3) differed significantly for developmental stages (Kruskal-Wallis-ANOVA, 3 d.f., N=25; 3-octanone (H=18,21, p=0.0004); 1-octen-3-ol (H=20,06, 3 d.f., p=0.0002); 3-octanol (H=18,46, p=0.0004). A considerable decrease can be observed after stage 2, the stage after which no more active growing tissue at the pore surface is available (Fig. 4). Nonetheless, all three remain detectable in dead sporophores, and level off at a headspace concentration around 0.05 ppbV for the two alcohols and 0.5 ppbV for the ketone. On average measured concentrations encompass about three orders of magnitude with the largest difference for 1-octen-3-ol. 3-Octanone dominates the headspace throughout all stages. For all compounds both living

stages 1 & 2 differ significantly or at least near significantly in concentrations from those constituted by the dead stages 3 & 4 (p-values PostHoc-Test "comparison of mean ranks"). Summing up, the headspace of living (1 & 2) and dead sporophores (2 & 3) differs considerably in absolute content of eight-carbon volatiles. However, the composition between living (1) and weakened (2) stages of *F. fomentarius* is qualitatively different. 1-octen-3-ol in relation to the others is highest in stage 1, but 3-octanone and 3-octanol come forward in stage 2. Comparing the relations, in stage 1 the concentration of 3-octanone : 1-octen-3-ol : 3-octanol is 50:10:1, while it is approximately 20:1:1 in all other stages (Fig. 4).

BOLITOPHAGUS - C8 electroantennographic recordings

In antecedent GC-EAD experiments (data not shown) only 1-octen-3-ol and 3-octanol elicited weak signals hardly above the high background noise of *B. reticulatus* antennae. We changed to EAG dose-response experiments with selected eight-carbon volatiles (Fig. 3), whereof 1-octen-3-ol provoked the overall highest absolute mV responses in *B. reticulatus* antennae (Fig. 5). Responses to the lowest dose tested (10^{-5} in paraffin w/w), were still significantly above those from solvent controls for 1-octen-3-ol and 3-octanol (t-test, $p<0.01$ for both sexes). Antennae of females gave overall higher absolute mV signals than those of males.

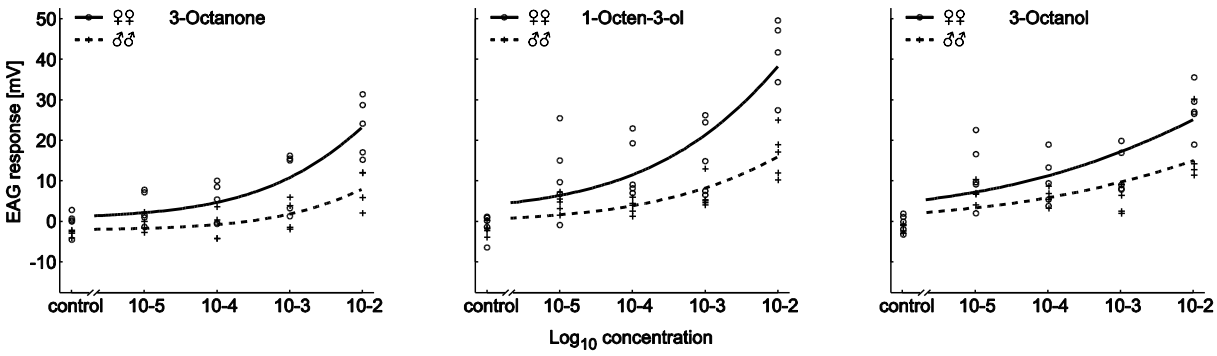

Fig. 5 Dose-EAG-response curves of female (○) and male (+) *Bolitophagus reticulatus* antennae to 3-octanon (left), 1-octen-3-ol (middle) and 3-octanol (right). Lines represent fitted logistic curves. Doses are shown as $\log_{10}$ concentrations in paraffin (w/w). Responses are absolute amplified values [mV].

However, from analysing the shape of fitted dose-response curves, it follows that derived sensitivity parameters (Table 1) were equal among sexes. While the half-response (ED_{50}) is within the same range around concentrations of 10^{-1} for all compounds and sexes, those representing ED_{10} are about 1.6 to 2.5 powers of magnitude lower for 3-octanol resulting in the broadest detection half-range and flattest curve for this compound.

Table 1. Sensitivity parameters[a] derived from fitted dose vs. EAG-response curves of male and female *Bolitophagus reticulatus.*

		Threshold ED_{10}		Half-response ED_{50}		Detection range ED_{50} ~ ED_{10}
Compound	Sex	Log_{10}conc. (w/w) ± SE	t	Log_{10}conc. (w/w) ± SE	t	Log_{10}conc. (w/w)
3-Octanone	f	-3.2 ± 0.6	1.0	-1.0 ± 0.7	1.2	2.2
	m	-1.6 ± 1.6		1.4 ± 1.8		3.0
1-Octen-3-ol	f	-3.2 ± 0.6	0.1	-0.9 ± 0.7	0.4	2.4
	m	-3.3 ± 0.5		-0.5 ± 0.5		2.8
3-Octanol	f	-4.8 ± 0.8	0.6	-0.9 ± 0.5	1.8	3.9
	m	-4.1 ± 1.0		0.6 ± 0.7		4.7

[a]Sensitivity parameters as defined in Material and Methods

Behavioural studies

In walking bioassays both sexes behaved rather similar (Fig. 6). *B. reticulatus* males and females preferred 3-octanone clearly over the control. The racemic mixture of 1-octen-3-ol appeared to be a strong repellent, when more than four fifth of the beetles favoured the control to avoid the compound. 3-octanol did not evoke a distinct behavioural activity in the tested dose.

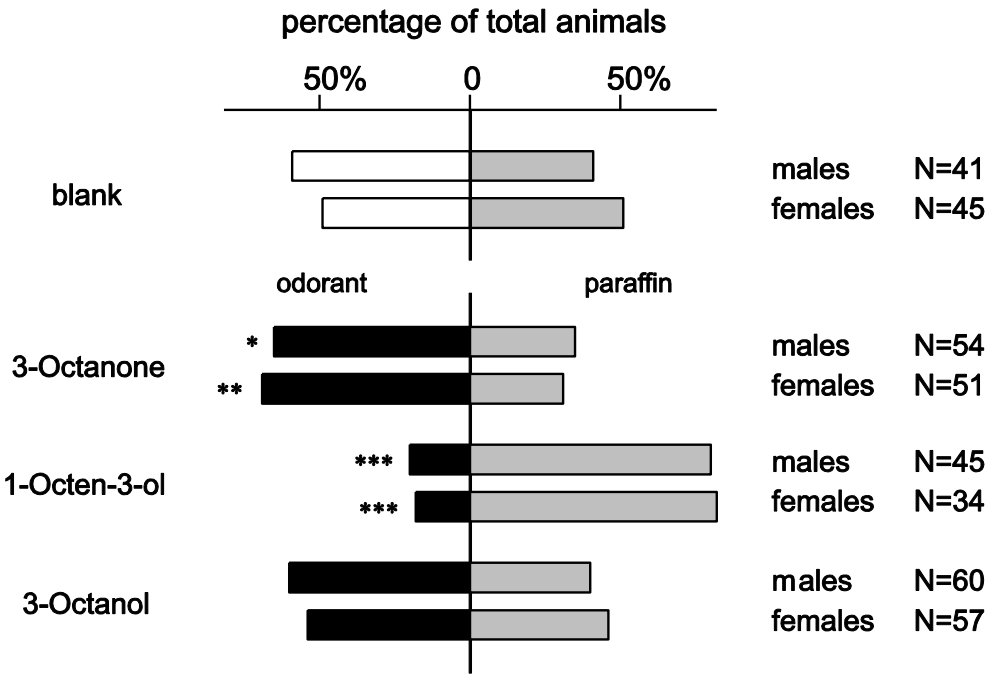

Fig. 6 Relative decisions of *Bolitophagus reticulatus* in dual-choice walking olfactometer preference tests. Males and females in groups of six beetles were given the choice between either 3-octanone, 1-octene-3-ol or 3-octanol dissolved in paraffin in a dose of $\log_{10}$-3 w/w (black) and paraffin only (grey). In a control experiment a blank pitfall (white) was tested against the solvent paraffin. Numbers of choosing individuals are given (χ^2 tests, * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Discussion

Fruiting bodies of *F. fomentarius* released nine eight-carbon volatiles in total, concurrently decreasing in late developmental stages when no more active hymenium is present. Moreover, our results show proportions among selected eight-carbon volatiles in the course of early sporophore development to be considerably different. Eight carbon volatiles of *F. fomentarius* thus appear concerted but subsequently independent. Furthermore we were able to demonstrate, that selected eight-carbon volatiles are infochemicals for the monophagous

fungivor *B. reticulatus*, evoking contrasting behavioural responses. The beetle has a differentiating olfactory sense for eight-carbon volatiles. Compounds and concentrations are well distinguished, as it is corroborated by sensitivity parameters derived from electroantennographic dose-response curves. With these skills, *B. reticulatus* should be capable of discriminating developmental stages of *F. fomentarius*. These, characterise optimal developmental conditions for each life stage of the beetle. Moreover, we found 1-octen-3-ol to be repellent to *B. reticulatus*. This may indicates a chemical defence of young fruiting bodies of *F. fomentarius*. With regard to the ubiquitous presence of eight-carbon volatiles in fungi, we discuss eight-carbon volatiles to be possible infochemicals for mycophagous animals associated to sporophore developmental stages and processes.

Three of the eight-carbon volatiles, 1-octen-3-ol, 3-octanone and 3-octanol, were further tested to investigate occurrence in sporophore developmental stages of *F. fomentarius* and their meaning for behavior and olfaction of *B. reticulatus*. The selected eight-carbon volatiles are the most prominent fungal volatiles in general (Combet et al., 2006), the metabolism of 1-Octen-3-ol is the best-known (Chen & Wu, 1984; Combet et al., 2006), whereas 3-Octanone is the most abundant in *Fomes fomentarius* in situ (Fäldt et al., 1999) and not least 3-Octanol appeared in preliminary GC-EAD studies to be perceived by *B. reticulatus* (Holighaus, pers. obs.). Enantiomeric composition of 1-octen-3-ol and 3-octanol was not determined.

FOMES - Fungal Volatiles

The volatiles found in *F. fomentarius* headspace are well known from many fungal species (Fäldt et al., 1999; Chiron & Michelot 2005). The aroma includes branched ketones and alcohols, terpenoids, organic acids and aldehydes, and chopped fruiting bodies are expectedly dominated by eight-carbon volatiles (Combet et al., 2006). The most prominent, 1-octen-3-ol and 3-octanone, 1-octanol and 3-octanol have been previously identified in *F. fomentarius* (Fäldt et al., 1999). Another six eight-carbon volatiles, namely octanal, 1-octen-3-one, 2-octenal, (5,Z)-octa-1,5-dien-3-ol, 2-octen-1-ol and two compounds with a nine-carbon backbone chain comprise to the blend. Those certainly derive from autoxidative and enzyme-catalyzed lipid oxidation processes via lipoxygenase or lipoxygenase-like enzymes and subsequent hydroperoxide lyases (Chen & Wu, 1984; Combet et al., 2006; Brodhun & Feussner, 2010). While the major part can be attributed to dominant linoleic acid among the polyunsaturated fatty acids in fungi (Cruz et al., 1997; Combet et al., 2006), (5,Z)-octa-1,5-dien-3-ol is probably the pendant to 1-octen-3-ol if linolenic acid is the precursor

(Wurzenberger & Grosch, 1986). Furthermore Wurzenberger and Grosch (1986) were able to show that an acid-catalyzed rearrangement of 10-hydroperoxy-E-8,Z-12-octadecadienoic acid (10-HPOD), a main intermediate in the fungal lipid oxidation process, resulted in production of 2-nonenal and furher nine-carbon volatiles as nonadienals. Therefore we assume, the nine-carbon volatiles we found in *F. fomentarius*, especially 2-nonenal, may have been formed due to the presence of organic acids in the fruiting bodies (Fäldt et al., 1999; Holighaus, pers. obs.), and can thus be considered as directly related to eight-carbon volatiles. Sesquiterpenes and terpenes were previously found to be emitted *in situ* from *F. fomentarius* (Fäldt et al., 1999). However, the terpene fraction (α-pinene, limonene, β-phellandrene, myrcene) probably originates from birch as the host tree in their study rather than fungal metabolism. We neither found the mentioned terpenes in chopped fruiting bodies grown on beech *F. sylvatica*, nor in beech bark or wood (Holighaus & Schütz, 2006; Holighaus, pers. obs.; Thakeow et al., 2006,2008), but they occur in birch bark, *Betula* spec. (Tollsten & Müller, 1996; Byers et al., 2000), the host in the study of Fäldt et al. (1999). Enzyme activity of *F. fomentarius* differs significantly whether the substrate is *Betula pendula* or *F. sylvatica* wood (Větrovský et al., 2010) and an influence of host species to fungal volatiles should be considered for future research. The mass spectrum of the tentatively identified protoillud-6-ene shows similarity to the sesquiterpene named "ST E" in the *F. fomentarius* analysis of Fäldt et al. (1999), but certainly needs further confirmation. Several protoilludane-type sesquiterpenes have been isolated from Basidiomycetes and protoillud-6-ene has been found in *Fomitopsis pinicola*, *Heterobasidion insulare* (Ayer & Browne, 1981; Rösecke et al., 2000) and *Gloeophyllum trabeum* (Thakeow et al., 2006).

