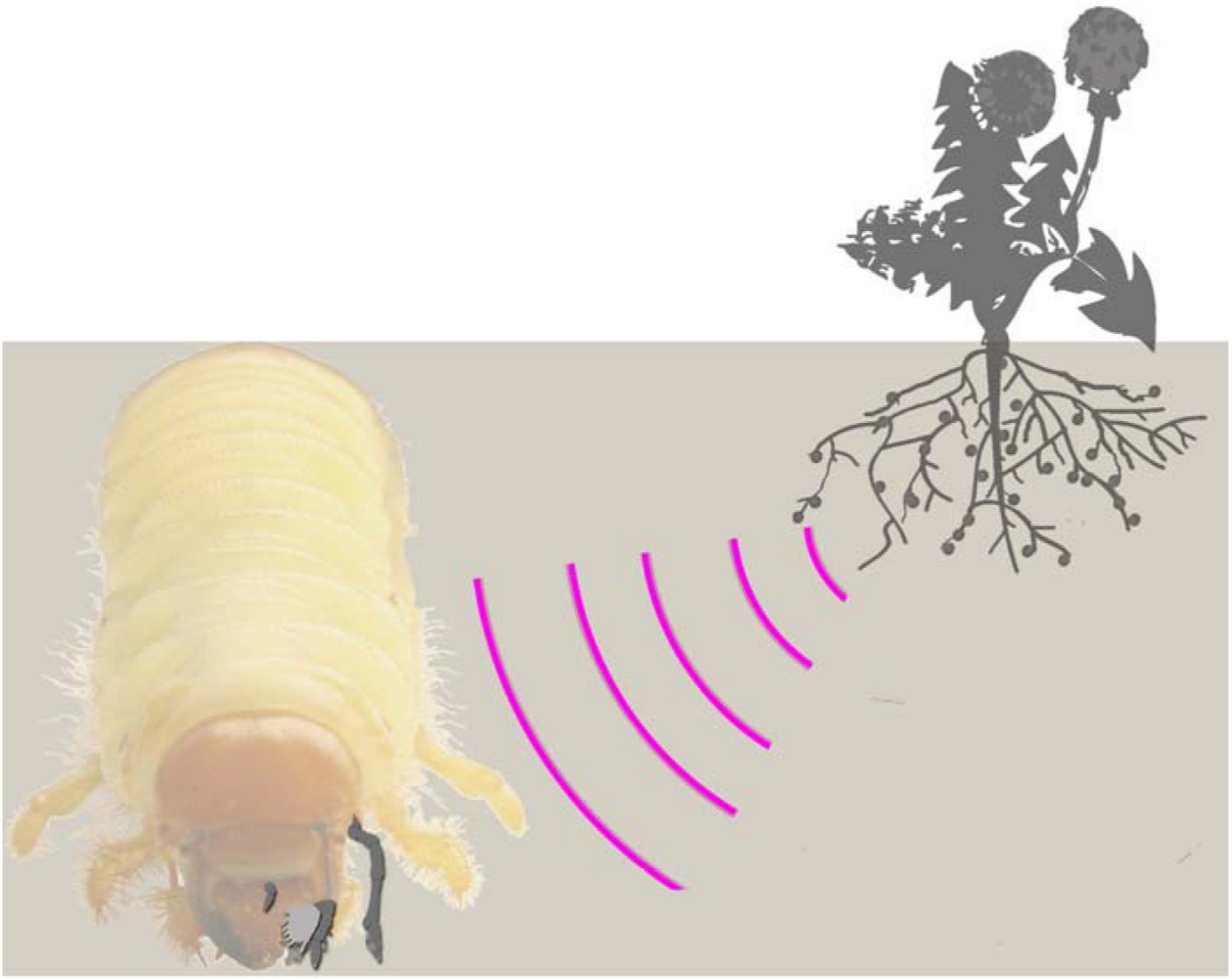
Elisabeth Johanna Eilers

Chemosensation and belowground host plant finding in *Melolontha melolontha* L. larvae



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Eilers E.J., Veit, D., Hansson B.S., Hilker M., Reinecke A. To find or not to find a tasty meal – a multifactorial model of ecological factors that render plant roots attractive to a rhizophagous insect. Manuscript.

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Eilers, E.J. Hansson B.S., Hilker M., Reinecke A. Behavioral responses of *M. melolontha* larvae to potential chemical cues. Manuscript.

References are listed at the end of each manuscript-based chapter. For the general introduction and discussion (chapters 1 and 5), a combined reference list is placed at the end of chapter 5.

Contents

List of figures

List of tables

Supplementary information figures and tables

Chapter 1

Introduction

1.1 Aims and scope

Below ground chemical orientation of rhizophagous insects is yet poorly understood. Using an economically important scarab beetle larva, *Melolontha melolontha* L. (Coleoptera, Scarabaeidae, Melolonthinae), and it's preferred host plant dandelion (*Taraxacum* sectio *ruderalia* Kirschner, Øllgaard et Stepanek), this PhD study aimed to elucidate the ecological, chemical and chemosensory factors that are important for orientation of soil-inhabiting insect larvae to host plant roots. The methods used were behavioral bioassays, chemical analyses of root exudates by GC-MS, electrophysiology and SEM and TEM of antennal morphology.

Special emphasis was paid to

- the elucidation of the sensory prerequisites the larvae possess for the detection of chemical cues present in the soil and exuded by host plant roots,
- understanding of the conditions that render plant roots attractive; hence, we analyzed the interactive effects of various biotic and abiotic factors on the attractiveness of plant roots to the rhizophagous larvae, and
- the identification of chemicals released by dandelion roots and physiologically or behaviorally active compounds derived from this as well as other plant rhizospheres.

From the chemosensory perspective, the chemosensory equipment on the mouthparts and antennae of the larvae was studied; furthermore, the electrophysiological responses of larvae to chemicals, of which some are released from dandelion roots, were tested (chapter 2). From an ecological point of view, this study focused especially on the impact of mycorrhizal

colonization, plant age, light intensity, and substrate type on the attractiveness of roots to *M. melolontha* larvae (chapter 3). From a chemical point of view, volatile and which non-volatile, water-soluble compounds that are exuded from dandelion roots were determined (chapter 4) and the behavioral response to electrophysiologically active compounds was tested (chapter 5).

1.2 The European cockchafer, *Melolontha melolontha*

The superfamily Scarabaeoidea is the largest subdivision of beetles, comprising approx. 30,000 species (Leal, 1998; Grebennikov and Scholtz, 2004; Smith, 2006; Smith *et al.*, 2006). Scarabaeid beetles are successful and ubiquitous pest insects, distributed in various habitats on every continent except Antarctica (Jackson and Klein, 2006).

European or common cockchafers, *Melolontha melolontha* L. (Coleoptera, Scarabaeidae, Melolonthinae) (Fig.1.1), have been studied extensively in the past and are also the subject of various poems, songs and children's books (e.g. Busch, 1960; Drews, 1991). Mass outbreaks of cockchafers have occurred approximately every 30-40 years in Switzerland and Germany during the last 200–300 years and were often accompanied by famine of the rural population (Keller, 1986; Jackson and Klein, 2006). Around the 1950s, numbers of cockchafers in Europe strongly declined, and mass occurrences of the European cockchafer and its sister species, the forest cockchafer *Melolontha hippocastani*, became scarce (Giannoulis *et al.*, 2011).

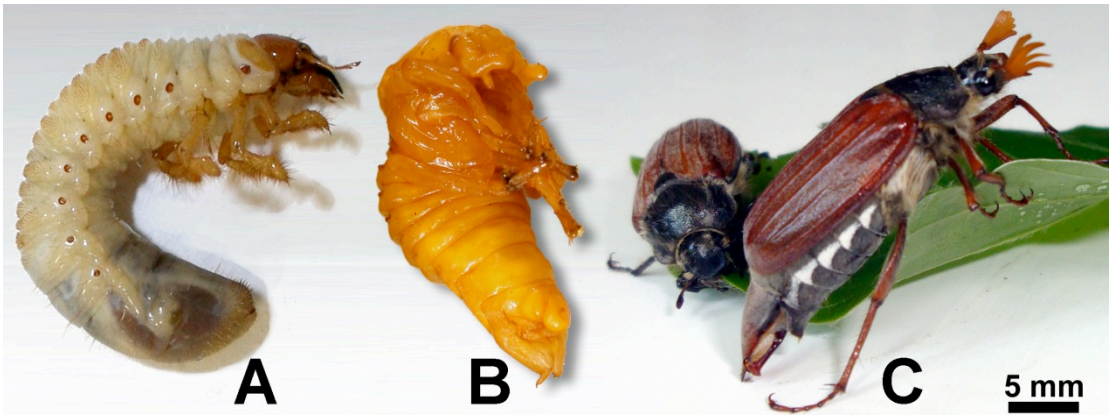


Figure 1. 1: left: soil-inhabiting third instar *M. melolontha* larva, center: pupa, right: adult male (in front) and female (behind) feeding on beech leaves.

However, since the 1980s, massive swarming events of both species have again been observed at regular intervals (Keller, 1986). The main ecological and agricultural damage caused by cockchafers, in fact, are ascribed to their root-feeding larvae (e.g. Keller, 1986; Ritcher, 1958; Harris, 1827). The soft, c-shaped body of the *M. melolontha* larva is equipped with three pairs of well-

developed legs and a sclerotized head capsule (Fig.1.1). This appearance is conserved among scarab larvae, which can perhaps be attributed to their similar, moist and dark habitats, namely soil, manure, wood, or decaying organic matter (Scholtz and Chown, 1995). *M. melolontha* larvae are polyphagous root-herbivores and feed for instance on roots or tubers of potato plants (Abendstein *et al.*, 2000), sugar beet, fruit trees, grapevine (Lüders, 1958), lettuce, strawberry, and various meadow grasses (Huiting *et al.*, 2006). However, dandelion (*Taraxacum* sectio *ruderalia* Kirschner, Øllgaard et Stepanek) is one of their most preferred host plants (Hauss and Schütte, 1977). This plant is an optimal food source for the larvae, both in terms of larval survival and weight gain (Hauss, 1975; Hauss and Schütte, 1976). Furthermore, the beetles oviposit preferentially in the vicinity of this plant (Hauss, 1975; Hauss and Schütte, 1977, 1978). Below ground orientation to host plants is widely restricted to chemical cues. As in other soil dwelling insects, *M. melolontha* larvae can pinpoint CO_2 sources belowground. In the larvae, attraction is even induced at weak gradients of 0.001%/cm (Hasler, 1986).

However, the chemical cues beyond CO_2 used by *M. melolontha* larvae for location of host plant roots have been largely unknown when starting this PhD study. Furthermore, knowledge of the factors shaping the release of these cues and the larval response to them has been scarce. Within this dissertation, dandelion is used as a model plant to study plant root exudation and belowground orientation of *M. melolontha* larvae (chapters 3, 4).