C8 changes and sporophore development

Considering the changes over developmental stages, and as we observed in *F. fomentarius*, an exponential decrease after the highest release of eight-carbon volatiles in general and 1-octen-3-ol in particular in the earliest phase of maturity or sporophore development has been observed in several mushrooms (*Volvariella volvacea* (Zhang et al., 2008), *Agaricus bisporus* (Wurzenberger & Grosch, 1983; Combet et al., 2009), *Fomitopsis pinicola* (Fäldt et al., 1999) *Coprinopsis cinerea* (Thakeow, 2008)). Macroscopic classifications of sporophore developmental stages (Hammond & Nichols, 1976; Kües, 2000) are probably incommensurable among species, especially because fruiting-body morphologies have multiple evolutionary origins (Hibbett & Binder, 2002). However, the few available studies of

eight-carbon volatile release suggest, if juxtaposed, different degrees of dependence on fruiting body development and differently directed correlations among 1-octen-3-ol, 3-octanol, 3-octanone and others and thus appear characteristical of fungal species (Mau et al., 1997; Zhang et al., 2008). This is consistent with our findings in *F. fomentarius*, where relations among these compounds change during developmental stages. The release of 3-octanone is positively correlated to 1-octen-3-ol in *C. cinerea* and *A. bisporus*, but negatively in *V. volvacea*, where an increase was observed in later stages - similar to what we observed in *F. fomentarius*. In *Pleurotus ostreatus*, the occurrence of 1-octen-3-ol and 3-octanone follows a third pattern, while their relation remains constant (Zhang et al., 2008). The relation of 3-octanol to both aforementioned eight carbon volatiles is even more obscure. More like 3-octanone in *F. fomentarius*, it follows 1-octen-3-ol and 3-octanone in early stages of *V. volvacea* (Mau et al., 1997) and *A. bisporus* (Zhang et al., 2008), but diverges in older sporophores. Chopping as a mechanical damage generally induces, and dramatically increases the release of eight-carbon volatiles as a whole (Wurzenberger & Grosch, 1983; Fäldt et al., 1999; Combet et al., 2009). Damage may affects qualitative release of eight-carbon volatiles to a certain degree in *F. fomentarius* (Fäldt et al., 1999). However, in case of *A. bisporus* the ratio of 1-octen-3-ol to 3-octanone has been shown unaffected of whether sporophores have been sliced or measured as a whole (Combet et al., 2009). In favour of measuring absolute concentrations effectively accumulating in the headspace, we selected an adsorbent free direct headspace technique, accepting inevitable impact of the preparation procedure. From an ecological point of view it is not a drawback, since beetles encounter disrupted tissue when gnawing within or on the surface of sporophores. Beside an expected decline of eight-carbon volatile release with loss of intact tissue in *F. fomentarius*, we were able to show qualitative differences among living sporophores of different viability for the first time.

BOLITOPHAGUS - C8 electroantennographic recordings & behavioural studies

The remaining question is whether a fungiorous insect can discriminate the observed differences. The answer is clear, when looking at the behaviour of *B. reticulatus*: The Black Tinder Fungus Beetle is either attracted (3-octanon), repelled (1-octen-3-ol) or does not react (3-octanol). Therefore it is evident that the olfactory system provides discrimination abilites for these chemically strikingly similar compounds. EAG is a crude measure, but the dose-EAG-response curves may allow assessing sensitivities (Grant & Lanier, 1982). When ranking the three compounds by vapour pressure (Shields & Hildebrand, 2001), neither

absolute antennal responses nor estimated sensitivity parameters as a relative response reflected their vapour pressure properties. This is in congruence with observations made on the level of single odorant receptor cells, e.g. in model insects as the moth *Manduca sexta*, where in some cases only very few molecules, and not even the most volatile compounds elicit the strongest responses (Shields & Hildebrand, 2001). EAG is incomparable to SCR recordings (Wibe, 2004), but it is generally accepted that coding of odour is likely to depend upon the multiplicity of responding ORNs (de Bruyne et al., 2001). Broad detection ranges, as observed for 3-octanol in *B. reticulatus*, could be achieved by responses of several ORNs, differently responding to a certain limited dose range (de Bruyne et al., 2001; Getz & Lánsky, 2001). We demonstrated effective headspace concentrations of *F. fomentarius* eight-carbon volatiles spanning magnitudes of the power of five, or even more when considering release rates of undamaged sporophores *in situ* (Fäldt et al., 1999). Essential in odour coding is the selectivity of insect ORNs to certain molecules (Todd & Baker, 1999), where the response mainly depend on chemical class and molecular complexity (Todd & Baker, 1999; Araneda et al., 2000; Shields & Hildebrand, 2001; Hallem & Carlson, 2006). Our selected eight-carbon volatiles are a prime example with an identic carbon backbone and branching, but a hydroxyl group substituted by a keto group (3-octanone and 3-octanol) and an additional terminal double bond (1-octen-3-ol) (Fig 3). Such chemophysical active centres are predisposed to be selectively processed by different ORNs. For the insect model organism *Drosophila*, it has been shown that already half of 24 known functional odorant receptors expressed in "the empty neuron" in *Drosophila melanogaster* antennae respond to 1-octen-3-ol (Hallem & Carlson, 2006). Further studies show significant differences in responses to 1-octen-3-ol compared to 3-octanol of excitatory and inhibitory manner (Galizia et al., 2010). Such complexity of olfaction in the fruit fly is observed predominantly among host odors (de Bruyne et al., 2001). Eight-carbon volatiles are certainly not less meaningful to fungivours such as *B. reticulatus*.

sporophore developmental stages & fungivores

It has been demonstrated that *B. reticulatus* effectively colonises fruiting bodies of *F. fomentarius* (Nadvornaya & Nadvornyy, 1991; Kaila et al., 1994; Nilsson, 1997b; Hågvar, 1999). *B. reticulatus* inhabits all fungal stages and is most abundant in weakened or freshly dead sporophores. Beetles avoid young fruiting bodies, but may occasionally deposit eggs. Large groups (max. >100 per sporophore) overwinter in old sporophores. Consequently,

abundance is to a large extent dependent on life stage and sporophore development (Nadvornaya & Nadvornyy, 1991; Nilsson, 1997a,b; Midtgaard et al.,1998; Jonsell et al., 1999; Jonsell et al., 2001, Jonsson et al., 2001). The whole *Fomes* fungivor species assemblage is shifted with fungal age, when monophagous specialists initiate colonisation and generalists follow subsequently (Kaila et al., 1994; Jonsell et al., 1999,2001; Rukke, 2002). This is most apparent in a field study of Jonsell et al. (2001), where *B. reticulatus* was included (Fig. 7). Its occurrence over sporophore developmental stages in the field demonstrated there is remarkably consistent with our chemical and behavioural findings: Fresh and young fruiting bodies, in our study shown to release the highest amounts of the repellent 1-octen-3-ol, yield the fewest beetles in the field. In contrast, the second stage of partially dead fruiting bodies, bearing the most specimen (Fig. 7), releases the highest amounts of the attractant 3-octanone (compare Fig.7, Fig. 4). To which degree eight-carbon release is independent or feeding damage caused the observed changes (Drilling & Dettner, 2009), has yet to be studied.

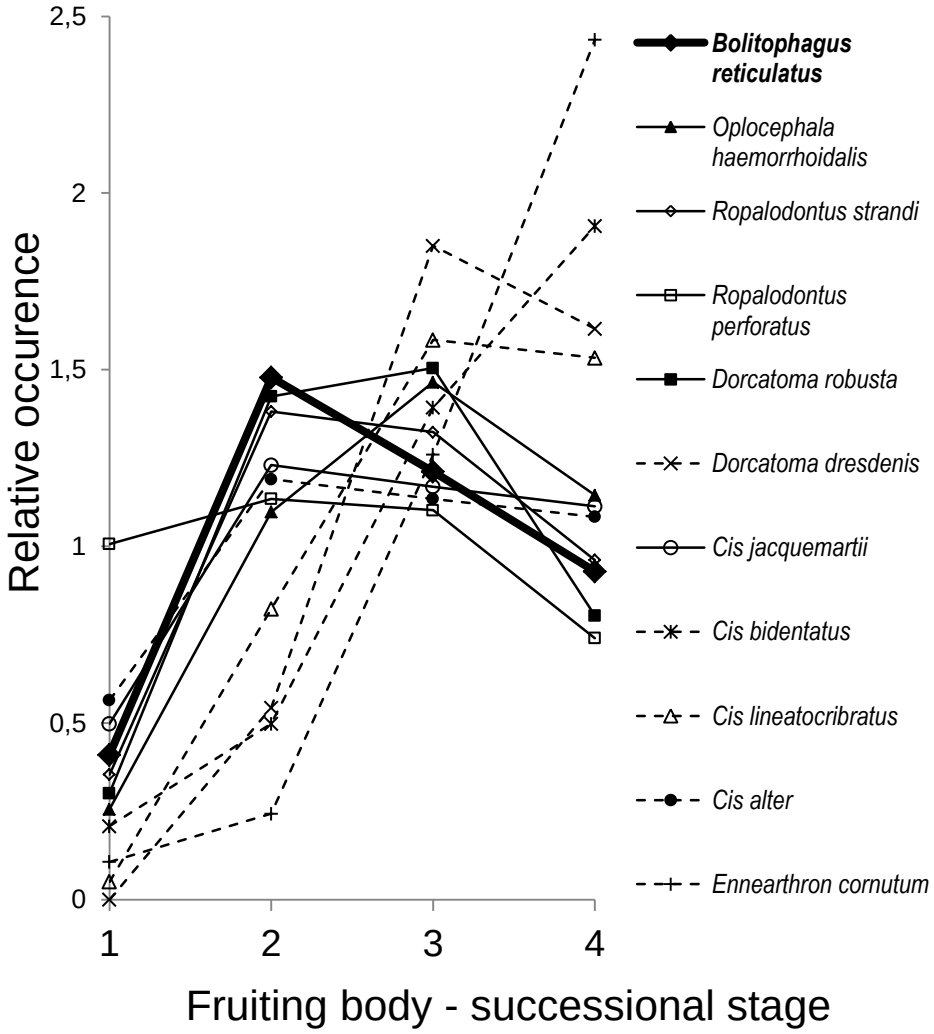

Fig. 7 Relative abundance of *Fomes fomentarius* fruiting bodies of four successional stages "living" (1), "newly dead" (2), "dead, with cracks & holes" (3), "falling apart" (4) with presence of fungivorous beetle species, classified as monophagous (solid lines) or polyphagous (dashed lines). Figure redrawn after Jonsell et al., 2001, Ecol. Bull. 49, Fig. 6, p. 188 with courtesy of Mats Jonsell.

Alternatively, the amount of vital, decayed or frayed tissue within a single sporophore could explain our results. The growth zone of a sporocarp (zone III, Fig. 1) above the pore surface normally inhabits the lowest numbers of larvae, eggs and adult beetles (Nadvornaya & Nadvornyy, 1991). Avoidance of such vital tissue could be due to a high content of 1-octen-3-ol, the strong repellent. Nevertheless, adults leave skin-deep depressions at the pore surface, probably while feeding fungal spores as an additional food source (Hågvar, 1999). A repellent effect of 1-octen-3-ol has been shown for several fungivores such as slugs (Wood et al. 2001), collembolans (Sawahata et al., 2008) and flies (French & Kline, 1989; Pfeil & Mumma 1993). Furthermore, vapour of 1-octen-3-ol is severely neurotoxic for the model insect *D. melanogaster*, while the eight-carbon ketone 2-octanone is noneffective (Inamdar et al., 2010). Our results for *B. reticulatus* may thus represent a more general pattern. Wound

activated chemical defence is well known in fungi (Spiteller, 2008) and a direct linkage to eight-carbon volatile release via DOX/LOX pathways is assumed (Spiteller, 2008, Rohlfs & Churchill 2011). For herbivores (Hildebrand et al., 1993; Bruce et al., 2005), plant oxylipins such as six-carbon volatiles (green leaf volatiles "GLVs"), have been documented to be repellents and also attractants. They are involved in a cascade of constitutive and induced plant defence (Frost et al., 2008; Holopainen & Gershenzon, 2010) including higher trophic levels (Allmann & Baldwin, 2010). Thus, analogous to volatile plant oxylipins (Prost et al., 2005), eight-carbon volatiles could be toxic themselves or be a volatile signal of an underlying chemical defence (Rohlfs & Churchill, 2011). A defence in *F. fomentarius* has been assumed for young fruiting bodies (Hanski, 1989; Jonsell et al., 2001). This assumption derived from the observation that monophagous beetles, hence evolutionary adapted to overcome the defence, dominate early stages, while later stages host generalists that are more generally more susceptible to defence compounds (Fig. 6). Among plants, changes in volatile oxylipins can alter the ability of herbivores to locate their host (Bruce et al., 2005). Beside similarities in the metabolic pathways of plant and fungal oxylipins, our results support a similar scenario among fungivores.

Matthewman & Pielou (1971) make one step ahead and claim for *B. cornutus*, the sister species replacing *B. reticulatus* on *F. fomentarius* in Northern America, to kill the fungus. Both have a strikingly similar ecology, an identical chemical defence against predators (Holighaus, unpublished; Holliday et al., 2009), and constitute large local metapopulations on tree-trunks covered with *F. fomentarius*. It is worth mentioning that eight-carbon volatiles are multifunctional, and that they are selective bactericides against natural fungal antagonists (Beltran-Garcia et al., 1997). However, for insects related to fungi, 1-octen-3-ol has also been shown to be an attractant, but merely when released in low concentrations (Pierce et al. 1991; Fäldt et al., 1999; Thakeow et al., 2008; Collatz, 2009; Drilling & Dettner, 2009). Therefore, the volatile mixture or environment affect the behavioural meaning among species, beside the concentration.

Several landscape ecology studies examining colonisation success of *B. reticulatus*, experimentally tested the attractiveness of entire fruiting bodies of *F. fomentarius* compared to natural ones in the field (Jonsell & Nordlander, 1995; Jonsson et al., 1997; Jonsell et al., 1999; Sverdrup-Thygeson, 2010). Shifts in favour of generalists and at the expense of monophagous specialists, particularly *B. reticulatus*, lead to controversial discussions on

beetle mobility and landscape fragmentation effects on the *F. fomentarius* fungivor community (for a review see Jonsell et al., 2003). From our chemical studies it seems to emerge that efforts to homogenise trap sporophores by quick-freezing to -70 °C seriously influenced volatile chemical host cues, including eight-carbon volatiles. Reductionistic field-trials applying 1-octen-3-ol, 3-octanone, both, or mixes of 1-octen-3-ol together with 1-octanol, 3-octanol and 1-nonanol did not attract *B. reticulatus* either (Fäldt et al., 1999). It was at least possible to show that ethanol attracts the Black Tinder Fungus Beetle, even more when combined with fruiting bodies as a lure (Jonsell et al., 2003). Wood-decay volatiles including ethanol and admixtures of aldehydes, eight-carbon volatiles and terpenes attracted modest numbers of *B. reticulatus* (Holighaus & Schütz, 2006). All compounds originate from either fruiting bodies (Fäldt et al., 1999) or host trunks (Holighaus & Schütz, 2006) or probably both. Trunks are strong emitters of ethanol (Gara et al., 1993) and mycelium-infected wood may contributes to attract beetles to sporophores (Johansson et al., 2006). Attractiveness of beetle derived compounds as pheromones (Holighaus, unpublished), and also behavioural observations (Nilsson, 1997a,b) suggest that it is not the sporophore alone indicating suitable host patches. Behavioural studies on feeding or egg deposition behaviour of *B. reticulatus* are scarce (Nadvornaya & Nadvornyy, 1991; Kaila et al., 1994; Nilsson, 1997a,b), and how eight-carbon volatiles contribute at certain life stages to the behavioural repertoire of *B. reticulatus* is discussable.

Host finding, host defence and the chemoecology of fungivorous beetles is generally little understood, but apparent analogies between herbivory and fungivory could direct approaches for future research. A better understanding of insect-fungus interactions will help to support nature conservation concerns, where deadwood insects are often evaluated and many rare species worth protecting exist.

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CHAPTER 7

PHEROMONAL FUNCTION OF DEFENSIVE SECRETIONS IN *Bolitophagus reticulatus* (COL., TENEBRIONIDAE).

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Abstract

The defensive secretion of *Bolitophagus reticulatus* was analyzed. Chemical composition of abdominal glands of this basal tenebrionid beetle is comparatively simple, consisting of both methyl- and ethyl- substituted benzoquinone, hydroquinone and phenol. The alkylated phenols are specific to *B. reticulatus* and gland extracts of male and female contain equal amounts of 3-methylphenol and 3-ethylphenol. However, only male beetles were significantly attracted to the phenols in walking bioassays and they were also more decisive. Dose-response experiments with an electroantennographic setup (EAG) demonstrate a higher sensitivity of male antennae to the phenols, and corroborate those findings. We summarize occurrence and multifunctionality of methylphenol and ethylphenol among Tenebrionidae. We also generally discuss aggregation pheromone or sex pheromone release along with defensive secretions in the context of spatial aggregation, widespread among fungivores, as it

is *B. reticulatus*. For *B. reticulatus* we propose a major function of 3-methylphenol and 3-ethylphenol as a sex pheromone or mating stimulant, the first demonstrated in Bolitophagini.

Keywords

allomone, secondary pheromone component, sex pheromone, semiochemical parsimony, cresol, quinones, antimicrobial, *Fomes fomentarius*, *Tribolium*, EAG, dose-response, metapopulation

Introduction

In arthropods, chemical defense mechanisms evolved numerously within diverse taxa. Their primary role is to keep predators from feeding und thus guarantee individual or at least increase conspecific survival (Roth & Eisner, 1962; Dettner, 1987). The so called defense compounds are diet-sequestered and/or *de novo* synthesised by many species, acting against predators or as antibiotics (Blum, 1996; Ruther et al., 2001a; Laurent, 2005; Pankewitz & Hilker, 2008). As a rule, many of these compounds are multifunctional (Dettner, 1987; Blum, 1996) and can act as intraspecific signals (Pasteels et al., 1983; Ruther et al., 2001a,b; Reinecke et al., 2002; Wertheim et al., 2004; Wertheim, 2005). Such examples are defense secretions of ants, which also work as alarm or trail pheromones (Morgan, 2009; Pasteels et al., 1983). Some arthropods have exocrine glands releasing sexual pheromones, although they likely originated as defensive glands, providing strong evidences of an evolutionary diversification of their original function (Peschke & Metzler, 1982; Peschke, 1983; Aldrich et al., 2000; Geiselhardt et al., 2009). Many darkling beetles (Tenebrionidae) have been found to possess prompt chemical defense mechanisms, enabling them to efficiently release poison chemicals, when they are under attac of predators. (Tschinkel, 1975a; Blum, 1981; Pasteels et al., 1983; Francke & Dettner, 2005; Laurent et al., 2005; Villaverde et al., 2007, 2009). Most tenebrionid species have abdominal defensive glands, but in some taxa prothoracic defensive glands are also present (Tschinkel, 1975c; Dettner, 1987). Several researches are currently focusing on the molecular mechanisms underlying the defensive system of the tenebrionid beetle *Tribolium castaneum*, since the genome of this beetle has been published (Miyatake et al., 2008; Richards et al., 2008; Michalczyk, 2010; Pedrini et al., 2010). Although the defense compounds are well described in many Tenebrionidae, only few pheromones are known within this family. They are terpenes (Bartelt et al., 2009), methylated alcohols and aldehydes

(Francke & Dettner, 2005), cuticular hydrocarbons and in a few cases irritant parts of the defensive secretions as quinones (Verheggen et al., 2007). The multifunctionality of defense compounds and pheromones has been repeatedly highlighted (Pasteels et al., 1983; Dettner, 1987; Blum, 1996; Wertheim et al., 2005), but more examples are needed to evaluate generalisability of their interdependence.

In our study, the chemical secretions and their behavioural meaning to the Black Tinder Fungus Beetle, *Bolitophagus reticulatus* (L.) (Coleoptera: Tenebrionidae), was examined. *B. reticulatus* belongs to the basal tribe Bolitophagini within the large superfamily Tenebrionoidea (Hunt, et al., 2007; Marvaldi et al., 2008; Matthews & Bouchard, 2008) containing only 170 species divided in 22 genera (Jung et al., 2007). The chemistry of defensive glands of Bolitophagini has been studied in detail for *Bolitotherus cornutus* (Tschinkel, 1975b; Holliday et al., 2009), and for chemotaxonomic purpose for *Eleates occidentalis* (Tschinkel, 1975b), *Byrsax egenus*, *Byrsax maclaeyi*, and a *Rhipidandrus* species (Brown et al., 1992). These species have two compounds, namely 2-methyl-1,4-benzoquinone and 2-ethyl-1,4-benzoquinone, in common, as it is the case for many Tenebrionidae. All species within the Bolitophagini are closely associated with perennial and woody fruiting bodies of fungi, particularly with Polyporales (Jung et al., 2007; Matthews & Bouchard, 2008). This is also the case for *B. reticulatus*, monophagous on sporocarps of the widespread Tinder Fungus *Fomes fomentarius* (L.) (Polyporales: Polyporaceae). Dead trees bearing tinder fungi are a scattered food source in a forest (Jonsell et al., 1999; Müller-Using & Bartsch, 2009). Resulting metapopulation structures of *B. reticulatus* colonies have been investigated intensively within nature conservation and spatial ecology studies (Nilsson, 1997a,b; Rukke & Midtgaardt, 1998; Sverdrup-Thygeson & Midtgaardt, 1998; Knutsen et al., 2000). Beetles often seek shelter in groups in old sporocarps and show a bimodal activity, peaking around midday and after midnight (Nadvornaya & Nadvornyy, 1991; Nilsson, 1997a; Hågvar, 1999). Adults feed on the pore surface of living sporocarps (Nilsson, 1997a; Hågvar, 1999) and by doing this inevitably encounter natural enemies (Aulén, 1991; Nadvornaya & Nadvornyy, 1991). The primary mode of defense response of *B. reticulatus* to avoid predators is the *thanatosis*. They fold their legs and if bothered continuously, likely triggered by signals as mammalian breath (Conner et al., 1985), they can exude a volatile defensive chemical secretion with an intensive phenolic smell. Hence, our study aimed to investigate the chemical composition of the defensive glands of *B. reticulatus* and unravel components which eventually contribute to intraspecific communication. Additionally we will shortly refer to the

modes of pheromonal side actions of defensive secretions, their origin and impact on behaviour of group living fungivores.

Material and Methods

Insects.

Specimen of *Bolitophagus reticulatus* (Coleptera: Tenebrionidae: Bolitophagini) have been collected from colonised sporocarps growing on beech trunks before flight period between February and April 2008 and 2010 at "Egge" and "Solling" mountains (North Rhine-Westphalia and southern Lower Saxony, Germany, respectively) . Sex defining characteristics are antennal setae as well as setae and apical thorns on meso and metatarsi, distinctly pronounced in males (Nilsson, 1997b). Beetles were kept at 8 °C, 70% relative humidity under darkness with a piece of sporocarp until one hour before use for further studies.

Chemical Standards and Abbreviations of Chemicals.

The following authentic standards were obtained with given purity from commercial sources: 2-methyl-1,4-benzoquinone (toluquinone) further referred to as "MBQ", CAS 553-97-9, 98% (TCI, Zwijndrecht, Belgium); 2-methyl-1,4-hydroquinone "MHQ", CAS 95-71-6, 99%; 2-ethyl-1,4-hydroquinone "EHQ", CAS 2349-70-4, 98%; 2-methylbutanal, CAS 96-17-3, 90% (Sigma-Aldrich, Steinheim, Germany); 3-methylphenol (m-cresol), CAS 108-39-4, 99%; 3-ethylphenol, CAS 620-17-7, 95% (ABCR, Karlsruhe, Germany). 2-ethyl-1,4-benzoquinone, CAS 4754-26-1 is hereafter referred to as "EBQ".

Sampling and Analysis of Defensive Secretion Extracts.

Pairs of abdominal defensive glands were dissected as described by Tschinkel (1975c) after having been dipped in liquid nitrogen. A pair of glands was transferred into 50 µl of dichloromethane (GC grade, 99.8% purity, Merck, Germany) and sonicated for 5 min. Six beetles of each sex were analyzed. All defensive gland extracts were analyzed with a 5973N quadrupole mass spectrometer with electron ionisation (EI, 70 eV) connected to a 6890N GC, both Agilent (Palo Alto, USA). A HP-5ms fused silica column (Agilent, 30 m, 0.25 mm ID, 0.25 µm coating thickness, 5% phenylmethylsiloxane) was used. An aliquot of 1 µl was injected into a split/splitless injector held at 250 °C. The temperature program was 50 °C for 1.5 min, followed by an increase of 7.5 °C/min to 250 °C, remaining at 250 °C for 5 min. The

MS was run in scan mode (m/z 20-345). Additionally, the identification of compounds was confirmed using a HP-INNOWax column (Agilent, 30 m, 0.25 mm ID, 0.25 µm coating thickness, polyethyleneglycol) on a similar setup. The constituents were identified by comparison of their mass spectra and GC retention values (LR_I) with those of reference compounds and those given in the mass spectral databases Wiley 9 combined with NIST ´08 (McLafferty, 2009). Additionally, for comparison and compound confirmation, we analyzed extracts of *Tribolium castaneum* defensive glands, of which defense chemicals are well characterised and reviewed by Unruh et al. (1998). For quantification of 3-methylphenol and 3-ethylphenol, standard solutions containing 10 ng, 100 ng, 1µg, 10 µg, 100 µg per 1 ml dichloromethane of each compound were measured with the same procedure as described for the gland extracts. Peak areas of 3-methylphenol (m/z 108) and 3-ethylphenol (m/z 107) from the extracts were calculated and related to those obtained from the standard solutions.