Larval development in *M. melolontha* is temperature-dependent and lasts 3-5 years (Schwerdtfeger, 1939; Brussaard *et al.*, 1997). In a 3-year lifecycle, the first larval instar molts after two months, the second instar molts after approx. 10 months and the third instar lives for approx. 1 year before it pupates (Fig. 1.2). The third instar larvae consume most root material and are thus most detrimental to their hosts. In addition to this quantitative difference in host damage, the different larval instars also show different host plant preferences (Ene, 1942; Hauss and Schütte, 1976). After their 8-week-long pupation period, the adults emerge and hibernate in soil before they start the

aboveground period of their life beginning of May. Past pupation, the cockchafer's ecological niche changes entirely: from a rhizophagous life in soil during their juvenile development to a phyllophagous life aboveground.

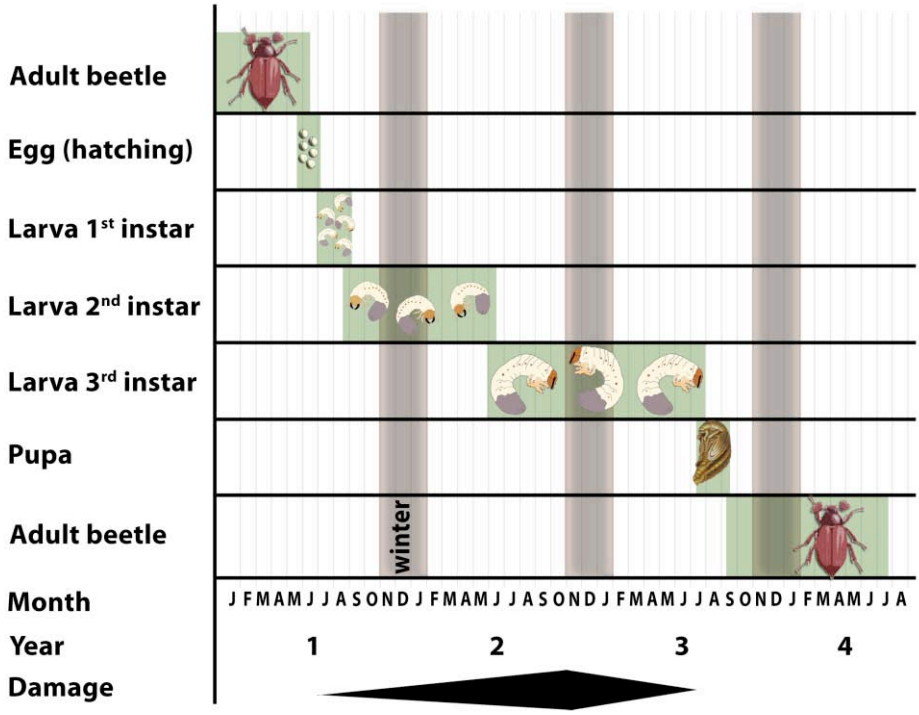


Figure 1. 2: Durations of egg, larval and pupal development and damage caused by respective stages. This example shows a 3-year life cycle of M. melolontha. Modified after (Oberhofer, 1979).

Adult *M. melolontha* are phyllophagous and feed e.g. on oak, beech, maple, and cherry leaves (Schneider, 1980). Several pheromones and plant derived semiochemicals have been shown to play a major role in host finding, aggregation, and mating of adult cockchafers (e.g. Ruther et al., 2000; Reinecke et al., 2002a; Ruther et al., 2002; Reinecke et al., 2005). For instance, toluquinone acts as a pheromone for *M. melolontha* (Reinecke *et al.*, 2002a) and its efficacy is strongly enhanced by **g**reen **l**eaf **v**olatiles (GLV), which are induced by leaf-feeding females. Furthermore, GLV alcohols alone are attractive to males and elicit approach behavior of males to a plant infested by feeding females (Reinecke *et al.*, 2002b).

1.3 Olfaction and taste in Scarabaeidae

Chemical senses (olfaction and taste) enable insects to locate food sources, mating partners, or oviposition sites, and are thus vitally important for the survival and reproduction of insects. By virtue of the outstanding data provided on the relationship of structure and function of insect chemosensory organs, so-called sensilla, e.g. reviewed by Altner (1977; Altner and Loftus, 1985), the function of a sensillum can be determined with near certainty, based on its external morphology and internal structures. For instance, minute wall pores penetrating the sensillum cuticle and leading to pore tubules or secretion-filled cuticular channels indicate olfactory function. In contrast, gustatory sensilla each possess only one terminal pore, which leads to a channel filled with a viscous substance (Altner and Loftus, 1985). Additional information is obtained from the innervation pattern of the sensilla. Dendrites forming a lamellated termination by flattening and folding (Altner and Loftus, 1985), indicate hygro-/thermoreception, whereas concentric lamellations have been described in osmosensory sensilla (Zacharuk and Shields, 1991).

Although Scarabaeid larvae mostly inhabit rather cryptic surroundings, the number of published studies on their sensory structures (e.g. Jepson, 1937; Ratcliffe and Chalumeau, 1980; Baker, 2005; Alekseev *et al*., 2006) is similar to that of their winged conspecifics. Among the larvae, several joint morphological characteristics have been found, which are used for the classification of the beetles into families and subfamilies (Grebennikov and Scholtz, 2004). Most prominent among them are the placoid structures on the apical antennal segment also referred to as sensory spots or pore plates. Unlike sensilla placoidea in most adult cockchafers, larval pore plates are smooth, but show a similar innervation (cp. review by Meinecke, 1975 and citations therein). The number of pore plates on the apical antennal segment of Scarabaeid larvae may range from one (Moron, 1991; Grebennikov and Scholtz, 2004) to more than a dozen (Jameson and Moron, 2001) in xylophagous larvae, but is always three on the apical antennal segment of rhizophagous scarabs: one at the dorsal and two at the ventral side (Habeck, 1963; Micó et al., 2001b, cp. chapter 2 and Fig.6.1 in general discussion).

This suggests that these structures play a major role in host location and are adapted to each respective lifestyle. In addition to the pore plates, bristles, pegs or setae have been described on antennae and mouthparts of larval Carabidae (Giglio *et al.*, 2003), Chrysomelidae (Mitchell *et al.*, 1979; Sen and Mitchell, 1987; Farazmand and Chaika, 2008), and Scarabaeidae as well (Jepson, 1937; Baker, 2005; Alekseev *et al.*, 2006).

However, ultrastructural data allowing for the annotation of structures to functions are sparse in both, the pore plates and the peg-like structures. Furthermore, studies combining morphological and electrophysiological data on the sensory organs of soil-inhabiting insects are scarce. A comprehensive study on morphology and electrophysiology of *M. melolontha* larval sensory organs is presented in chapter 2 of this dissertation.

Ultrastructural research on antennae and mouthparts of adult Scarabaeidae has been a popular field of research, resulting in a multitude of available descriptions and illustrations of sensory organs, established within the past decades (e.g. by Meinecke, 1975; Allsopp, 1990; Hansson *et al.*, 1999; Kim and Leal, 2000; Romero-Lopez *et al.*, 2004; Tanaka *et al.*, 2006).

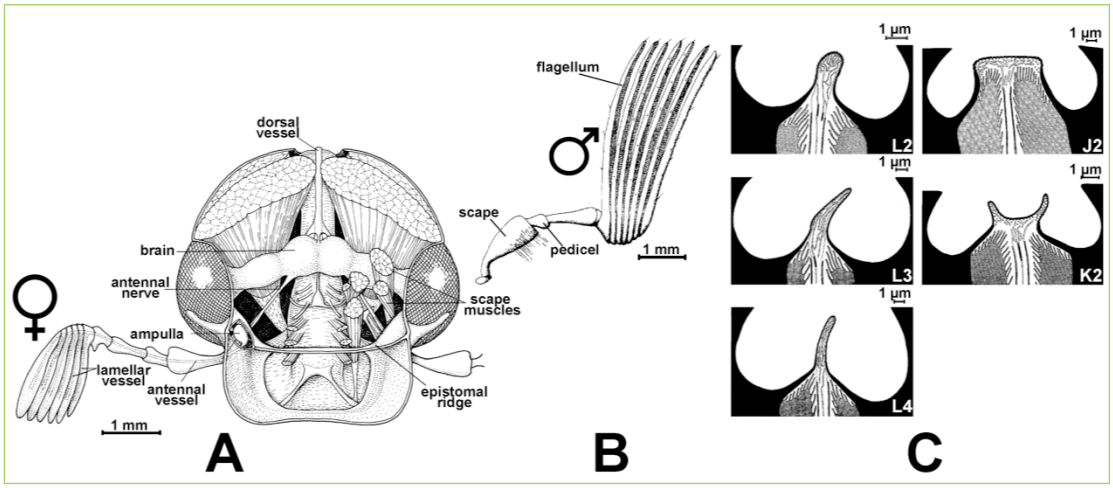


Figure 1. 3: Antennae and sensilla in adult *M. melolontha*. Opened head of a female cockchafer, tracheal system removed (A); male antennal club (B); and sensilla types (C). Modified after Pass (1980) and Meinecke (1975).

The primary olfactory organ in adult *M. Melolontha* is comprised by a pair of antennae, which possess an enlarged surface due to lamellation of the distal

flagellomere (Hansson, 1999). Antennal morphology in *M. melolontha* underlies a conspicuous sexual dimorphism: the males possess 7 large distal limbs and the female antennae bear only 6 smaller limbs (Meinecke, 1975, Fig.1.3A &B). The large antennal surface is irregularly covered with approx. 30 000 sensilla, most of which are located on the ventral surfaces of the lamellae (Meinecke, 1975). The placoid sensilla have been shown to have an olfactory function and respond to pheromones (Kim and Leal, 2000). They are located in minute cavities and the perforated inner surface may be cone- (type L), socket- (type J) or bowl-shaped (type K, Fig.1.3C).

1.4 Beyond CO_2: Chemical cues in root-feeding insects

A tremendous number of insects spend at least some portion of their lives in the soil, where they contribute to vital ecosystem services, such as decomposition of organic matter, nutrient cycling and bioturbation (Brussaard *et al.*, 1997). Fascination for soil ecological research has increased among various international research groups within the past decade (e.g. De Deyn *et al.*, 2003; Sugden *et al.*, 2004; Rasmann *et al.*, 2005).

Soil is a multiphase system, consisting of mineral and organic (living and decaying) matter, water (vapor, soil solution) and air (Bardgett, 2005). In contrast to aerial insect life, belowground insect life is associated with darkness and a dense matrix, which decelerates locomotion and renders it more costly. Spatial and temporal dynamics of developing gradients of potential chemical cues in soil, gaseous or volatile (and soluble), are strongly influenced by the matrix and thus differ drastically from gradients of the same substances in air (Jones, 2009). Yet, soil chemical research raises difficulties: the major constraints are low concentrations of signaling compounds, high decomposition rates, and high levels of extracted non-target compounds from the surrounding matrix (Tang and Young, 1982; Kuzyakov and Domanski, 2000).