Direct Headspace Analysis.

Six alive, unsexed beetles were put into a 20 ml glas vial, closed with twisted caps and fitted with PTFE cladded silicone septae. In order to provoke gland eversion, they were shortly shaken. The direct headspace syringe of 2.5 ml was flushed with helium and held at 100 °C before uptake. The sample was incubated for 2 min at 35 °C and subsequently one stroke of 2 ml was injected with a speed of 0.1 ml/sec into a cooled PTV injector (programmed temperature vapouriser; CIS 4, Gerstel, Mühlheim, Germany). The PTV was equipped with a Tenax TA© filled liner and held at -40 °C in solvent vent mode during injection, followed by an increase of 12 °C/sec up to 260 °C. The temperature program for the GC oven was 50 °C (2.5 min), followed by an increase of 6.2 °C/min to 220 °C. The constituents were identified by comparison of a full scan (m/z 15-300) with the aforementioned sources.

Electroantennographic Recordings.

Dose-response experiments with selected defensive gland components were carried out using an EAG setup as described by Weissbecker et al. (2004). Negative controls (synthetic air only) and solvent controls (synthetic air; filter paper of 1 cm^2 soaked with 50 µl paraffin - Uvasol®, spectrosc. qual., high visc., Merck, Darmstadt, Germany) were puffed over the antenna first. In each case, a piece of filter paper was soaked with 50 µl of the directly prepared dilution of a pure test compound (concentrations 0.00001, 0.0001, 0.001and 0.01 (w/w) in paraffin) and put immediately into a 10-ml glass syringe (Poulten & Graf GmbH,

Wertheim, Germany). The syringe was then flushed with synthetic air and after a short equilibration time a stimulus was applied. Reproducibility was achieved by each time puffing 3 ml of syringe headspace over the antenna, after a standard resting time of 2 min. Each set of experiments always started with the lowest concentration, each puff was repeated twice. Every compound was tested in the five given concentrations for 10 beetles, five of each sex.

Behavioral Studies.

The behavior evoked by pure gland components was tested in dual-choice walking pitfall bioassays at room temperature (20 °C) and daylight conditions. The walking arena, placed on a dark table, consisted of an open Petri dish (20 x 140 mm diam., polystyrene) with two drilled bores (90 mm apart, 20 mm diam.) supported by, and connected to two 100 ml Erlenmeyer flasks as pitfalls. Pure test compounds were dissolved in paraffin (Uvasol®, spectrosc. qual., high visc., Merck, Darmstadt, Germany) to concentrations of 10^{-3} w/w. Five droplets (equal to ca. 60 mg) of this solution were filled into a 1.5 ml glass vial which was put into one Erlenmeyer flask. Pure paraffin was applied similarly as a control at the opposite flask. Flasks were arranged randomly. The full arena setup was rinsed, dried in a ventilated oven for 30 min at 60 °C between experiments. A single male or female beetle was placed in the middle of an arena. When the beetle tumbled down into one of the pitfalls, treatment or control as the type of choice was noted. After a maximum experimental time of 60 min, beetles lingering at the rim of a pitfall-hole were also accounted for choice. Otherwise, if the beetle remained elsewhere in the arena, it was noted as non-responding. Arenas were replaced betimes, and a maximum of about 20 arenas were run in parallel. A set of experiments ended, after a minimum of thirty beetles made a positive or negative choice. Beetles were interchanged from a stock of 130 specimen held at 8 °C to equalise conditions and avoid pseudoreplications.

Data Analyzes of Electroantennographic Recordings.

The mV-response of each initial calibration puff with synthetic air was subtracted from all response values. Therefore, data for five doses (blank, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} (w/w) in paraffin) were obtained to describe a dose-response relationship for each tested substance. EAG responses were characterised by nonlinear curve fitting using the software package "drc" generally designed for analysis of multiple dose response curves within the software environment "R" (Ritz & Streibig, 2005). We applied logisitic non-linear models to calculate

an appropriate dose-response-curve (Getz & Lánsky, 2001), using the "drc" package. An Akaike's information criterion (AIC) was calculated to select the minimal adequate model of a three-, four- or five-parameter logistic model (details see Ritz & Streibig, 2005). Established parameters relating to particular characteristics of the sigmoid curve were calculated (van Giessen et al., 1994; Getz & Lánsky, 2001; Jordan et al., 2009; Nichols, 2010): The ED_{50} is the effective dose which provokes a response halfway (50%) between zero and maximum response and is the inflection point of the curve. ED_{10} (10% response), we took in our sole discretion as a comparable measure with the meaning of an odour detection threshold. Accordingly, ED_{90} - ED_{10} dimensions a detection range (compare van Giessen et al., 1994; Cometto-Muñiz & Abraham, 2010).

Statistical Analyses of Behavioral Studies.

In order to test independence of the olfactometer setup from ambient conditions, we tested the solvent paraffin against paraffin itself or against an empty pitfall. The proportion of choices was equal to both sides and so we performed a χ^2*-test* for an expected proportion of 1:1 for all choice experiments, using a common spreadsheet program.

Results

Defensive Secretions

The dichloromethane extracts of the abdominal glands from both females and males contained six aromatic components (Table 1). Together they comprise 97.4% of the secretion detected by GC-MS. Similar to most tenebrionid beetles, MBQ and EBQ were dominant and yielded three quarters of the extract. The corresponding hydroquinones MHQ and EHQ were also present. Additionally 3-methylphenol and 3-ethylphenol were detected and contribute with one fifth to the secretion.

The phenols 3-methylphenol and 3-ethylphenol appeared to be species specific for *Bolitophagus reticulatus* and we consequently examined them in further detail. A pair of glands yielded an average of 21 µg 3-methylphenol (18.9±29 µg females, 22.6±20.7 µg males; average ±SD) and 3.4 µg of 3-ethylphenol (2.4±1.5 µg females, 4.4 ±2.7 µg males).

Table 1 - Abdominal defensive gland composition of female and male *Bolitophagus reticulatus.*

		Relative amount (%) ± SE	
Chemical	Abbreviation	Females	Males
2-Methyl-1,4-benzoquinone	(MBQ)	18.4 ± 2.0	20.0 ± 0.7
3-Methylphenol	(m-Cresol)	19.9 ± 3.2	21.0 ± 1.6
2-Ethyl-1,4-benzoquinone	(EBQ)	51.5 ± 3.4	50.0 ± 1.5
3-Ethylphenol		1.0 ± 0.2	1.2 ± 0.2
2-Methyl-1,4-hydroquinone	(MHQ)	1.2 ± 0.1	1.2 ± 0.1
2-Ethyl-1,4-hydroquinone	(EHQ)	5.4 ± 0.7	4.5 ± 0.4

There was no statistical difference between sexes (*t-test*, 3-methylphenol -0.26, $P = 0.8$; 3-ethylphenol -1.56, $P = 0.14$; n = 6/sex). As a result, the absolute individual amount varied considerably while the relative variation of the compounds is comparably low (Table 1).

When the vapour space over six stimulated beetles was analyzed by direct headspace technique (Figure 1), all six compounds present in the extracts were recovered, but additionally 2-methylbutanal (compound 1, fig. 1) and an unknown compound (compound 8, fig. 1) with the following fragmentation was found: MS (70 eV): m/z (%) = 137 (2), 136 (27) [M^+], 135 (3), 134 (1), 121 (1), 108 (2), 107 (10), 106 (1), 91 (1), 90 (1), 89 (1), 82 (2), 81 (1), 80 (2), 79 (9), 78 (2), 77 (5), 68 (1), 66 (1), 65 (1), 63 (1), 62 (1), 55 (2), 54 (2), 53 (3), 52 (2), 51 (2), 50 (1), 43 (1), 39 (2), 27 (1), 26 (1).

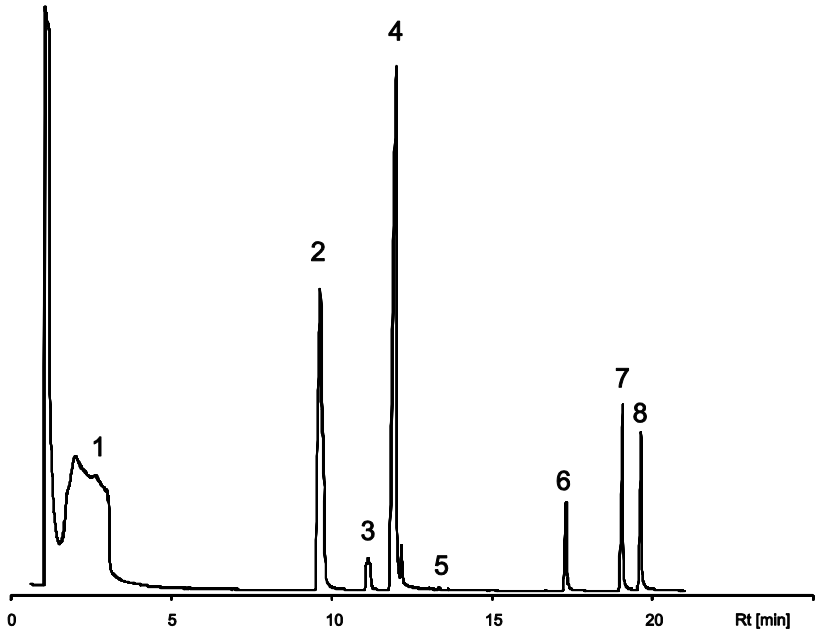

Fig.1. GC-MS total ion chromatogram obtained by direct headspace analysis of six abdominal gland pairs of *Bolitophagus reticulatus*. Numbered compounds were identified as 2-methylbutanal (1), 2-methyl-1,4-benzoquinone "MBQ" (2), 3-methylphenol "m-cresol" (3), 2-ethyl-1,4-benzoquinone "EBQ" (4), 3-ethylphenol (5), 2-methyl-1,4-hydroquinone "MHQ" (6), 2-ethyl-1,4-hydroquinone "EHQ" (7), unknown compound (8).

Electroantennographic Recordings

Non-linear regression of dose-response measures for 3-methylphenol and 3-ethylphenol resulted in typical sigmoid dose-response curves, distinctive for the compounds and male and female *B. reticulatus* (Figure 2).

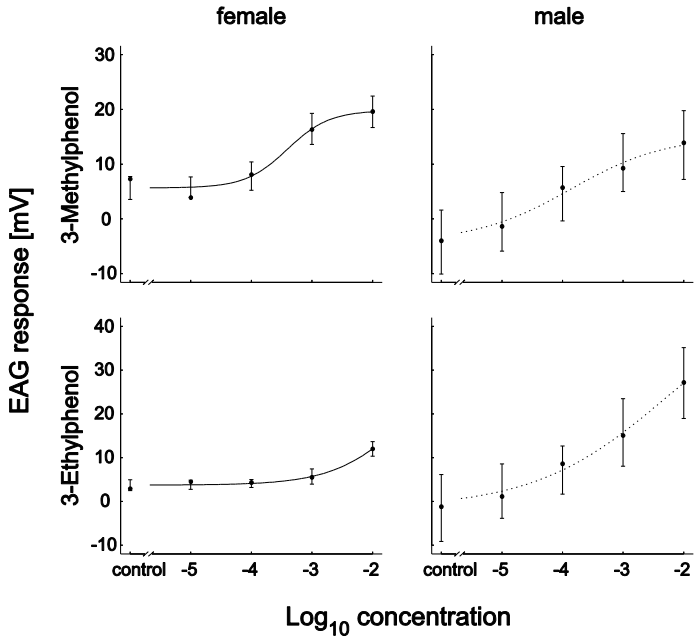

Fig. 2. Dose-EAG-response curves of female (left) and male (right) *Bolitophagus reticulatus* to 3-methylphenol (top) and 3-ethylphenol (bottom). Points are means of recordings from 5 beetles, error bars are the SE of the fitted logistic curve. Doses are shown as $\log_{10}$ concentrations in paraffin (w/w). Responses are absolute amplified values [mV].

Table 2 - Sensitivity parameters[a] derived from fitted dose vs. EAG-response curves of male and female *Bolitophagus reticulatus*

		Threshold ED_{10}		Half-response ED_{50}		Detection range ED_{50} ~ ED_{10}
Compound	Sex	Log_{10}conc. (w/w) ± SE	t	Log_{10}conc. (w/w) ± SE	t	Log_{10}conc. (w/w)
3-Methylphenol	f	-4.2 ± 0.2	2	-3.4 ± 0	0.7	0.8
	m	-5.6 ± 0.7		-3.9 ± 0.6		1.7
3-Ethylphenol	f	-2.8 ± 0.8	1.8	-1.5 ± 0.9	0.5	1.3
	m	-4.7 ± 0.7		-2.3 ± 1.2		2.4

Male antennae had an overall higher sensitivity for 3-methylphenol and 3-ethylphenol, reflected in lower thresholds (ED_{10}) between 1.4 to 1.9 powers of magnitude lower than females (Table 2). Since the half-response ED_{50} was in a similar dose range for both sexes, a broader detection range of males becomes evident. Comparing the compounds, the detection half range was broader for 3-ethylphenol than for 3-methylphenol (Table 2).

Behavioral Studies

Comparing the activity of both sexes, there were more non-responders among females (21%) than among males (9%) ($\chi^2 = 6.4$, $P < 0.05$). For those which were decisive in the dual-choice arena, males showed a clear preference for 3-ethylphenol ($\chi^2 = 11.11$, $P < 0.001$, Fig. 3) and less pronounced for 3-methylphenol ($\chi^2 = 6.39$, $P < 0.05$, Fig. 3) *vs*. the solvent control.

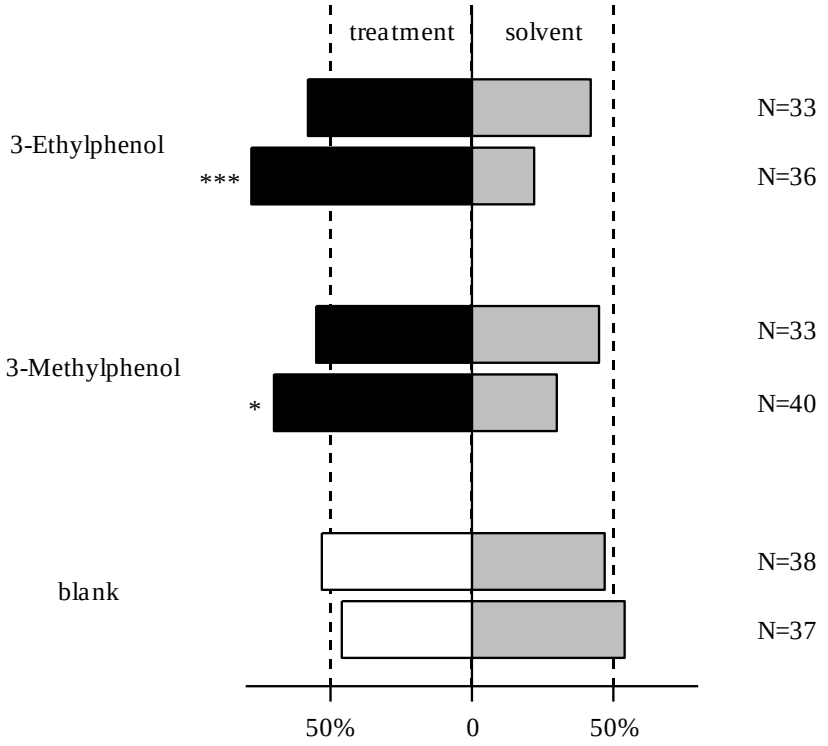

Fig. 3. Relative decisions of *Bolitophagus reticulatus* in dual-choice walking olfactometer preference tests. Males and females were given the choice between either 3-ethylphenol or 3-methylphenol dissolved in paraffin (left) and paraffin only (right). In a control experiment a blank pitfall was tested against the solvent paraffin. Numbers of tested individuals are given, asterisks designate p-levels of Chi-square tests, for details see text.

Discussion

Our results show that the glands of both sexes of *B. reticulatus* contain three pairs of either methyl- or ethylsubstituted benzoquinone, hydroquinone and phenol. All compounds are released when beetles are stimulated. With our behavioral assays we demonstrate that both species-specific alkylated phenols, albeit released by both sexes, considerably attract male beetles in a walking bioassay. Due to the fact that virtually no females are attracted to these compounds, and their decisiveness is significantly lower compared to male *B. reticulatus*, it is therefore possible to consider 3-ethylphenol and 3-methylphenol as sex pheromones, rather than aggregation pheromones. Electrophysiological measures revealed that sensitivities of males and females to these odorants diverge and corroborate our behavioural findings.

The composition of the pygidial defensive secretions of *Bolitophagus reticulatus* is consistent to previous findings in tenebrionid beetles. MBQ and EBQ are considered as ubiquitous, and many species secrete the corresponding hydroquinones MBQ and MHQ (Tschinkel, 1975b; Brown et al., 1992). The secretion of phenols is comparably rare among at least 300 chemically analyzed Tenebrionidae (Dettner, 1987; Francke & Dettner, 2005). Methyl- or ethyl-phenol occur most often in species of Tenebrioninae, some Diaperinae (Tschinkel, 1969,1975b; Dettner, 1987; Brown et al., 1992; Attygalle et al., 1991; Villaverde et al., 2007; Geiselhardt et al., 2009) and assumptively Coelometopinae (*Rhinandrus* spec., see Tschinkel, 1975b). All included tribes represent basal Tenebrionidae (Hunt et al., 2007; Löbl & Smetana, 2008; Matthews & Bouchard, 2008). An exception are some Australian Lagriinae (Brown et al., 1992), a subfamily where defensive glands may have evolved independently from all other Tenebrionidae (Dettner, 1987). However, the quinones are well known defense compounds effective against several natural enemies of beetles (Blum, 1981; Francke & Dettner, 2005). Regarding the phenols, 3-methylphenol has been proven to be an irritant repelling ants (Jefson et al., 1983); and is the primary agent of a carabid defensive secretion (Eisner et al., 1963). We did not examine whether the gland content of phenols of *B. reticulatus* in the low µg-range acts as an irritant itself. However, for vertebrates they have an unmistakable and unpleasant odour, qualifying for an appropriate alerting signal. Mice and ants are affected by the defense secretion of the tenebrionid beetle *Bolitotherus cornutus* (Conner et al., 1985), a near relative and also a member of the Bolitophagini. This nearctic equivalent of *B. reticulatus* is similar in many respects, inhabiting the same ecological niche (Matthewman & Pielou, 1971), sharing *F. fomentarius* as a host species (Liles, 1956),

showing similar defense behaviour (Conner et al., 1985) and most notably the same defense chemicals (Holliday et al., 2009). Both species have the aforementioned six main compounds in common. Compared to SPME-headspace analysis of stimulated *B. cornutus* (Holliday et al., 2009), the dichloromethane extracts of *B. reticulatus* glands yield a higher proportion of the alkylphenols and no p-benzoquinone. Comparisons across different methods should be drawn very cautiously. Direct headspace analysis of a dissected gland (see compound 1 & 8, fig. 1) shows yet other compounds being secreted by *B. reticulatus* not yet determined.

To our knowledge the presented study is the first report of a pheromone in Bolitophagini. Even for the closely related *B. cornutus* no pheromone activities were suggested, although the same two alkylphenols were part of its gland secretion (Holliday et al., 2009). Interestingly, 3-ethylphenol and 3-methylphenol have been recently described as male sex pheromone components in the desert tenebrionid beetle *Parastizopus armaticeps* (Geiselhardt et al., 2008). Males release the full pheromone blend well directed from sex specific aedeagal glands to attract females. In contrast to *B. reticulatus*, in this species males are releasing the sex pheromone, and this secretion does not originate from abdominal defensive glands. In turn, prothoracic glands of the tenebrionid *Zophobas rugipes* secrete considerable amounts of both alkyphenols when provoked, suggesting a defense function (Tschinkel, 1969). Therefore, to sum up, there are cases where either abdominal, or prothoracic or aedagal glands of Tenebrionidae secrete 3-ethylphenol and 3-methylphenol, acting as allomones or as male or female pheromones. Generally, pheromones affecting only one sex are regularly secreted by the other. However, in few cases sex pheromones are secreted by both sexes, and appear to have an influence only on the male. Peschke & Metzler (1982,1983) described defense compounds of the staphylinid beetle *Aleochara curtula* as a supplementary mating stimulant for males, even though they are produced by both sexes. Similar modes within complex pheromone mediated mating systems (Bryning et al., 2005) are discussed for the tenebrionid *Tenebrio molitor* (Tschinkel et al., 1967; August, 1971) and *Tribolium* species (Keville & Kannowski, 1975; Arnaud et al., 2002). At first it was hardly suspected that defense compounds could serve as a pheromone at all (Roth, 1962). Available studies suggest so far, that it is probably a matter of concentration whether secretions act as intraspecific aggregation signals or as irritants and therefore repell the receiver, probably even conspecifics (Peschke, 1983; Blum, 1996; Verheggen et al., 2007). Such a binary dose-response phenomenon of low-dose stimulation of a compound producing higher-dose inhibition is termed ‘hormesis’ (Hadley, 2003), and is probably typical of defense chemicals with secondary pheromonal

function. However, the dose we selected for walking assays is in the lower sensitivity range of the beetles antennae, between ED_{10} and ED_{50} (Table 2) and thus likely below irritant concentrations. We observed in our stock population, that male copulation attempts increased after beetles were slightly disturbed. Such a phenomenon has been described for the staphylinid beetle *Aleochara curtula* (Peschke, 1983), where disturbance in groups of mixed sex induced a male grasping response, in male groups even followed by occurrence of homosexual behaviour. Similar observations were made for *Zophobas rugipes*, *Tribolium castaneum* and *T. confusum* (Tschinkel et al., 1967; Keville & Kannowski, 1975). Thus, we assume that 3-ethylphenol and 3-methylphenol act over short distances, and that their major role is sexual excitation of the males. Additional research is required to determine if further compounds are involved concerning the mating behaviour of *B. reticulatus*. However, defensive secretions acting as sex pheromones seem to be rare (Peschke, 1983; Aldrich et al., 2000). It is more common for aggregation pheromones and has been discussed as an evolutionary origin of such intraspecific signals (Wertheim et al., 2004; Wertheim, 2005). To the best of our knowledge, secondary pheromonal functions of defense secretions have been shown among Tenebrionidae only for the basal Tenebrioninae (Tschinkel et al., 1967,1969; August, 1971; Dettner, 1987). Bolitophagini within, or at least close to the Tenebrioninae show many primitive features (Matthews & Bouchard, 2008), including morphology of their defense glands (Tschinkel, 1975c; Tschinkel & Doyen, 1980) and defense chemistry (Tschinkel, 1969,1975b; Brown et al., 1992). The secondary pheromonal function of the defensive secretion of *B. reticulatus*, thus might be added as a primitive functionality of a pheromone, derived from a defensive secretion as proposed by Wertheim et al. (2005) and highlights once again the multifunctionality of such compounds (Wertheim et al., 2004; Wertheim, 2005; Blum, 1996). However, only a few tenebrionids show intraspecific differences in their gland constituents (Dettner, 1987; Tschinkel, 1975b). Thus, in the case that secretions do not solely have a defensive function, pheromones could have been overlooked in search of sexual differences in their chemical composition. Concerning multifunctionality, we should also mention that phenols have strong antibiotic properties (Russell, 2002). *B. reticulatus* has gregarious phases, most notably in overwintering clutches (Nadvornaya & Nadvornyy, 1991). Antibiotics could be vital for group living *B. reticulatus* in detritus of tree trunks and fruiting bodies (Ruther et al., 2001a,b). A chemical defense favours group living, either to reach effective doses (Blum, 1996), or to instill distastefulness in predators, which in turn brings forth longevity (Pasteels et al., 1983). Aggregation is a central

mechanism within this interdepencies, and the sex pheromone or copulation stimulant we have shown here is probably only functional or at least effective in agglomerate populations. Such intraspecific cohesion may also effects larger spatial scales, since the metapopulation structure among *B. reticulatus* populations has been controversially discussed (Nilsson, 1997a,b; Rukke & Midtgaardt, 1998; Sverdrup-Thygeson & Midtgaardt, 1998; Hågvar, 1999; Knutsen et al., 2000; Rukke 2002), still lacking knowledge of mechanisms which influence genetic isolation, dispersal, colonisation or extinction. Chemical defense is widespread among fungivorous beetles (e.g. Tenebrionoidea, Erotylidae s.l., Scarabaeoidea or Staphylinidae) and aggregation has been hypothesised as the primary factor contributing to mycophagous beetle diversity (Pasteels et al., 1983; Wertheim et al., 2000; Persiani et al., 2010). A better understanding of intraspecific signals, their multifunctionality and their relation to host infochemicals might help then to bridge the gap from chemical to population ecology (Vet, 1999).