Carbon dioxide (CO_2) is commonly described as a chemical cue for root-feeding insects. However, the gas is ubiquitous in the rhizosphere and therefore a very unspecific cue. Indeed, *M. melolontha* larvae themselves release CO_2, which is utilized by entomopathogenic nematodes to locate them (Gaugler, 1991). Besides CO_2, plant roots may release a bouquet of many different airborne and water-soluble compounds (e.g. Szmigielska *et al.*, 1995; Gransee and Wittenmayer, 2000; Fan *et al.*, 2001 and reviews by Rovira, 1969; Bertin *et al.*, 2003; Bais *et al*., 2006; Dennis *et al.*, 2010, Fig.1.4). The composition of these so-called root exudates varies according to plant species, cultivar, age, and biotic and abiotic stresses (Bertin *et al.*, 2003). The light intensity, which the shoot is exposed to, strongly influences the release

of CO_2 and sugar from the roots e.g. (Kasperbauer and Hunt, 1992; Nagel *et al.*, 2006; Kuzyakov and Gavrichkova, 2010).

Figure 1. 4: Organic compounds released from plant roots. After the review of Dennis *et al.* 2010.

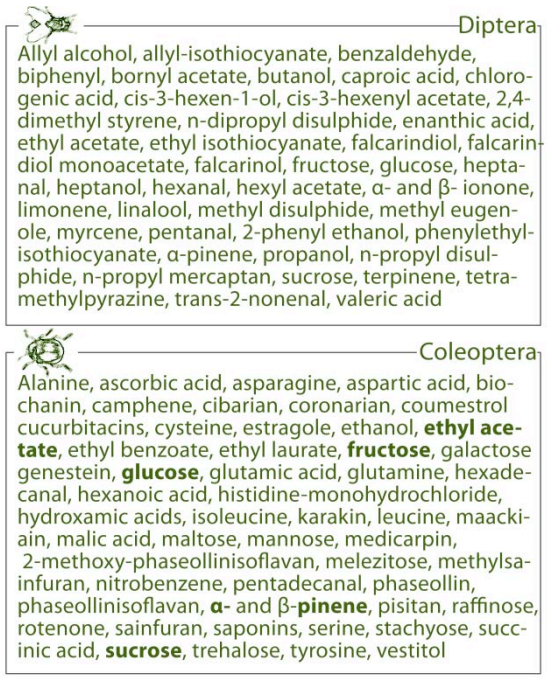


Figure 1. 5: Volatile and soluble chemical cues for root feeding dipterans and coleopterans. Consistencies of both insect orders are highlighted in bold. After the review by Johnson and Gregory, 2006.

Furthermore, a plant´s association with **a**rbuscular **m**ycorrhizal **f**ungi (AMF) may alter plant traits, such as root architecture (Pozo *et al.*, 2010; Wu *et al.*, 2011), defense signaling pathways (Kuzyakov and Gavrichkova, 2010; Oddsdottir *et al.*, 2010) and root exudation (Bodker *et al.*, 1998; Vannette and Hunter, 2009). In fact, the majority of vascular plant families are associated to AMF, which are members of the Glomeromycota (e.g. Fitter and Moyersoen, 1996; Schüssler *et al.*, 2001). The fungi are obligate symbionts, incapable of completing their life cycle without the plant symbiont (Read and Perez-Moreno, 2003; Akiyama and Hayashi, 2006). In return, the fungus provides hardly accessible nutrients to its host, particularly phosphorus (e.g. Dodd *et al.*, 1987; Habte and Manjunath, 1987) and nitrogen (Veresoglou *et al.*, 2012). The additional nutrient support may assist the plant to compensate herbivore feeding damage. Furthermore, AMF have been described as being

antagonistic to root-feeders in dandelion (Gange *et al.*, 1994). The increased resistance to root-feeders mediated by AMF may be caused by AMF-induced changes in plant defence signaling pathways (Vannette and Hunter, 2009). Besides feeding-induced mechanisms, AMF may preemptively circumvent root herbivory by masking attractive root-derived substances or by excreting repellent substances. However, little is known of how the fungus alters root exudation. The symbiosis is however not in all cases mutualistic: the costs for the plant may as well outweigh the benefits provided by the fungus (Bethlenfalvay *et al.*, 1982; Hendrix *et al.*, 1992; Johnson *et al.*, 1997; Brundrett, 2004).

Little is known on how the intraspecific plasticity in root exudation, mediated by AMF symbiosis, plant age and abiotic factors, affects root herbivore host location. Chapter 4 of this dissertation deals with intraspecific chemical diversity of root exudation and in chapter 3, the impact thereof on *M. melolontha* host finding is addressed.

So-called **g**reen **l**eaf **v**olatiles (GLVs), such as C6-alcohols, -esters, -aldehydes, but also terpenoids are commonly used by herbivorous insects for aboveground host detection (cp. chapter 1.2). These types of compounds are also known as chemical cues used by soil-dwelling insects for host location (Fig.1.5, Johnson and Gregory, 2006). Specific information may be conveyed over long distances belowground *via* root-derived **v**olatile **o**rganic **c**ompounds (VOCs)(Wall *et al.*, 2006). Hence, these compounds may be particularly suitable chemical cues for root herbivores (Kai, 2008; Wenke *et al.*, 2010). According to Johnson and Nielsen (2012), short-chain-alcohols, -aldehydes and -esters are commonly observed to initiate attraction behavior, whereas long-chain hydrocarbons are mostly repellent to belowground insects.

Due to the humid surroundings of root herbivores, semi-volatile or nonvolatile, water-soluble components (e.g. sugars, amino and organic acids) may also function as chemical host cues. However, these components have largely been neglected in chemo-ecological research on root herbivores.

In search for potential chemical cues for host location by *M. melolontha* larvae, we tested electrophysiological (chapter 2) and behavioral (chapter 5) larval responses to a large set of volatile and nonvolatile substances; we chose substances that were detected in root exudates of dandelion (chapter 4) or other plants (Dennis *et al.*, 2010).

Chapter 2

Sensing the underground – ultrastructure and function of sensory organs in root-feeding *Melolontha melolontha* (Coleoptera: Scarabaeinae) larvae

2.1 Abstract

Introduction:

Below ground orientation in insects relies mainly on olfaction and taste. The economic impact of plant root feeding scarab beetle larvae gave rise to numerous phylogenetic and ecological studies. Detailed knowledge of the sensory capacities of these larvae is nevertheless lacking. Here, we present an atlas of the sensory organs on larval head appendages of *Melolontha melolontha*. Our ultrastructural and electrophysiological investigations allow annotation of functions to various sensory structures.

Results:

Three out of 17 ascertained sensillum types have olfactory, and 7 gustatory function. These sensillum types are unevenly distributed between antennae and palps. The most prominent chemosensory organs are antennal pore plates that in total are innervated by approximately one thousand olfactory sensory neurons grouped into functional units of three-to-four. In contrast, only two olfactory sensory neurons innervate one sensillum basiconicum on each of the palps. Gustatory sensilla chaetica dominate the apices of all head appendages, while only the palps bear thermo-/hygroreceptors. Electrophysiological responses to CO_2, an attractant for many root feeders, are exclusively observed in the antennae. Out of 54 relevant volatile compounds, various alcohols, acids, amines, esters, aldehydes, ketones and monoterpenes elicit responses in antennae and palps. All head appendages are characterized by distinct olfactory response profiles that are even enantiomer specific for some compounds.

Conclusions:

Chemosensory capacities in *M. melolontha* larvae are as highly developed as in many adult insects. We interpret the functional sensory units underneath the antennal pore plates as cryptic sensilla placodea and suggest that these perceive a broad range of secondary plant metabolites together with CO_2. Responses to olfactory stimulation of the labial and maxillary palps indicate that typical contact chemo-sensilla have a dual gustatory and olfactory function.

2.2 Introduction

Below ground interactions between plants and herbivores have gained increased attention over the past years (e.g. Van Dam, 2009; Watts *et al.*, 2011). Little knowledge is, however, available regarding how rhizophagous herbivores such as scarab beetle larvae locate host roots. In the absence of visual stimuli, olfaction and taste are the core sensory modalities to orient below ground.

Sensory head appendages of rhizophagous larvae have been described from phylogenetic perspectives in scarab beetles (Grebennikov and Scholtz, 2004), or studied from a functional point of view in other model or pest organisms (Albert *et al.*, 1993; Keil, 1996; Gerber and Stocker, 2007). Despite the presence of many pest species within the superfamily Scarabaeoidea, comprising 25,000-to-35,000 species in 8-to-14 families (Leal, 1998; Grebennikov and Scholtz, 2004; Smith, 2006; Smith *et al.*, 2006), a comprehensive inventory of sensory organs on larval antennae, labial, and maxillary palps is missing. The scarcity of data becomes even more apparent when searching for studies linking morphology, physiology and ecology of insect larvae in general and scarab larvae in particular. Out of ten basic sensillum types that have been described in adult insects, all except the sensilla squamiformia have also been found in insect larvae (Zacharuk and Shields, 1991).

Common sensory structures among coleopteran and lepidopteran larvae are placoid structures on apical antennal segments (Giglio *et al.*, 2008) and maxillary palps (Faucheux, 1995), digitiform organs on maxillary palps (e.g. Guse and Honomichl, 1980; Honomichl and Guse, 1981) and peg-like sensilla on apices of antennae and palps (e.g. Doane and Klingler, 1978; Shields, 2009; cp. Table S1). The conjoint occurrence in various coleopteran and lepidopteran taxa of a broad geographical range, diverse habitats and diets, indicates a highly conserved nature of these structures. Between taxa they differ in number, size and location on head appendages. Pore plates on larval antennae with hypothesized olfactory function have been demonstrated in Carabidae (Giglio *et al.*, 2008). Similar structures have olfactory function in adult scarab (Kim and Leal, 2000) and Dynastidae beetles (Renou *et al.*, 1998). Furthermore, peg-like sensilla of unknown function have been identified on apices of antennae (Jepson, 1937), labial and maxillary palps (Alekseev *et al.*, 2006) in Scarabaeidae and other Coleoptera (see Table S1). Finally, digitiform organs have been described in larvae of Carabidae (Giglio *et al.*, 2003), Chrysomelidae (Farazmand and Chaika, 2008), Curculionidae (Dosdall and McFarlane, 2004) and Elateridae (Doane and Klingler, 1978) (Table S1). The putative function of the digitiform organ is hygro-/thermo- (Guse and Honomichl, 1980), or CO_2-reception (Honomichl and Guse, 1981), and in lepidopteran larvae mechanoreception (Devitt and Smith, 1982). Most reference studies, however, are purely descriptive, lacking physiological and ultrastructural investigations of sensory function and organization.

In our model insect *Melolontha melolontha* (L., 1758) (Scarabaeidae: Melolonthinae) it has been postulated that CO_2 is the only or main attractant below ground (Klingler, 1957; Hasler, 1986). However, CO_2 receptive structures have not been identified yet (Hasler, 1986). In wireworm larvae, CO_2 receptive sensilla are suspected to be located on both palpal apices (Doane and Klingler, 1978). Recent findings indicate that other compounds of the rhizosphere contribute to orientation or interact with CO_2 in *Melolontha* larvae (Reinecke *et al.*, 2008). In addition to CO_2, which is an ubiquitous gas produced by respiring roots and other soil (micro)organisms, plant roots

release various water-soluble substances into the soil, such as sugars, organic acids, and amino acids (reviews by Dakora and Phillips, 2002; Bertin *et al.*, 2003; Dennis *et al.*, 2010, and references therein). Gustatory discrimination of food sources based on sugars, amino acids, and isoflavonoids has been shown in rhizophagous clover root weevil and scarab larvae (Wensler and Dudzinski, 1972; Johnson *et al.*, 2005). Volatile compounds are secreted in comparatively limited diversity and quantity from plant roots (Steeghs *et al.*, 2004). However, these compounds act as attractants or deterrents in various scarab larvae (Osborne and Boyd, 1974; Sutherland and Hillier, 1974).