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CHAPTER 8

ODOUR SIGNALS RELEVANT TO BEETLES IN DEADWOOD HABITATS: RESULTS AND GENERAL CONCLUSIONS

Deadwood - diversity of odours and insects - *literature review Chapter 2*

Odours are specific signals of their originators and provide information of their identity and activity. Although as a principle of nature's parsimony, many compounds are released from a wide range of originators, due to shared fundamental metabolic or chemical processes (Bruce et al., 2005; Schoonhoven et al., 2005; Kües & Navarro-Gonzales, 2009). This results in typical chemical profiles not only of certain species but also of certain deadwood types or decay stages (Chapter 2 & 4). Many of the diverse insects in deadwood habitats are dependent on certain decay stages (Grove, 2002; Jonsell, 2008; Stokland et al., 2012) and are directed by volatile chemical profiles to find and identify suitable hosts (Bruce et al., 2005). Technically, organic trace analysis is capable of separation, analysis and identification of such odour profiles. It was possible to show, that using a bionics approach of odour analysis is a versatile technique to assess wood as a construction material and also wood quality (Chapter 2 & 3). It is also emphasised that analytical techniques can be used to understand chemical processes of wood decay, its progress and actual states but also to recognise involved organisms (see also Chapter 5). The loss of wood quality is, technically spoken, also the loss of integrity or defence in nature - the ultimate signal for diverse wood-consuming organisms to seize their chance (Chapter 2). The types of deadwood are manifold; they are explored, inhabited and also dramatically altered by countless insect-, or fungal species but also inconspicuous organisms (Stokland et al., 2012). In order to make use of the valuable information of volatile chemistry, it has been and will be indispensable in future research to distinguish between types of wood and deadwood, to describe the major chemical classes and functions of detectable odours according to attributable *originators*. Such a classification is proposed in Chapter 2: 1) Volatile organic compounds (VOC) of *living trees* as an unavoidable result of plant metabolisms, as mevalonate and lipoxygenase pathways producing terpenoids and green leaf volatiles (GLV), both infochemicals which maintain plant-insect communication; 2) *trunks and deadwood*, releasing defence compounds which are typical wood odours to the

human nose but also indicating the collapse of cell functions, a progressive oxidative regime breaking down chemical structures and depletion of constitutive defence pools; 3) VOCs of *wood and wooden products*, outgassings of liquid and vapourable cell contents; 4) volatile metabolites of *fungi* such as yeast fermentative products like alcohols, acids, aldehydes and esters, also metabolites of higher fungi, again lipoxygenase products of lipid oxidation as eight-carbon volatiles, but also terpenoids, and S-/N-containing compounds; 5) degradation products released from *fungus-infested wood* as a consequence of chemical and enzymatic digestion. It could be concluded that the sources of VOCs released from deadwood are manifold. They have to be analysed and assigned to the source, if those relevant to deadwood insects have to be ascertained. The systematic insect groups present on trees and the wood types mentioned above, forming the deadwood habitats together with all their associations have been reviewed and the information content of VOC for each examined.

Food specialisations, deadwood types and host odours – *experimental studies Chapter 3-7*

The understanding of insect ecology is essential, as well. Feeding habits of insects depend on digestive abilities and food source, often known or at least assumed for most species. Feeding habits in the deadwood context are subsumed under the generic term *saproxylophagy* (Grove, 2002). According to the major food sources, xylophagy, mycophagy, or hybrid forms are the dominating habits, as long as wood structures are recognisable, finally ending up in saprophagy. Such feeding habits have been contrasted in the single experimental studies (*xylophagy* - Chapter 3, *mycophagy* - Chapter 6, *xylomycetophagy* - Chapter 5) and conclusions focus on the general relevance of volatile organic compounds as infochemicals among these. It is emphasised here and with all experiments (except Chapter 3) that fungal participation in tree/wood-insect-interactions is not an exception, but rather the normal case. As long as fungal associations do not involve large, fleshy fruiting bodies or fungal breeding habitats of perennial sporophores of lignicolous species (Chapter 6 & 7), entomologists have been rarely able to identify fungal species in the field (Stokland et al., 2012). Endosymbiotic associations with fungi and bacteria but also ectosymbiotic associations with microscopic fungi as yeasts are much less apparent (Buchner, 1953; Rosa & Péter, 2006) and probably less known (Blackwell, 2010). Indeed, sophisticated ectosymbiotic associations of the ambrosia feeding habit and fungal agriculture have been described for bark beetles with Ascomycetes (Chapter 4 & 5) (Francke-Grosmann, 1967). However, most fungal and in particular bacterial associations are unexplained in deadwood insects, and because of evaluation difficulties,

probably often dismissed (Blackwell 2010,2011). As a consequence from the studies presented here, it is also suggested to accept the challenge to unravel such microbial associations in detail. With genetic tools nowadays available, like for example DNA-barcoding or enzymatic assays, a significant upturn in understanding insect interactions in the *Saproxylobios* could be generated (Blackwell, 2011). The results presented in the respective chapters lead to the assumption that fungal odours are critical signals in the insect-wood-fungus relationship. This is especially supported by a situation similar to that of plant odours, with odours released from almost all species and others more specific to certain taxa (Hansson et al., 1999; Bruce et al., 2005; Schoonhoven et al., 2005). It has been demonstrated that such general informational chemicals occur also in the deadwood environment with those present in most wooden substrates (Chapter 3) or the majority of fungal species (Chapter 5 & 6), but also others specific to certain fungal taxa or substrates (Chapter 5 & 3). A widespread occurrence of such infochemicals in insect-wood-fungal interactions can be postulated, since volatile release on which examined beetles adapted their olfactoric sense, is either unavoidable in the case of autoxidative processes and involvement in essential primary and secondary metabolisms of trees (Chapter 3) and fungi (Chapter 6), or - even more sophisticated - purposeful as a reciprocal adaptation or coevolution (Chapter 5). Among ubiquitous infochemicals that emerged from the subsequent studies, lipid oxidation products in the form of aliphatic aldehydes (Chapter 3) or fungal oxylipins as eight-carbon volatiles (analogue to six-carbon - green leaf volatiles of plants) (Chapter 6), but also products of primary and intermediary fungal metabolism (Chapter 5), are of particular importance. Not least, and in contrast, exceptional specificity has been demonstrated for a terpenoid released by symbiotic fungi (Chapter 5). A general conclusion is that, just as for herbivores (Bruce et al. 2005), host recognition in deadwood habitats occurs by using host (species) specific compounds, but probably much more by the use of ubiquitous host volatiles pertinent to many wood or fungal species. This should not be dismissed in future research. However, beyond the emphasis on fungal odours and general infochemicals, significant importance should also be accorded to intraspecific chemical signals affecting saproxylic insects (Chapter 7). Since group living and spatial aggregation is a characteristic feature and maintains diversity in resource-limited insect communities on patchy resources (Wertheim et al., 2005), unravelling intraspecific communication via infochemicals contributes a lot to the understanding of species diversity in the deadwood environment.

Analysis of wood odours - *Chapter 3*

Compared to plant volatile profiles, the volatile chemistry of deadwood is highly complex. Methods available to analytically separate such profiles are for example solvent extractions, pre-column separation, multidimensional separation with GC-GC couplings, but most acquire large sample amounts, or are expensive and technically delicate (Goodner et al., 2011). In order to analyse and reproduce natural volatile release rates exactly as they are perceived by insects, and to increase separational and discriminative power, new analytical instruments have been employed. The presented studies were also the suitability test of a recently developed GC-MS/EAD setup (Weissbecker et al., 2004), a separating gas chromatograph combining a mass spectrometer and a simultaneous electroantennographic detector. It was possible to demonstrate that the setup is capable of analysing highly complex chemical profiles of deadwood and allows assignment and identification of perceived compounds in a single step. This is a significant advancement over separately conducted GC-EAD and GC-MS analysis, the common practice in Chemical Ecology, and improves infochemical analysis in chemically complex environments.

Xylophagy: aldehydes as general infochemicals - *Chapter 3*

The xylophagous representative of the given studies (Chapter 3), the Old House Borer, *Hylotrupes bajulus*, preferably infests dead and parched conifers and is thus a major pest of roof structures, an excellent secondary habitat (Fettköther et al., 2000). Terpenes are the major compound class released by boards and wooden beams, especially from softwoods, and are known to attract the Old House Borer, but also many other woodboring insects (Fettköther et al., 2000; Allison et al., 2004). A high sensitivity of the Old House Borer to aliphatic aldehydes has been demonstrated here for the first time. Strongest responses to hexanal and nonanal also demonstrate a peculiar selectivity (Fig. 2). Such a specialised olfactory system with several receptors independently detecting certain aliphatic aldehydes is an evolutionary adaptation having its cause, and its generality in comparable deadwood habitats is proposed because of the following reasons.

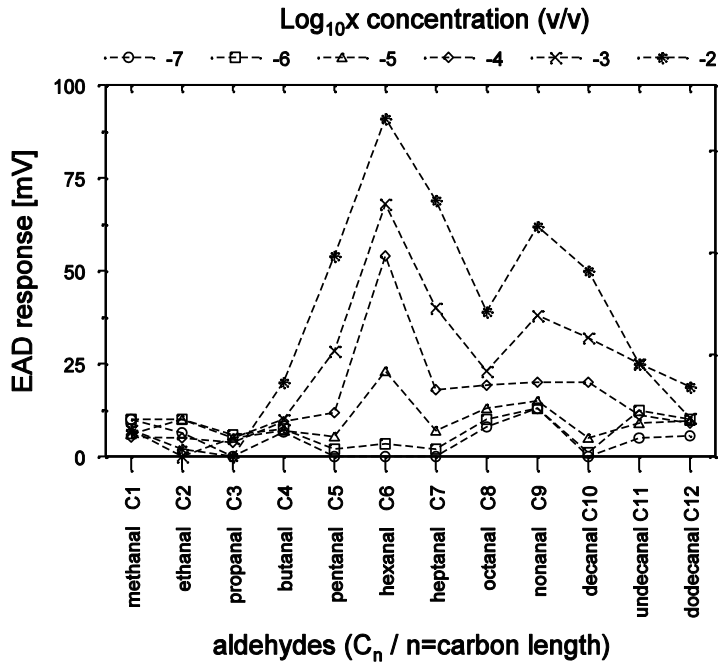

Fig.2: Dose-response relationship of antennae of the European house borer *Hylotrupes bajulus* for a homologous series of aliphatic aldehydes from C_1 to C_{12} demonstrating the sensitivity of the beetle olfactory system to aliphatic aldehydes (C_4 - C_{11}) and selectivity to hexanal and nonanal (C_6 & C_9). Doses are shown as $\log_{10}$ concentrations in paraffin (v/v). Antennal responses (EAD) are absolute amplified values [mV].

Aldehydes and ketones are autoxidative products of lipids in a series of radical reactions, finally resulting in aldehydes with chain lengths of mainly 6 to 10 carbons (Risholm-Sundman et al., 1998; Roffael, 2006). Lipids are major constituents of wooden cell walls. However, enzymatic pathways as plant lipoxygenase activity are a major source of certain aldehydes (Feussner & Wasternack, 2002). With the most abundant linoleic acid in wood as the substrate (Demirbaş, 1991; Fischer & Höll, 1992), lipoxygenases cleave it into major products such as hexanal and nonenal and further C6 and C9 fragments (Spiteller et al., 2001). Because of this dependency on key enzymes involved in many environmental responses, the aldehydes might carry information on the suitability of wood as the beetles breeding substrate. Finally the aldehydes dependent on several factors, such as lipoxygenase activity, which is upregulated under plant stress and plant defence (Rosahl, 1996), cell wall condition and depletion of lipids and free fatty acids and probably also fungal activity. Wood lipids have been shown to decrease during fungal decay, while fungal lipids increase during wood degradation (Gutiérrez et al., 2002). For Scots Pine (*Pinus sylvestris*), the preferred host tree of *H. bajulus*, the release of nonanal and decanal in comparison to hexanal, follows at least two different, but overlapping pathways (Wildt et al., 2003). In conclusion, independent

emission mechanisms seem to exist for individual aldehydes in deadwood or dying trees, which are plant or even fungal enzymatic activity, and probably autoxidation processes. Here, reactive oxygen species as O_2^- entering the cells, or H_2O_2, indicating oxidative burst and cell death (Lamb & Dixon, 1997), might play a role in a aldehyde formation in a dying tree. Phytophagous insects are particularly sensitive and discriminative to volatile lipoxygenase and subsequent enzymatic products, also known as green leaf volatiles (GLV) (Bruce et al., 2005), which include only hexanal among the aldehydes (Matsui, 2006). However, wood releases further aldehydes in great quantities, an observation also made in wood quality assessment and wood drying (Manninen et al., 2002), caused by oxidation mechanisms or other yet unknown processes. Interestingly, other beetles considered as occasionally saprophagous show a quite similar pattern. Grain beetles of the genus *Oryzaephilus* and *Sitophilus* are generally attracted by aliphatic aldehydes, strongest to hexanal and nonanal (Pierce et al., 1990; Germinara et al., 2008). Aldehydes are ubiquitous natural lipid oxidation products of cereals and are indicative of deteriorating grain stores (Magan & Evans, 2000; Loiseau et al., 2001). Utilisation of lipid oxidation products as host-finding kairomones by grain beetles has been proposed (Germinara et al., 2008). Such aldehydes might be an important class of infochemicals also for deadwood insects, especially woodborers damaging timber constructions.

Living trees and deadwood odours - *Chapter 4*

Ambrosia bark beetles exhibit complex fungal associations and are attributed to the xylomycetophagous feeding habit (Francke-Grosmann, 1967). They are secondary pests, exclusively infesting weakened or dead trees. Several species have recently developed pathogenicity, attack apparently healthy trees and threaten forest ecosystems (Kühnholz et al., 2001; Hulcr & Dunn, 2011). The examination of living trees (Chapter 4) surprisingly infested by *Trypodendron domesticum* - which is a species that might go astray - revealed no volatile compounds explaining such behaviour. Their existence is certainly not denied. Quite contrary, their original habitat has been analysed and it could be proposed that they were involved in attack on living trees. Volatile analysis of felled trunks, the ideal habitat of this beetle, revealed a spatially and chronologically distinctive pattern of volatile release. Localising the infochemicals perceived by the beetle, it has been shown that a trunk is not emitting attractive or repellent compounds as a whole. Beginning at bark scars or cut surfaces, small spots undergo a continuous change and expansion in quality and quantity of infochemicals. For

T. domesticum, they are mainly short chained alcohols, aldehydes, phenolics and a terpene oxidation product. It is generally known that ethanol is an attractant for many ambrosia beetles and also *T. domesticum* (Bouget et al., 2009). Ethanol is produced within stems of stressed trees (Kimmerer & Stringer, 1988), and therefore it might have happened, that a yet unknown stress caused a temporary release of this attractive volatile. Attraction of secondary bark beetles to ethanol is almost a paradigm (Bouget et al., 2009), but the results given here suggest that yet other volatiles contribute to the attraction of beetles on host search flight. Measured compounds indicate, that even a severely weakened tree might not be a good host, or at least does not release the volatiles characterising the ideal breeding tree. It is therefore unlikely that beetles switched to an aggressive behaviour, but host finding is a decision-making process based on fuzzy signals. Certain infochemicals might have attracted the beetles to a putatively dead or severely weakened tree, and reasons should be sought after on the plant side. However, it is known that fungal associates of ambrosia beetles, especially of the genus *Ceratocystis* also include aggressive and pathogenic strains that generate attractive volatiles (Hanssen, 1993), and endophytes might also contribute if revived in a weakened tree (Sieber, 2007).

Xylomycetophagy: wood versus fungal odours - *Chapter 5*

The xylomycetophagous feeding habit combines both worlds of plant and fungi, and also revealed the most unexpected results of this work and advancements in understanding insect-fungus-wood interactions. *Elateroides dermestoides*, formerly known as *Hylecoetus dermestoides* (Cuccodoro, 2002), is a representative of the enigmatic ship timber beetles (Lymexylidae). They are named after wreaking havocs in wood stocks of shipyards, in the old times when ships were made of oak beams (Ratzeburg, 1839). Ambrosia beetles are sub-social fungal farmers, however, fungal agriculture in non-social beetles has been rarely described, such as among Lymexyloidea (Francke-Grosmann, 1967) and recently for Erotylidae (Toki, 2012). *E. dermestoides* is notedly polyphagous, respective to host tree selection (Kurir, 1972). Larvae are assigned commonly to the xylomycetophagous feeding habit and once they have bored into the xylem, they establish fungal gardens, and in fact feed on their cultivar, the ascomycetous yeastlike fungus *Ascoidea hylecoeti* (Batra & Francke-Grosmann, 1961). Until now, no effective attractants are available to control this beetle, and existence of an aggregation pheromone has been denied or at least put into question (Klimetzek et al., 1986; Brattli et al., 1989). Almost thirty compounds eliciting signals in

E. dermestoides (Chapter 5) have been identified. Trap experiments rendered possible the identification of effective long-range attractants. They are constituents of an effective lure subsequently developed and lead to a patent application, thus they are not designated here. However, the results allowed further insights into basic principles of host finding in deadwood beetles. For phytophagous insects it is generally hypothesised that polyphagy could either be explained by perception of merely general volatiles present in all hosts, or by integration of many tree specific host signals (Bruce et al., 2005; Schoonhoven et al., 2005). It could be demonstrated that *E. dermestoides*, unlike many other bark beetles (Byers, 2004), perceives almost no tree specific volatiles, although many are emitted. This necessitates other signals to be present, which indicate suitable host trees. It could also be shown that the majority of volatiles perceived by *E. dermestoides* are typical products of fungal or bacterial metabolism. Many of them are produced by *Ascoidea hylecoeti*, the fungal symbiont of *E. dermestoides* (Chapter 5, Francke & Brümmer, 1978a,b), which is a surprising result. Particular compounds have been demonstrably dispersed through the bark. They evaporate from trunks as odour plumes in concentrations, which attracted female *E. dermestoides* in trap experiments already from long distance. This attraction is highly effective and gives rise to argue that aggregation pheromone systems known for bark beetles have to be extended by a further principle. It is known that there are comparable primitive pheromone systems, relying on products which are also autoxidatively produced (Francke & Dettner, 2005; Erbilgin et al., 2008), but the aggregation of *E. dermestoides* has a novel aspect: strong aggregation to a chemical exclusively released from the fungal symbiont. It fulfils the same function, in other beetle species achieved by nonsexual *de novo* produced pheromones: aggregation to conspecifics. Aggregation pheromones are obligatory in true bark beetles and very common in saproxylic insects (Byers, 2004; Francke & Dettner, 2005). It is also an adaptation to the scattered spatial nature of deadwood habitats (Wertheim et al., 2005). However, in case of *E. dermestoides* the pheromone definition can only be applied if an extended terminology is accepted, based on cost-benefit to the emitter and receiver and not the origin of compounds (Dicke & Sabelis, 1988), since the releaser is another species, the symbiotic fungus. The pheromone aggregation, common in many species, might be fully replaced here by the symbiont aggregation mechanism. Attempts to identify at least long range aggregation pheromones have not been successful in this case (Redlich et al., 1981; Brattli et al., 1998). However, unique sexual dimorphic sensory palp organs of Lymexylidae and also *E. dermestoides* suggest the existence of a sexual pheromone (Wheeler, 1986). Male

E. dermestoides are not attracted to the fungal odour, although they can perceive it (Holighaus, unpublished results), and copulation does not take place at the host, as it is common in bark beetles (Ratzeburg, 1839). There is also no brood-care or subsocial behaviour as in most other fungal gardeners (Francke-Grosmann, 1967). A possible scenario is that pheromones in bark beetles often mediate conspecific aggregation and mating function at the same time in the same place (Byers, 2004; Wertheim et al., 2005), while this is separated in *E. dermestoides*. Mating, triggered by a sex pheromone might be divided by open ground from host aggregation maintained by fungal volatiles. This might have basically evolved because larvae alone are the fungal gardeners in Lymexylidae, which is a major difference to all fungal gardeners across beetles, ants and termites (Mueller et al., 2005). Females on host search flights are attracted to trunks where fungal gardens of larvae are already established. Of course, this is also supported by the preference-performance hypothesis, which states that female beetles will evolve to oviposit on hosts on which their offspring fare best (Gripenberg et al., 2010). Larval performance might be simply enhanced due to the presence of conspecific larvae and their fungal gardens. Whether this is due to fungal properties remains unknown but the symbiont is very competitive against weed fungi as growth experiments have shown (Zimmermann, 1973). Invaders of fungal symbioses are a common threat of horizontally transmitted symbionts as in ants and termites (Mueller et al., 2005). However, the transfer from one generation to the next as in *E. dermestoides* via a female mycetangium, inoculating the eggs with the symbiont, called vertical transmission, is much less prone to invading fungi (Francke-Grosmann, 1967; Mueller et al., 2005). If fungal transmission fails, the offspring would benefit if females preferably laid eggs where conspecifics were already present. The fungal gardens have to be kept clean, often via parental brood care, as in scolytine ambrosia beetles, ants and termites (Mueller et al., 2005). In galleries of *E. dermestoides*, several fungal species also occur irregularly, and are considered as „weed“ fungi (Batra & Francke-Grosmann, 1961; Zimmermann, 1973). They are members of the ophiostomatoid genus *Ceratoystis* and can be partly assigned to trunk cohabitants, primarily ambrosia bark beetles of the genus *Trypodendron* and *Anisandrus/Taphrorychus* and also produce general yeast fermentative products (Chapter 5, Zimmermann, 1973). Their volatile products are similar but also species specific to a certain degree (Holighaus, unpublished data; Hanssen, 1993). This leads to the hypothesis that fungal odours might be signals essentially released to attract common beetle vectors for spreading or to bring together fungal mating types (Kües & Navarro-Gonzales, 2009). This only makes

sense as long as the fungi are sexual. Many vertically transmitted fungal symbionts of ambrosia beetles lost the sexual stage (Francke-Grosmann, 1967; Mueller et al., 2005). However, the symbiont of *E. dermestoides*, the ascomycetous yeast *Ascoidea hylecoeti* forms sexual ascospores, albeit only rarely observed in lab cultures and until now not in fungal galleries yet (Batra & Francke-Grosmann, 1961). Aware of the specificity of volatile signals of certain fungi and also the presence of general fungal metabolites, it can be concluded that *E. dermestoides* and fungal gardeners generally distinguish well between species of filamentous and yeastlike fungi, among these symbionts, commensals, or antagonists, if needed. General fungal volatiles are of great importance and informational value as infochemicals in the deadwood environment. It has however been shown, that (xylo-) mycetophagous beetles also exploit tree signals such as stress markers (Kühnholz et al., 2001; Allison et al., 2004; Francke & Dettner, 2005), and reproductive isolation among ambrosia beetles has been demonstrated to be maintained by host tree volatiles which explain tree specificity in certain species (Kühnholz et al., 2001). However, many fungal metabolites, products of lipid oxidation and also lignin and cellulose degradation products are perceived, to increase information on the suitability of the substrate (Chapter 4) and might direct future research.

Mycophagy: eight-carbon volatiles as general infochemicals - *Chapter 6*

The mycophagous feeding habit of *Bolitophagus reticulatus* is apparent (Chapter 6 & 7). The long lasting sporophores of the Tinder Fungus *Fomes fomentarius* provide a full breeding subhabitat within the deadwood habitat of dead beech trunks (Martikainen & Kaila, 2004). It is a model species of nature conservation studies evaluating landscape fragmentation effects on deadwood insects (Jonsson et al., 2005). Stimulated by those studies demonstrating attractiveness of fruiting bodies, the potential of eight-carbon volatiles, the major metabolic products of *F. fomentarius* and almost all higher fungi (Fäldt et al., 1999; Combet et al., 2006), could be demonstrated to control behaviour of *B. reticulatus* (Chapter 6). Their general importance in insect-fungal associations is suggested. The Black Tinder Fungus Beetle is either attracted (3-octanon), repelled (1-octen-3-ol) or does not react (3-octanol) to the most abundant eight-carbon volatiles. They are oxylipins, lipid oxidation products of primary fungal metabolism. Because of the key functions of oxylipins in regulation of fungal growth and development (Tsitsigiannis & Keller, 2007; Kües & Navarro-Gonzales, 2009), the general importance of eight-carbon volatiles is proposed as infochemicals for insects which encounter

fungi. It could be shown that their release changes with *F. fomentarius* fruiting body development or at least successional stages of beetle infestation. Abundance of *B. reticulatus* in different stages of sporophore development in the field (Jonsell et al., 2001), correlates with stage dependent release of attractant and repellent eight-carbon volatiles. It has also been possible to demonstrate that the olfactory system provides discrimination abilities for these chemically strikingly similar structures and this corroborates that such discrimination is probably common in insects faced with fungi. Oxylipins have multiple signalling functions on individual, species and kingdom level in many organisms and both, fungi and plants (Kües & Navarro-Gonzales, 2009; Feussner & Wasternack, 2002). Volatile oxylipins, the eight-carbon volatiles of fungi and the green leaf volatiles (GLV) of plants, have marked similarities in pathways and also function (Combet et al., 2006). We therefore discuss the potential of eight-carbon volatiles as general infochemicals for fungivorous insects, such as insect-inducible fungal defence signals, which have until now only been speculated about (Rohlfs & Churchill, 2011). 1-Octen-3-ol has a certain toxicity towards insects in general (Inamdar et al., 2010), and at least its repellence in high concentration for *B. reticulatus* has been demonstrated here. Once more, an important class of infochemicals in the deadwood context is highlighted: the ubiquitous eight-carbon volatiles in fungi. Some might be at least detected by probably almost all insects (Bruce et al., 2005) as a cue on moulded food, but fungivores might have evolved discriminative power as demonstrated here and benefit from their informational value on fungal host quality or even species. Such functions are well known concerning the green leaf odours, major volatile oxylipins in the plant kingdom (Bruce et al., 2005). Host finding, host defence and the chemoecology of fungivorous beetles is generally little understood, but apparent analogies between herbivory and fungivory could direct approaches for future research.