In this study we establish a comprehensive inventory of the sensory structures on the head appendages of *M. melolontha* larvae by scanning and transmission electron microscopy. We present a functional interpretation of our ultrastructural data and an assessment of olfactory responses to compounds known to be behaviorally active in soil dwelling insects, to be present in the rhizosphere of potential host plants, or to structural analogues of these compounds.

2.3 Materials and Methods

2.3.1 Animals

Melolontha melolontha (Linnaeus, 1758) larvae were collected in May 2010 and April 2011 from a meadow in Hessenthal, Bavaria, Germany (49°93’ N, 9°26’O). Larvae were kept individually in small pots filled with clay substrate (Klasmann-Deilmann GmbH, Geeste, Germany) in a climate chamber under dark conditions at 14°C and 70% humidity and fed carrots *ad libitum*. Third instar larvae were used in all experiments. Collected second instar larvae were allowed to molt before use.

2.3.2 Scanning electron microscopy (SEM)

After rinsing with tap water, five specimens were decapitated, and the heads were submerged in Sörensen phosphate buffer (0.1M, pH 7.2, 1.8% sucrose) before antennae, labial and maxillary palps were removed and placed in 50%

ethanol. Samples were dehydrated in ethanol (EtOH) (60, 70, 80% each step twice for 10 minutes; 90%, 96% for 10 minutes each, absolute EtOH overnight). Subsequently, the specimens were critical point-dried using a BAL-TEC CPD 030, mounted on aluminium stubs with adhesive film, and sputter coated with gold on a BAL-TEC SCD005 prior examination with a LEO 1450 VP scanning electron microscope.

2.3.3 Transmission electron microscopy (TEM)

After rinsing and decapitation, antennae and palps from two specimens were dissected in chilled Sörensen phosphate buffer (0.1M, pH 7.2, 1,8 % sucrose). Antennae were divided into antennal tip, rest of the first apical segment, and proximal half of post-apical segment; tips of palps and cylinder of apical segment of maxillary palps were dissected. Samples were fixed for 12 hours with 2.5% glutaraldehyde in phosphate buffer at 4°C. Samples were rinsed two times for 10 minutes with chilled phosphate buffer before the buffer was replaced by 2% phosphate buffered osmium tetroxide and stored for 12 hours at 4°C. After rinsing three times for 10 minutes with chilled phosphate buffer, the samples were dehydrated in EtOH in ascending concentrations (see above). Dehydrated samples were embedded in Spurr´s resin (Spurr, 1969) and polymerized for 24 hours at 65°C. Ultrathin sections (50-70 nm) were cut with a Diatome diamond knife (Ultra 35°) on a Reichert Ultracut microtome. Sections were collected on Pioloform®-coated mesh or single slot copper grids and examined without additional staining with a Zeiss CEM 902A (with a TVIPS FastScan camera) or a JEOL JEM 1011 (with a Olympus Megaview III camera) transmission electron microscope.

2.3.4 Electroantennograms (EAGs) and electropalpograms (EPGs)

White grubs were fixed in slit silicone tubes (ca. 2cm long ID=6mm) supported by a bandage of Parafilm (Pechiney Plastic Packaging), leaving the head appendages and hindmost part of the abdomen free. Microcapillary glass electrodes (tip OD ca. 3µm) with Ringer´s solution and a silver wire provided electrical contact via a Syntech 10x universal probe pre-amplifier (Ockenfels SYNTECH GmbH, Kirchzarten, Germany) to a Syntech IDAC 4 D/A-

converter. The indifferent electrode was inserted into the larval abdomen (Rumbo, 1988). The measuring electrode was positioned laterally on the apical segment of the respective head appendage without penetration of the cuticle. Sensilla on the tip of all appendages, antennal pore plates and the digitiform organ on the maxillary palps were not covered by the electrode. Signals were recorded on a PC using Syntech EAG Software with 50/60Hz electric noise suppression and the 'EAG-filter' activated. Larval head appendages were subjected to a constant flow (1 L/min) of charcoal-filtered, humidified air through a stainless steel tube (ID 8mm) terminating 1cm from the preparation and with two lateral holes (2 mm ID) about 1 cm upstream of the outlet. Stimuli were applied by puffing charcoal filtered air (500mL/min, 0.5 s per stimulus, 4mL in total) through Pasteur-pipettes with odor-laden round filter paper discs (12 mm diameter) into one of the holes. To ensure constant total flow and humidity (65% r.h., 24°C) prior and during stimulation the alternating second flow channel of a Syntech CS-05 Stimulus Controller was connected via identical tubing and pipettes to the other hole. The humidity was measured at the tube outlet prior recordings, using a digital thermo-hygrometer (P330, Tematec GmbH, Hennef, Germany). Compounds to be tested were applied to the filter paper discs in 10µl solvent, which was allowed to evaporate for 1min prior to stimulation. CO_2 was applied by filling a Pasteur-pipette (2.5mL) with 20% CO_2, through which 4mL air were pushed during stimulation and mixed with 8mL air from the constant flow, resulting in a final concentration of approximately 4%. When water was used as solvent or stimulus, humidity increased to 66% r.h. at 24°C during stimulation. Prior to stimulation and after each 10th puff, the vigor of the preparation was tested. Breath was used as positive control, as contained humidity and CO_2 elicited reliable responses. The average lifetime of the preparations exceeded 10hrs, but preparations were discarded earlier if the response to breath fell below 80% of the initial response, or after all compounds had been tested three times. All stimuli (see below) were applied in randomized order. In total, every compound was tested 15 times on 6 animals (1-3 replicates per animal). For statistical analysis and graphical display responses to the respective solvent were subtracted from responses to the stimuli. Statistical analysis and

graphical charts were implemented using the statistic program “R” (R version 2.9.2, 2009-08-24). Square-root transformed data showed optimally reduced variance heterogeneity among treatments and were successfully tested for normality (“R” command “qqnorm”). Transformed data of EAG/EPG responses were compared separately for each head appendage to responses to the respective solvent, applying Welch two sample t-tests.

2.3.5 Test compounds and solvents

Stimulants are selected by their known ecological function in soil-inhabiting insects or occurrence in plant root exudates, and by their structure and carbon chain length in order to test a broad range of chemically diverse compounds. Exponents given for each chemical indicate the purchasing source mentioned below.

(1) Compounds attractive or repellent to other soil-dwelling insects. Gases: CO_2, terpenoids: (+)-camphene[1)], (-)-camphene[2)], β-elemene[3)], α- and β-farnesene (mix of isomeres)[1)], (-)-limonene[1)], (+)-limonene[2)], linalool (mix of enantiomers)[1)], β-myrcene[2)], α-pinene[2)], β-pinene[1)], α-terpinene[2)], α-phellandrene[1)]; others: benzaldehyde[1)], ethanol[4)], ethyl acetate[1)], hexyl acetate[1)] (Johnson and Gregory, 2006);

(2) Compounds commonly released by plant roots. Acids: acetic acid[1)], citric acid[1)], formic acid[2)], fumaric acid[5)], lactic acid[2)], malic acid[4)], oxalic acid[1)], propionic acid[1)] (Bertin *et al.*, 2003; Dennis *et al.*, 2010); terpenoids: β-caryophyllene[2)], eucalyptol (1,8-cineol)[2)], γ-terpinene[1)] (Kai *et al.*, 2009).

(3) Other compounds: acetone[1)], 2-butanone[1)], butyl acetate[2)], butylamine[2)], α-(-)-cedrene[2)], cinnamaldehyde (cinnamal)[1)], hexylamine[2)], hydrochloric acid[4)], ethanal[1)], methanal[4)], methanol[4)], methyl acetate[2)], 1-nonanol[2)], 1-octanol[1)], pentylamine[2)], propanal[1)], 1-propanol[4)], propyl acetate[2)], propylamine[1)], pyridine[6)], sulcatone[1)].

Acids were dissolved in dichloromethane (DCM) supplemented by 20% water to increase solubility (the applied concentration was 1µg/µl). Remaining compounds other than CO_2 were diluted in DCM[4)] and used at 1µg/µl. DCM supplemented by 20% water (for acids), clean filter paper (for undiluted compounds and CO_2) and DCM (for remaining compounds) served as

controls, respectively. Components were purchased from [1)] Sigma Aldrich (Steinheim, Germany), [2)] Fluka (Steinheim, Germany), [3)] Aapin Chemicals Limites (Abingdon, Oxfordshire, UK), [4)] Roth (Karlsruhe, Germany), [5)] Alfa Aesar (Karlsruhe, Germany) and [6)] Merck (Darmstadt, Germany).

2.4 Results

2.4.1 Scanning and transmission electron microscopy (SEM& TEM)

The antennae of third instar *M. melolontha* consist of five, and the maxillary and labial palps consist of four and three segments, respectively (length ratio antenna: maxillary palp: labial palp = 20:7:4) (Fig. 2.1B). While all appendages possess conspicuous crown-like apical sensillum fields (Figs. 2.1C-H), only antennae and maxillary palps carry additional subapical sensilla, namely three pore plates on the sides of the apical antennal segment (Figs. 2.1C, E), small peg-like sensilla and one pore plate on a cuticular protrusion of the post-apical antennal segment and the digitiform organ on maxillary palps. In total, 17 different sensory organs are present on larval head appendages (see Table 2.1).

2.4.2 Digitiform organ and adjacent sensilla (S13 and S14)

The digitiform organ, which is presumably a hygro-thermoreceptor (cp. Table 2.1), is located on the lateral surface of the apical segment of the larval maxillary palps (Fig. 2.1E). It consists of a long, distally slightly tapering seta, which lays flat in a longish oval recess of the palpal cuticle (Fig. 2.2A, B). Its blunt tip points towards the apex of the maxillary palp, and it consists of a massive, poreless cuticle (tip: Fig. 2.2E) with longitudinal channels (shaft: Figs. 2.2F, G). Subapically, the shaft lumen contains a thin dendritic sheath without dendritic structures (Fig. 2.2F). However, numerous flat dendritic profiles, partly arranged in a lamellar way, reside inside the dendritic sheath in the center of the organ (Figs. 2.2G, H). Their number is reduced towards the base of the shaft, but several profiles gain in diameter (Figs. 2.2I-L). Finally, only one ensheathed outer dendritic segment is present in the socket (Figs. 2.2M, N). All profiles in the shaft are branches of this single dendrite. The

socket does not show flexible cuticle areas (Fig. 2.2M). The integument of the recess does not show any structures, indicative of additional sensory functions (Fig. 2.2O).