Intraspecific signals of deadwood colonisers - *Chapter 7*

The general occurrence of aggregation in deadwood insects as an adaptation to the scattered distribution of deadwood has already been emphasised (Wertheim et al., 2005). Fungi and especially fruiting bodies of Agaricomycetes are an ephemeral food source, whose appearance is spatially and seasonally unpredictable for an insect (Hanski, 1989). Aggregation is a successful strategy to overcome this trait and has been hypothesised as a major factor contributing to mycophagous beetle diversity (Wertheim et al., 2005). Aggregation calls enemies into action and chemical defence is thus widespread among fungivorous beetles

(Pasteels et al., 1983). It was possible to demonstrate (Chapter 7) that constituents of defensive beetle secretions are sexual pheromones or copulation stimulants. They act on short distances and would only be reasonable and effective in gregarious insects. *B. reticulatus* constitutes multitudinous metapopulations on single trunks (Jonsson et al., 2005). A chemical defence favours group living, either to reach effective doses, or to create distastefulness to predators (Pasteels et al., 1983; Wertheim et al., 2005). Aggregation or in other words connectivity may also effects larger spatial scales, since metapopulation dynamics are influenced by dispersal and colonisation (Jonsson et al., 2005). Those in turn are mediated by volatile signals as host cues and intraspecific signals among individuals within a beetle population. Consequently chemoecological studies as presented here might also support population ecology (Vet, 1999). The defensive secretions of *B. reticulatus* are also antibiotics and could be vital for group living in detritus of tree trunks and fruiting bodies, once again highlighting the multifunctionality of defence compounds (Blum, 1996). In addition to the odours relevant in deadwood habitats aforementioned, it has to be kept in mind that intraspecific communication among saproxylic insects is always present and considerably influencing insect-host interactions and behaviour.

As an overall conclusion it is necessary in deadwood colonising insects to reassess the concept of a host and subsequently also host-volatiles, as long as host terms for example a tree species. Deadwood is a concept that is only at first sight clear: conventional usage of the term is confined to dead and decaying trees or woody debris, while wood is the material that constitutes trees or trunks, or products such as timber (Thakeow et al., 2007). However, whenever the concept is in use, it is applied with tacit inclusion of the entire process of dying of a tree (Grove, 2002; McElhinny et al., 2005). This could imply the situation of a living tree with huge parts already dead. In other usages of the term, a tree has been felled and is considered to be dead, but cell functionality fades away slowly and some trees still survive, shoot and form new roots. The process from a living tree to dead wood is a continuum of changes (Stokland et al., 2012), also expressed in words such as aging, weakening, decaying, rotting, moulding, weathering, and seasoning to name only a few. Similarly, terms as putrescence, decomposition, deterioration, or degradation provide additional information as the period of time, changes in properties or originators of the dying process. While within the progress of wood decay, decay types and species assemblages fan out and diversify, it has been observed that host specificity (conventional usage of the term) generally declines in saproxylic insects (Hanski, 1989; Jonsell et al., 2001; Stokland et al., 2012). But it has to be

admitted, that “host volatiles” characterise certain decay states, species successions or assemblages instead of a single (e.g. tree) species. Of course the “host” is no biological species in this case, and might also not be satisfactorily described by common nomenclatures assigning saproxylic insects to tree compartments as phloem, cambium, xylem or vitality of the substrate (Bouget et al., 2005) – not to mention that fungal associations including yeast and probably also bacteria are to a great extend not examined (Blackwell, 2010,2011). However, it has been demonstrated with this work that among the originators of deadwood “host” volatiles are filamentous fungi, yeast fungi, bacteria, saproxylic and saprotrophic insects or arthropods, and not least degradation products of organic matter. They overlay and significantly influence or even constitute behaviour of beetles in deadwood habitats. These pioneer studies on the chemical ecology of a wide choice of deadwood colonising beetles brought out manifold novel results relevant to insect host location. Consequential trendsetting ideas on ecological functions of volatiles in insect-fungus-plant interactions could be developed that are waiting to be approached and hopefully approved in upcoming research.

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KURZFASSUNG

Duftsignale bedeutend für Käfer in Totholzhabitaten
- Duftstoffe, Wahrnehmung und Verhalten -

Ziel der vorliegenden Arbeit war die Untersuchung und Zuordnung flüchtiger chemischer Markersubstanzen, sogenannter Infochemikalien, die Insekt-Holz-Interaktionen beeinflussen. Das Totholz als Habitat schließt Insektenarten ein, die den größten Teil ihres Lebens in allen Arten von Holz aller Zerfallsstadien oder davon abhängigen Pilzen verbringen oder sich dort reproduzieren. Um dort flüchtige Verbindungen, deren Ursprung und deren Bedeutung als Infochemikalien zu identifizieren, wurde ein Gaschromatograph mit massenspektrometrisch-elektroantennographischer Detektion (GC-MS/EAD) vorgestellt. Das Hauptaugenmerk galt insbesondere Wirtsduftstoffen (Kairomone), aber auch Allomonen und Pheromonen ausgewählter Käferarten mit einer xylophagen, mykophagen oder xylomycetophagen Ernährungsweise.

Komplexe chemische Gemische aus Holz-, Totholz- und Pilzproben wurden analysiert. So wurden in trockenem Kiefernholz dreißig Substanzen identifiziert, von denen die Hälfte Antworten von Antennen des Hausbocks *Hylotrupes bajulus* im elektroantennographischen Aufbau auslösten. Aldehyde, wenngleich sie ubiquitär sind und nicht ausschließlich von Wirtsbaumarten abgegeben werden, wurden besonders sensitiv wahrgenommen. Sie werden als potentielle Infochemikalien vorgeschlagen und vervollständigen die Reihe bereits bekannter terpenoider Wirts-Kairomone für diese xylophage Art.

Die Analyse gefällter Buchenstämme zeigte, dass sich Volatile im zeitlichen Verlauf verändern. Das Absterben am Stamm verläuft räumlich sehr unterschiedlich. Für den Buchen-Nutzholzbborkenkäfer *Trypodendron domesticum*, ein xylomycetophager Ambrosia-Käfer, wurden potentielle Wirtskairomone, darunter verzweigte Alkohole, Aldehyde und phenolische Verbindungen, bestimmt, die eine integrierte Schädlingsbekämpfung und Risikoeinschätzung ermöglichen. *T. domesticum* als sekundärer Borkenkäfer befällt gelegentlich augenscheinlich gesunde Bäume und ist eine der Arten für die eine zunehmende Pathogenität innerhalb von Insekt-Pilzsymbiosen beobachtet wurde.

Antennen des ebenso xylomycetophagen Sägehörnigen Werftkäfers *Elateroides dermestoides*, aus der Gruppe der Lymexyloidea, wurden auf ihre Sensitivität für volatile Inhaltsstoffe verschiedener befallener Wirtsbaumarten, darunter Laub- und Nadelhölzer, untersucht. Die Baumarten unterschieden sich erheblich in den emittierten Stoffen, die Käfer jedoch nehmen fast ausschließlich solche Substanzen war, die in allen Baumarten vorkommen, was mit dem breiten Wirtsspektrum der Art übereinstimmt. Die meisten Substanzen entstammen dem Intermediärstoffwechsel von Pilzen, wie die Analyse von isolierten Pilzstämmen aus den Gängen des Käfers zeigte. Die Wahrnehmung dieser Pilze, aber auch ubiquitärer Hefen, viel mehr jedoch die Möglichkeit der Erkennung des Hauptsymbionten *A. hylecoeti*, der sich von allen anderen nachgewiesenen Arten durch eine artspezifische Substanz unterscheidet, wurde bislang nie so deutlich gezeigt. Wiederum wird jedoch die besondere Bedeutung ubiquitärer Stoffwechselprodukte, in diesem Falle pilzlichen Ursprungs, für die Wirtswahl hervorgehoben. Die Bedeutung von Infochemikalien in Symbiosen und Insekt-Pilz-Interaktionen ist allgemein nur wenig untersucht und ihr sollte zukünftig mehr Aufmerksamkeit geschenkt werden.

Fruchtkörper von Baumpilzen sind charakteristisch für Totholzhabitate und der Spezialisierungsgrad ihrer Besiedler lässt typischerweise mit zunehmendem Alter der Pilze nach. Flüchtige Bestandteile alternder Fruchtkörper des Zunderschwamms *Fomes fomentarius* wurden analysiert und quantitative Änderungen in der Abgabe von C8-Komponenten, pilzliche Oxylipine, treten auf. Diese lösen als Infochemikalien unterschiedliches Verhalten des auf *F. fomentarius* spezialisierten Tenebrioniden *Bolitophagus reticulatus* aus. Das verfügbare Wissen um C8-Oxylipine als Aromastoffe von Pilzen und deren Einfluss auf Insekten wird diskutiert. Eine möglicherweise ähnlich große Bedeutung für mykophage- und Totholz-Insekten, wie pflanzliche Oxylipine wie Grünblattalkohole für herbivore Insekten haben, wird zur Diskussion gestellt. Wirtserkennung mittels ubiquitärer Infochemikalien, im Gegensatz zur Erkennung über artspezifische Stoffe, wurde für Herbivore vielfach gezeigt. Die in dieser Arbeit gezeigten Parallelen legen nahe, diese Hypothese zur Wirtserkennung auch in den Lebensraum Totholz zu übertragen und zu schlussfolgern, dass dort Wirtserkennung ebenso mit Hilfe (Art-) spezifischer Wirtsduftstoffe geschieht, die Bedeutung ubiquitärer Duftstoffe die als generelle Stoffwechselprodukte vielen Holz- oder Pilztaxa gemeinsam sind, jedoch möglicherweise überwiegt.

Neben Wirtskairomonen wurden Abwehrsekrete von *B. reticulatus* untersucht. Eine Multifunktionalität einzelner Bestandteile die zusätzlich als Pheromon fungieren wurde gezeigt. Artspezifische phenolische Inhaltsstoffe, die von beiden Geschlechtern als Abwehrsekret abgegeben werden, zeigen eine anlockende Wirkung ausschließlich auf männliche Käfer, die diese auch sensitiver wahrnehmen. Sie stellen das erste nachgewiesene Pheromon innerhalb der Bolitophagini dar. Die Ergebnisse werden in den Kontext der Insekten-Chemoökologie, Totholzökologie und Symbioseforschung gestellt, und ihre Anwendbarkeit in integrierten Bekämpfungsmethoden und Holztechnologie diskutiert. Mit modernen analytischen Methoden und der Auseinandersetzung mit vergleichsweise gut untersuchten Insektenarten konnte gezeigt werden, dass die Chemische Ökologie eine vielversprechende Disziplin der Totholzforschung sein kann. Wertvolle Einsichten auch in generelle Prinzipien, die effizient zwischen den Reichen der Pflanzen, Pilze und Insekten moderieren, konnten so gewonnen werden.

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PUBLICATIONS

Publications designated with * are part of the PhD Thesis

Book chapters:

Thakeow, P., **Holighaus, G.** & Schütz, S. (2007).* Volatile organic compounds for wood assessment. In: Kües, U. (Ed.) Wood Production, Wood Technology, and Biotechnological Impacts. Publisher: Universitätsverlag Göttingen / Universitätsschriften, Göttingen, pp. 197-228.

Holighaus, G. & Schütz, S. (2006). Strategie der olfaktorischen Wirtsfindung von *Trypodendron domesticum* L.. Mitteilungen aus der Forschungsanstalt für Waldökologie und Forstwirtschaft Rheinland-Pfalz Nr. 59/06, 119-128.

Refereed publications:

v. Fragstein, M., **Holighaus, G.**, Tscharntke, T. & Schütz, S. (08/2011 invited for resubmission Journal of Chemical Ecology). Porous defense in a tritrophic system: Odour perception reflects specialisation degree of potter wasps (Hymenoptera: Eumeninae). 21 pp.

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Manuscripts prepared for submission:

Holighaus, G. & Schütz, S.* (manuscript). Electrophysiological responses of a deadwood beetle to trunk volatiles: No matter of tree species. 16 pp.

Holighaus, G., Weissbecker, B., v. Fragstein, M. & Schütz, S.* (manuscript). Eight-carbon volatiles are infochemicals for a specialist fungivore and characterise successional stages of basidiocarps during beetle colonisation. 28 pp.

Holighaus, G., Angeli, S., v. Fragstein, M. & Schütz, S.* (manuscript). Pheromonal function of defensive secretions in *Bolitophagus reticulatus* (Col., Tenebrionidae). 19 pp.