Adjacent to the digitiform organ on the maxillary palps two further sensillum types are identified: the S13 and S14 sensillum (Fig. 2.1E; 2.2A). The S13 sensillum is characterized by a small, flat cuticular depression (Fig. 2.2B). A single, ensheathed outer dendritic segment, terminating in a large tubular body is projecting through a cuticular channel towards the cuticular depression (Figs. 2.2B-D). The dendritic sheath terminates in the matrix of the endocuticle (Fig. 2.2C). The putative S14 sensilla represent a group of bent cuticular furrows above the digitiform organ (Figs. 2.1E; 2.2A). Their ultrastructure is not known.

2.4.3 Pore plates

Four olfactory pore plates are present on the antennae of third instar *M. melolontha* larvae. Three with average diameters of about 100-200µm are located on the ventral and dorsal surfaces of the apical segment (Figs. 2.1C; 2.3A) and one of about (25µm in width and 70µm in length) is located on the inner surface of the lateral protrusion of the subapical segment (Fig. 2.3B). Sections show that the cuticle of a pore plate is almost six times thinner than adjacent parts of the antennal cuticle (Fig. 2.3D). A large tissue cluster of distinct cell types is present below each pore plate (Fig. 2.3E). Among them are numerous sensorial units, each consisting of a bundle of ensheathed dendrites, projecting radially towards the thin pore plate cuticle (Fig. 2.3F). These more or less columnar sensory units are surrounded and separated by support cells (Figs. 2.3E, F). The average distance between adjacent dendrite bundles is about 15µm. Over all, the sensory units exhibit a clear stratified arrangement (Figs. 2.3E, F-Q). Numerous fine pores penetrate the pore plate cuticle (Figs. 2.3F, G). Contrary to the name of this structure, surface openings appear to be sparse (Fig. 2.3C). However, dozens of fine pores are detectable in each ultrathin section (Fig. 2.3F). Electron-dense tubules are associated with the pores (Fig. 2.3G). These tubules extend into the space

below the cuticle (Fig. 2.3H), where they get in close vicinity to hundreds of fine dendritic branches with diameters between 0.1-0.3µm (Fig. 2.3I). They form a flat, lenticular receptor area directly below a fraction of the pore plate (Fig. 2.3F, I). These fine branches originate from medium sized dendritic branches with diameters between 0.5-1µm (Figs. 2.3I, J). The latter branch off from the inflated apices of three-to-four outer dendritic segments (Fig. 2.3F, J-M). A thin dendritic sheath surrounds the outer dendritic segments, which do not have ciliary character (Figs. 2.3F, K-M). The sheath is formed in the region where the outer dendritic segments project as short cilia out of the inner dendritic segments (Fig. 2.3F, N). The inner dendritic segments originate from clusters of sensory cell bodies that are located close to the central hemolymphatic space of the antennae at the base of the tissue cluster below the pore plate (Figs. 2.3O-Q). The aforementioned wider openings (Fig. 2.3C) are often plugged or sealed (Fig. 2.4A, B). The pore plate cuticle is penetrated by hour-glass-like ducts, in which the sealing material can often be seen in the outer part (Fig. 2.4C). The ducts are relatively narrow in the middle of the cuticle (Fig. 2.4D). Outer dendritic segments project into the inner openings of the ducts (Figs. 2.4E-F). Often cuticular threads protrude from the duct lumen between the outer dendritic segments (Figs. 2.4F, G). Close to these ducts, punctual contacts between support cells and the pore plate cuticle occur (Fig. 2.4H). Electron-dense material and mitochondria are concentrated in such contact areas (Fig. 2.4I) and desmosome-like densities are visible at the apical membrane (Fig. 2.4J).

2.4.4 Peg-like sensilla on apical fields and in antennal protrusion

The S1 sensillum is the longest sensillum of the antennae and occurs in the centre of the apical antennal sensilla field (Fig. 2.5A). The single, slightly bent seta has a bifurcated tip (Figs. 2.1D; 2.5A). A spongiform lumen is observed in the distal two thirds of its slender, poreless shaft (Figs. 2.5B-E). The cuticle becomes denser in the basal third (Fig. 2.5F). Shortly above the socket, two ensheathed outer dendritic segments occur inside the narrow lumen (Fig. 2.5G). Following the innervation deeper does not reveal numeric changes in the dendritic pattern (Figs. 2.5H-K). The socket itself bears areas with flexible

cuticle (Figs. 2.5I, J). A tormogen cell with a well-developed apical microvilli border surrounds the dendrite below the socket (Fig. 2.5K).

The S2 sensillum, which is the only sensillum type in common of all three head appendages (Figs. 2.1D, F, H), is relatively small. It occurs once in the centre of the apical sensillum field of the antennae (Figs. 2.1D; 2.5A), 14 times in the periphery of the apical sensillum field of the maxillary (Fig. 2.1F) and 7 times in the periphery of the apical sensillum field of the labial palps (Fig. 2.1H). Preparation artifacts may account for minor variations of tips and surfaces among appendages (Figs. 2.5L-N). However, all sensilla classified as S2 are of similar size and have a single terminal pore (Figs. 2.5L-N) and a poreless shaft (Figs. 2.5O, T) in common. The terminal pore is formed by densely arranged finger-like cuticular protrusions (Fig. 2.5P). Slit-like interspaces between the protrusions (Fig. 2.5Q) merge in the central lumen of the sensillum (Fig. 2.5R). Thin cuticular threads project from the protrusions into the lumen (Figs. 2.5P, R). A subapical transverse section reveals a thin dendritic sheath without dendritic segments inside the narrow lumen (Figs. 2.5O, S). Further basally, the lumen becomes wider and the dendritic sheath houses dendritic segments (Figs. 2.5O, T). Four-to-five outer dendritic segments innervate the S2 sensillum (Figs. 2.5U-W). One of them always terminates as a tubular body (Figs. 2.5U, V), attached to flexible cuticle areas of the socket (Fig. 2.5V). An individual dendritic sheath always separates the single tubular body-forming dendrite from the other ones (Figs. 2.5U-W), which proceed into the shaft (Fig. 2.5O, V).

The S3 sensillum is relatively large and exclusively located in the centre of the antennal apex (Figs. 2.1D; 2.5A). Its blunt tip bears a laterally shifted subterminal pore (Fig. 2.6A). The poreless shaft consists of thick cuticle (Figs. 2.6B, C). Apically, the narrow lumen houses a dendritic sheath (Fig. 2.6B). Further basally, the lumen is wider and the dendritic sheath follows a lateral fold in the shaft cuticle (Fig. 2.6C). Four-to-five outer dendritic segments innervate this sensillum (Figs. 2.6D-F). Some dendritic segments show

numerous microtubules. Interestingly, very small profiles containing microtubules can be observed as well (Fig. 2.6F).

The thick, cylindrical S4 sensillum also occurs exclusively on the antenna and constitutes the peripheral ring of the apical sensilla field (Fig. 2.1D). Pore structures are hardly visible (Fig. 2.6G) but a small terminal pore becomes visible in sections (Fig. 2.6H). Similar to the S2 sensillum, the S4 terminal pore possesses small finger-like protrusions and thin cuticular threads (inset in Fig. 2.6H). Furthermore, the subapical dendritic sheath and outer dendritic segments are present in the narrow lumen of the massive, poreless shaft (Figs. 2.6I, J). Close above the socket, the dendritic sheath is paralleled by two cuticular lamellae (Fig. 2.6K). Four-to-five outer dendritic segments extend into the shaft lumen (inset in Fig. 2.6K). Inside the socket, the dendritic sheath is attached to flexible cuticle parts (Fig. 2.6L). A dense tubular body is formed by one separated dendrite (Figs. 2.6L, M). Protrusions of the sheath producing thecogen cell can be observed below the socket (Fig. 2.6N).

Sensillum types S5, S6 and S7 are located inside the lateral protrusion of the subapical antennal segment, close to the pore plate (Fig. 2.3B). S5 is a small, egg-shaped sensillum in a comparatively large circular socket (Fig. 2.7A). It possesses a terminal pore surrounded by fine finger-like protrusions, similar to those of the S2 sensillum. The S6 sensillum is also very small, but its socket is inconspicuous (Fig. 2.7C). The ultrastructure of S5 and S6 is not yet known. The S7 sensillum is a short, slightly bent, conical seta with a slightly sculptured surface (Fig. 2.7D). Sections reveal the porous shaft structure of this sensillum (Fig. 2.6E). At least three outer dendritic segments could be observed inside the shaft lumen (Fig. 2.6E).

S8 is the largest sensillum type on maxillary and labial palps. It occurs twice in the central area of the apical sensillum fields of both appendages (Figs. 2.1F, H). A peculiar tip, formed by a nearly spherical apex, which is surrounded by a cuticular collar, characterizes this sensillum (Fig. 2.8A). Besides a relatively inconspicuous terminal pore surrounded by finger-like protrusions (Figs. 2.8A-

C), these sensilla show conspicuous cuticular openings (Fig. 2.8A), which turn out to be only deep cuticular folds (Fig. 2.8D, E). The terminal pore merges into the central lumen of the shaft where a dendritic sheath is present (Figs. 2.8E, F). Subapically, membranous structures are present inside the sheath (Figs. 2.8G-J). The thick shaft cuticle is poreless (Figs. 2.6G, J). Longitudinal channels are present in the cuticle (Fig. 2.8J). Basally, the sheath is guided by a cuticular lamella (Figs. 2.8I, J). Four-to-five outer dendritic segments innervate the S8 sensillum (Fig. 2.8K). Although one of them contains densely arranged microtubules, clear evidence for the presence of a mechanosensory tubular body is lacking.

The second largest sensillum on both palps belongs to type S9. Although structurally very similar among the appendages, two morphological variations of this type could be identified: the large S9a and smaller S9b. Five-to-six S9a occur on the maxillary palp (Fig. 2.1F) and two on the labial palp (Fig. 2.1H). The smaller S9b occurs twice on the labial palp (Fig. 2.1H). All S9 possess terminal pores, often inconspicuous (Fig. 2.8L), but sometimes a little elevated (Fig. 2.8M). The terminal pores bear finger-like protrusions, but unlike in the previously described sensilla, interspaces between these protrusions contain electron-dense tubules (Fig. 2.8N). The tubules from the terminal pore extend into the central lumen (Figs. 2.8O, P). A peculiar feature of these sensilla is the presence of additional channels with tubules that originate laterally of the terminal pore and project radially from the tip towards the central lumen of the shaft (Figs. 2.8Q, R). A dendritic sheath is attached to a cuticular lamella in the lumen (Fig. 2.8R). Outer dendritic segments are present in the basal part of the sensillum (Fig. 2.8S). Up to seven dendrites, one in a separate sheath innervate S9 sensilla (Fig. 2.8T). Comparing this with the findings for sensilla S2 (see Figs. 2.5U, W) and S4 (see Fig. 2.6M) indicates that the separated dendrite may contain a tubular body in its tip.

The small, conical S10 sensillum is present once on maxillary and once on labial palps (Figs. 2.1F, H). The sensillum surface is slightly sculptured (Fig. 2.9A), but sections reveal the porous character of the shaft (Figs. 2.9B-E).

Many fine dendritic profiles occur in the apical part of the sensillum (Fig. 2.9B). They get in close contact with pore tubules (Figs. 2.9C, E). Large, most likely inflated dendritic profiles can be seen in the basal portion of the shaft (Fig. 2.9D). The fine profiles branch off from these large profiles (Fig. 2.9F). The sensillum socket comprises 18 outer dendritic segments, joined by loose fibers of a dendritic sheath (Figs. 2.9G, H). At deeper section levels the number of dendrites decreases to two and the sheath becomes more and more condensed (Figs. 2.9 I-K).

S11 is another small, conical sensillum of the maxillary palps (Fig. 2.1E). The tip is usually fine (Fig. 2.10A) but occasionally blunt types are found (Fig. 2.10B). The shaft lacks any sensory structures (Figs. 2.10C, D). It merges in a socket with large areas of flexible cuticle (Fig. 2.10E). A single, large tubular body, surrounded by a thick dendritic sheath, is attached to the flexible cuticle of the socket (Fig. 2.10F). Below the socket, the corresponding dendritic sheath shows conspicuous radial folds, which divide the periphery of the outer dendritic segment (Fig. 2.10G) and vary in quantity at different section levels (inset in Fig. 2.10G).

The S12 sensillum is a single small, slender sensillum, which is exclusively located in the apical sensillum field of the labial palps (Fig. 2.1H). It is poreless and bears a subterminal (Fig. 2.10H) or terminal pore (Fig. 2.10I). The lumen contains lamellated dendritic branches surrounded by a thin sheath (Fig. 2.10J). Further basally, only two dendritic branches are visible (Fig. 2.10K). The sensillum is innervated by one ensheathed outer dendritic segment, which enters the shaft before it starts to lamellate (Figs. 2.10L, M).

2.4.5 Electroantennograms (EAG) and Electropalpograms (EPG)

Electrophysiological recordings are conducted on 3rd instar *M. melolontha* larvae antennae (EAG), maxillary and labial palps (EPG). The mean responses to tested compounds range from 0.03mV ± 0.01mV (solvent DCM) to 8.81 ± 0.86mV (water) in labial palps, 0.11± 0.02mV (empty pipette) to 6.89 ± 1.7mV (water) in maxillary palps, and 0.06mV ± 0.016mV (solvent DCM) to 5.7 ± 1.05mV (ethanol) in antennae. Overall, significant responses were found

for compounds from all tested chemical classes, i.e. alcohols, aldehydes, ketones (Fig. 2.11A), CO_2 and water (Fig. 2.11B), acids, amines, esters (Fig. 2.11C) and terpenoids (Fig. 2.11D). However, none of the head appendages respond to the tested sesquiterpenes β-elemene, β-caryophyllene, α-cedrene, and farnesene isomeres. In contrast, all appendages respond to propanal, acetone, methanal, propyl- butyl- and hexylamine, and α-terpinene. Both palps respond to changes in humidity, to butylamine and ethanal. Antennae and labial palps both respond to 1-butanol, 1-propanol, citric and acetic acid, methyl ethyl and propyl acetate, γ-terpinene and α-pinene. Interestingly, β-pinene elicits no response on these appendages. Moreover, (+)-camphene and α-terpinene elicit responses in maxillary palps, whereas no significant response is observed to (-)-camphene and γ-terpinene. This observation indicates enantio- and isomer-specific perception of these compounds. Other than antennae and maxillary palps, the labial palps respond significantly to cinnamaldehyde, benzaldehyde, linalool and (-)-camphene. Responses to CO_2 (4%), 2-butanone, 1-hexanol, fumaric, propionic, oxalic and hydrochloric acid, (±)-limonene and β-myrcene are restricted to the antennae.

Butyl acetate is the only tested component eliciting responses exclusively in the maxillary palps, but not coevally on antennae or labial palps.

Table 2. 1: Hypothesized function, abundance external morphology and dendritic structure of sensilla on antennae (A) and maxillary (M) and labial palps (L) in *M. melolontha* larvae.
(ODS: outer dendritic segment, TB: tubular body)

Hypothesized function	Sensillum (type, figure)	Number (location)	Structure	Surface	Pores	Dendrites	Reference
Olfaction	Pore plates (placoid, Figs. 2.1, 2.3, 2.4)	4 A (3 lateral on apical segment, 1 on subapical segment)	large, shallow cuticular depression	smooth	perforated with minute and larger pores	approx. 300 bundles of 3-4 branching ODS	CO_2 (Keil, 1996); olfaction (Kim and Leal, 2000; Alekseev *et al.*, 2006; Giglio *et al.*), this study
	S7 (basiconic, Figs. 2.3, 2.7)	1 A (subapical segment)	small, distally tapering seta	slightly sculptured	wall pores	2-3 ODS (branches?)	this study
	S10 (basiconic, Figs. 2.1, 2.9)	1 M (apex), 1 L (apex)	small, conical seta	slightly sculptured	wall pores	2 branching ODS	this study
Contact chemo-reception or bimodal (tubular body indicates additional mechano-reception)	S2 (chaetic, Figs. 2.1, 2.5)	1 A (apex), 14 M (apex), 7 L (apex)	small, slightly conical seta with blunt tip	smooth to slightly sculptured	terminal pore	4-5 ODS (one with TB)	this study
	S3 (chaetic, Figs. 2.1, 2.6)	1 A (apex)	large, slender, conical seta with blunt tip	smooth	terminal pore	4-5 ODS	this study
	S4 (chaetic, Figs. 2.1, 2.6)	7-8 A (apex)	large, thick, cylindrical seta with blunt tip	slightly sculptured	terminal pore	5-6 ODS (one with TB)	this study
	S5 (chaetic, Figs. 2.3, 2.7)	5 A (subapical segment)	small, egg-shaped seta in large circular socket	smooth	terminal pore	unknown	this study
	S8 (chaetic, Figs. 2.1, 2.8)	2 M (apex), 2 L (apex)	large, thick, conical seta with spherical apex and collar	slightly sculptured	terminal pore	4-5 ODS (TB not observed)	this study
	S9a (chaetic, Figs. 2.1, 2.8)	5-6 M (apex), 2 L (apex)	large, slender, conical seta with blunt tip	smooth	terminal pore and lateral molting pore	7 ODS (one likely with TB)	this study
	S9b (chaetic, Figs. 2.1, 2.8)	2 L (apex)	small, slender, conical seta with blunt tip	smooth	terminal pore	7 ODS (one likely with TB)	this study
Hygro-/thermo-reception	Digitiform organ (coeloconic, Fig. 2.2)	1 M (lateral on apical segment)	sunken seta in longish cuticular recess	smooth	aporous	1 ODS (distally lamellate)	CO_2 (Honomichl and Guse, 1981); mechanoreception (Devitt and Smith, 1982; Farazmand and Chaika, 2008); hygro-/thermo-reception (Guse and Honomichl, 1980; Giglio *et al.*, 2003), this study
	S12 (chaetic, Figs. 2.1, 2.10)	1 L (apex)	small, slender, cylindrical seta with	smooth	subterminal pore	1 ODS (distally lamellate)	this study
Mechanor-eception	S1 (trichoid, Figs.2.1, 2.5)	1 A (apex)	thin, bent seta with bifurcated tip	slightly grooved	basal molting pore	2 ODS (TB not observed)	this study
	S11 (trichoid, Figs. 2.1, 2.10)	1 M (apex)	small, distally tapering seta	smooth	aporous	1 ODS with TB	this study
	S13 (not classi-fiable, Fig. 2.2)	1 M (lateral on apical segment)	small pit	smooth	aporous	1 ODS with TB	this study
	S14 (not classi-f iable, Fig. 2.2)	3-4 M (lateral on apical segment)	flat, bent cuticular furrows	smooth	unknown	unknown	this study
Unknown	S6 (chaetic, Figs. 2.3, 2.7)	1 A (subapical segment)	small, blunt seta	smooth	unknown	unknown	this study

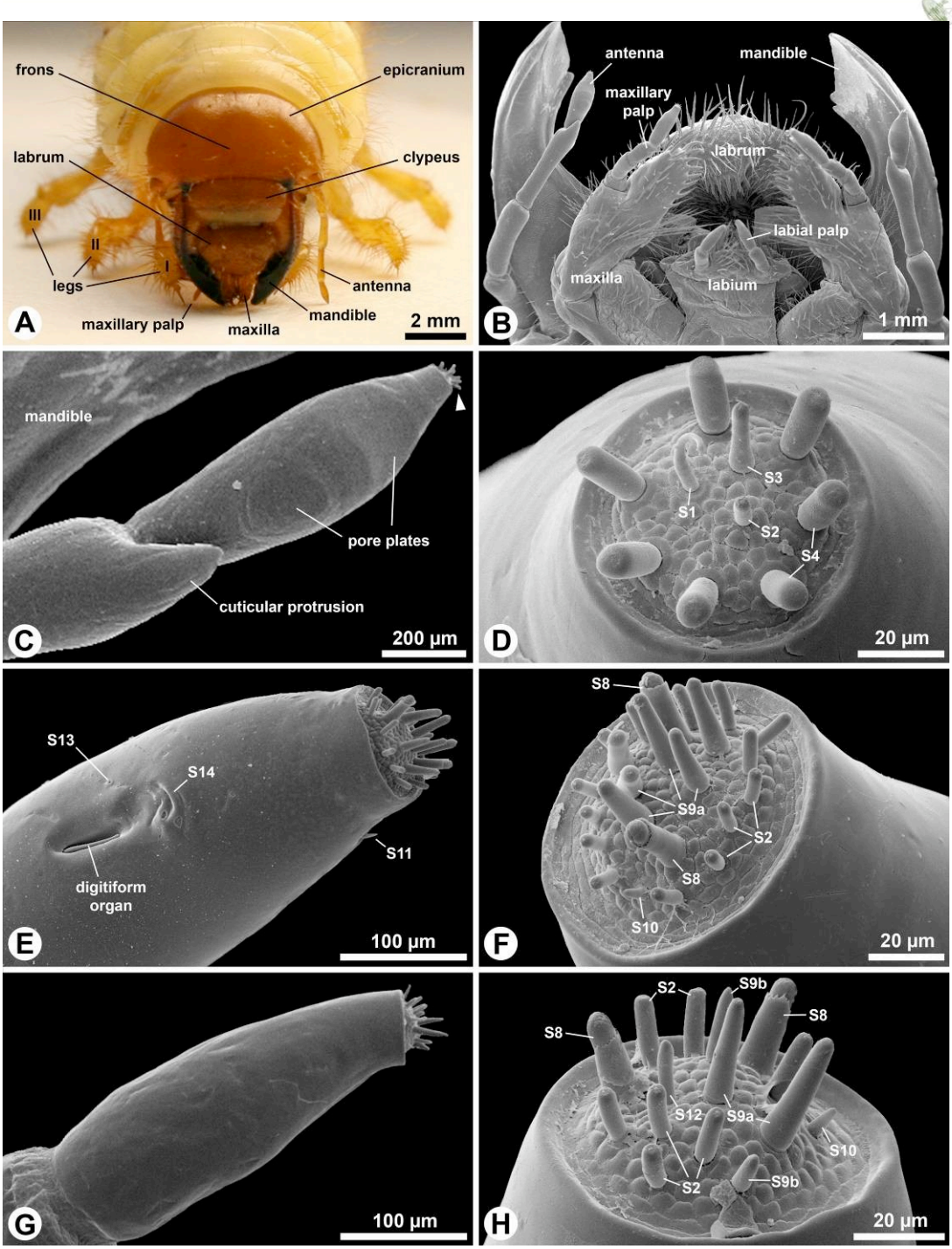


Figure 2. 1: Gross morphology of head and mouthpart appendages of third instar *M. melolontha* larvae.

A: Macro photograph. Frontal view on the head and the anterior body. B-H: SEM. B: Ventral view on the larval mouthparts showing labium and maxillae with their palps. In this specimen, the antennae are held below the opened mandibles, thus they become visible in this viewing angle. C: The apical segments of the antenna. The subapical segment bears a conical cuticular protrusion on its antero-lateral margin. Note the small apical sensilla field (arrowhead). Pore plates are hardly visible. D: Frontal view on the apical sensilla field of the antenna. This specimen possesses seven S4 sensilla. E: Tip of the apical segment of the maxillary palp. On this appendage, several different sensilla occur also below the apical sensilla field. F: The apical sensilla field of the maxillary palps bears the highest number of sensilla among the head sensory organs. G: The apical segment of the small labial palps. H: The apical sensilla field of the labial palps.

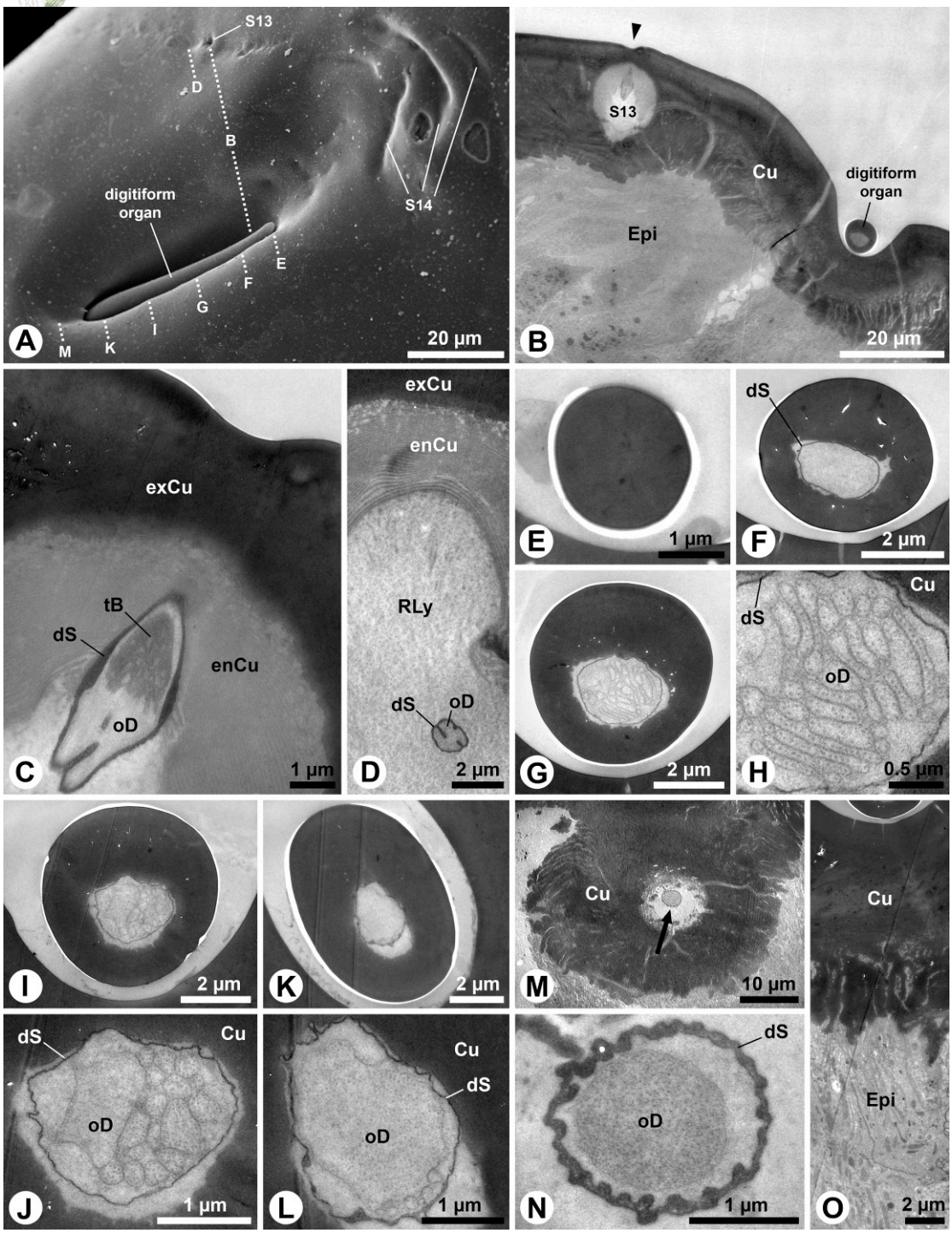


Figure 2. 2: Digitiform organ and adjacent sensilla on apical segment of maxillary palps

A: SEM. The digitiform organ is situated on the bottom of a cuticular depression. Note a row of flat pits (S13) and bent furrows (S14). Dotted lines indicate approximate cutting planes of transverse sections shown in figures B, D-G, I, K and M. B-O: TEM. B: Section on the level of the anterior third of the digitiform organ. In addition to the digitiform organ, one S13 is cut obliquely (arrowhead: flat cuticular pit above S13). C: Magnification of S13. An ensheathed tubular body is embedded in the matrix of the endocuticle. D: A further posterior section shows the single ensheathed outer dendritic segment of the S13 sensilla projecting through its receptor lymph cavity. E: Transverse section of the massive aporous tip of the digitiform organ. F: Posterior of the tip, the shaft lumen houses a thin dendritic sheath, which is empty at this section level. G: Outer dendritic segments occur within the middle portion of the digitiform organ. H: Note the lamellar arrangement of the flattened outer dendritic segments. I: Further posteriorly, the number of outer dendritic segments is reduced. J: The profiles of the

outer dendritic segments are either round or enlarged polygons. K, L: Close to the base only few outer dendritic segments are observable, M: The socket of the digitiform organ is formed by sclerotized cuticle. Note the outer dendritc segment in the central lumen (arrow). N: Only one outer dendritic segment is present, surrounded by a thick and slightly folded dendritic sheath. O: The integument below the digitiform organ. Abbr.: Cu, cuticle; dS, dendritic sheath; Epi, epidermis; enCu, endocuticle; exCu, exocuticle; oD, outer dendritic segment; RLy, receptor lymph; S13-14, sensilla 1-14; tB, tubular body.

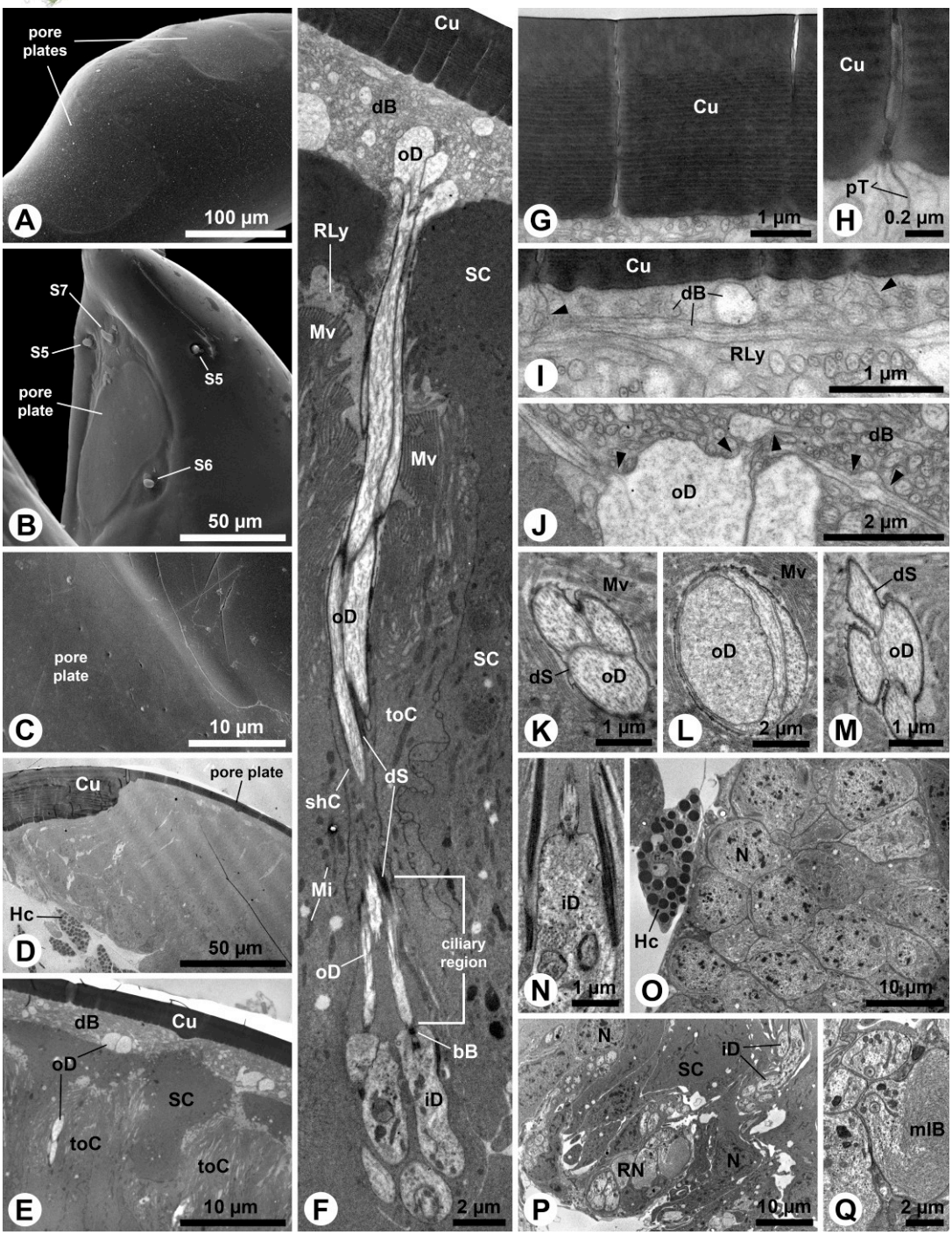


Figure 2. 3: Antennal pore plates

A-C: SEM. A: Two pore plates on the apical segment. B: Pore plate and adjacent sensilla (S5-7) in the lateral protrusion of the subapical segment. C: Pore plate and adjacent cuticle intersection. Apart from occasional openings (see Fig. 2.4), the surface of the pore plate appears smooth. D-Q: TEM. D: Panoramic view of a transverse section, displaying the thin pore plate cuticle and the large tissue cluster below. E: Layered arrangement of different cell types below the pore plate cuticle. F: Three outer dendritic segments, originating from the inner dendritic segments, deflect towards the pore plate. Note the relatively short ciliary portion of the outer dendritic segments. G: The pore plate cuticle, penetrated by narrow channels. H: Internally, each channel exhibits a bundle of tubules. I: The tubules contact small dendritic branches (arrowheads). Note the horizontal dendritic branch, originating from a larger profile (bottom right). J: Dendritic profiles with different diameters and branching points (arrowheads) below the pore plate cuticle. K-M: Transverse sections of outer dendritic segment bundles, showing profiles of varying number, diameter and shape. N: Formation of

the dendritic sheath around the apex of an inner dendritic segment. O: Cluster of receptor neuron somata close to the central hemolymph space of the apical antennal segment containing a hemocyte. P: Supporting cells surround somata and inner dendritic segments. Q: Region of the receptor somata from where inner dendritic segments protrude with large multilamellar body. Abbr.: bB, basal body; Cu, cuticle; dB, dendritic branch; dS, dendritic sheath; HC, hemocyte; iD; inner dendritic segment; Mi, mitochondrion; mlB, multilamellar body; Mv, microvilli; N, nucleus; oD, outer dendritic segment; pT, pore tubule; RLy, receptor lymph; RN, receptor neuron; S13-14, sensilla 13-14; shC, sheath producing cell; SC, support cell; toC, tormogen cell.

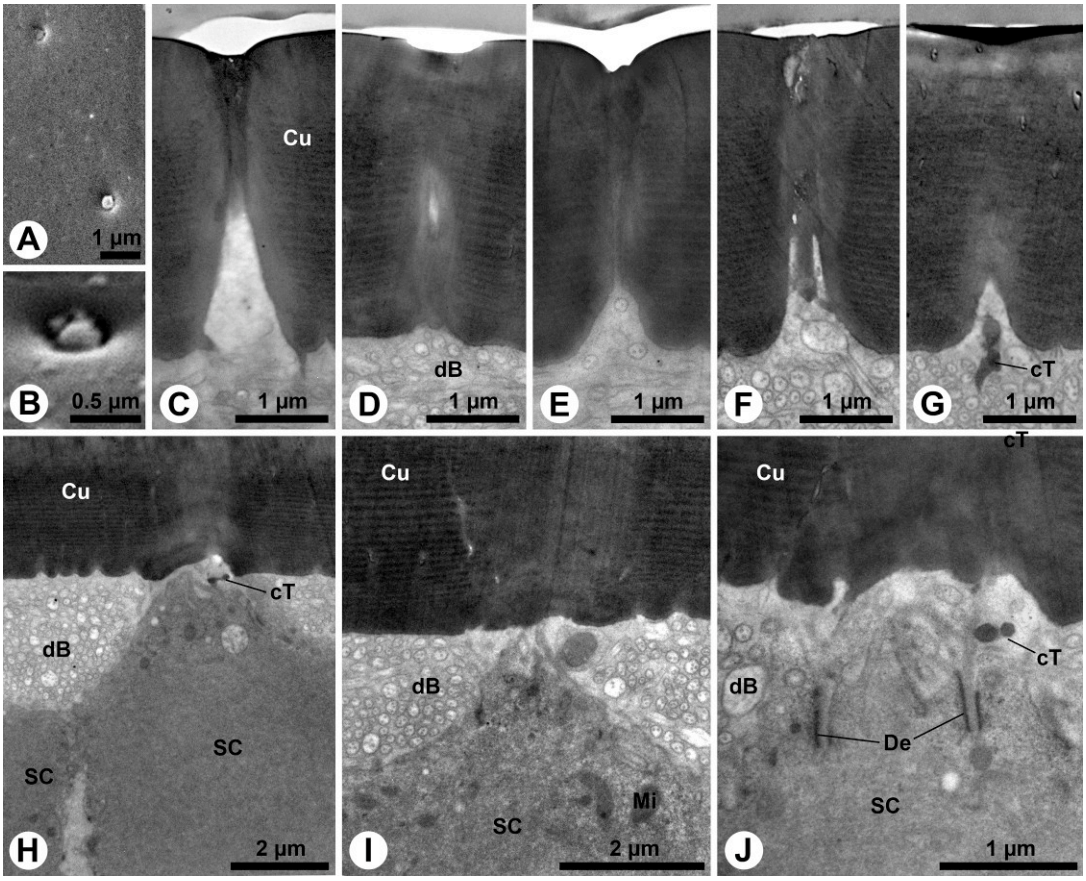


Figure 2. 4: Structure of the pore-like openings and support cells of antennal pore plates

A, B: SEM. A: Here the pore-like openings are plugged. Note small dark spots spread over the surface. B: Higher magnification of a plug within a pore-like opening. C-J: TEM. C: Longitudinal section of a pore-like opening. Although the pore-plate cuticle is fully ruptured by the hour-glass-like duct, its outer half seems to be sealed. D: In this oblique section the duct appears somewhat oval. E: Dendritic branches project into the inner half of the duct. F: This section shows a cuticular protrusion in the duct. G: This protrusion extends as a cuticular thread between the dendritic branches. H: The epidermal support cells have punctual contacts with the pore-plate cuticle. This separates adjacent areas with dendritic branches. I: Mitochondria and electron-dense material are concentrated in the contact areas of the support cells. J: Desmosome-like densities can be observed in the apical membranes of the support cells. Abbr.: cT, cuticular thread; Cu, cuticle; dB, dendritic branch; De, desmosome; Mi, mitochondrion; SC, support cell.

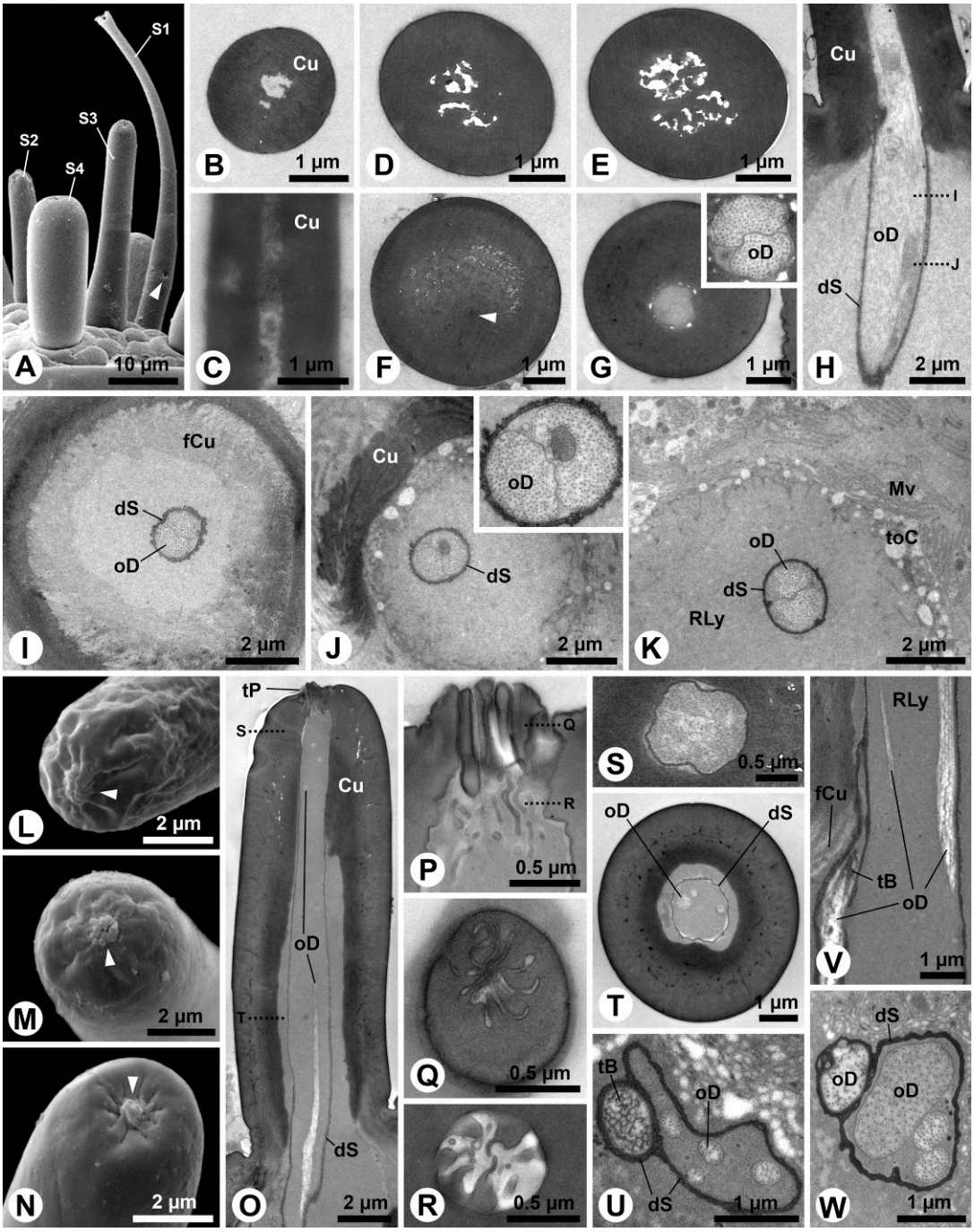


Figure 2. 5: S1 and S2 sensilla on antennae

S1 and S2 sensilla of *M. melolontha* larval antennae. A: SEM. Four different setiform sensilla on the apical sensilla field with putative molting-pore (arrowhead) on S1. B-K: TEM. B: Transverse sections of apical S1. The empty lumen is irregularly shaped. C: Transverse section of S1 center with spongiously hollow shaft. D, E: Further basally, the spongious area enlarges. F: Closely above the socket, the cuticle expands, reducing spongious areas. Note the electron dense spot (arrowhead). G: Closely above the socket, two outer dendritic segments are present (inset: 2x magnification of dendrites). H: This oblique longitudinal section shows the innervation of the sensillum base (dotted lines: approximate cutting planes for Figure I, J).I: Transverse section of the S1 socket revealing its flexible cuticle. J: An electron-dense structure, most likely a vesicle filled with granular material (compare with Chu and Axtell, 1971) is present in one dendrite (inset: 2.5x magnification). K: Transverse section below the socket. L-N: SEM. L-N: Tips of S2 on antenna, maxillary palp and labial palp with finger-like protrusions (arrowheads). O-W: TEM. O, P: Longitudinal section of labial palp S2 (dotted lines: approximate cutting planes for Figures S, T) and magnification of the pore

region (dotted lines: approximate cutting planes for Figures Q, R). Q: Transverse section of S2 apex. R: Transverse section below the pore demonstrating lumen bound cuticular threads. S: An empty dendritic sheath is present in the lumen. T: Outer dendritic segments at the base of the shaft. U: Five outer dendritic segments in a S2 socket, one containing a tubular body. V: Longitudinal section depicting the attachment of the tubular body to the socket cuticle. W: Four outer dendritic segments are present in this S2. Abbr.: Cu, cuticle; dS, dendritic sheath; fCu, flexible cuticle; Mv, microvilli; oD, outer dendritic segment; RLy, receptor lymph; S1-4, sensilla 1-4; tB, tubular body; toC, tormogen cell; tP, terminal pore.

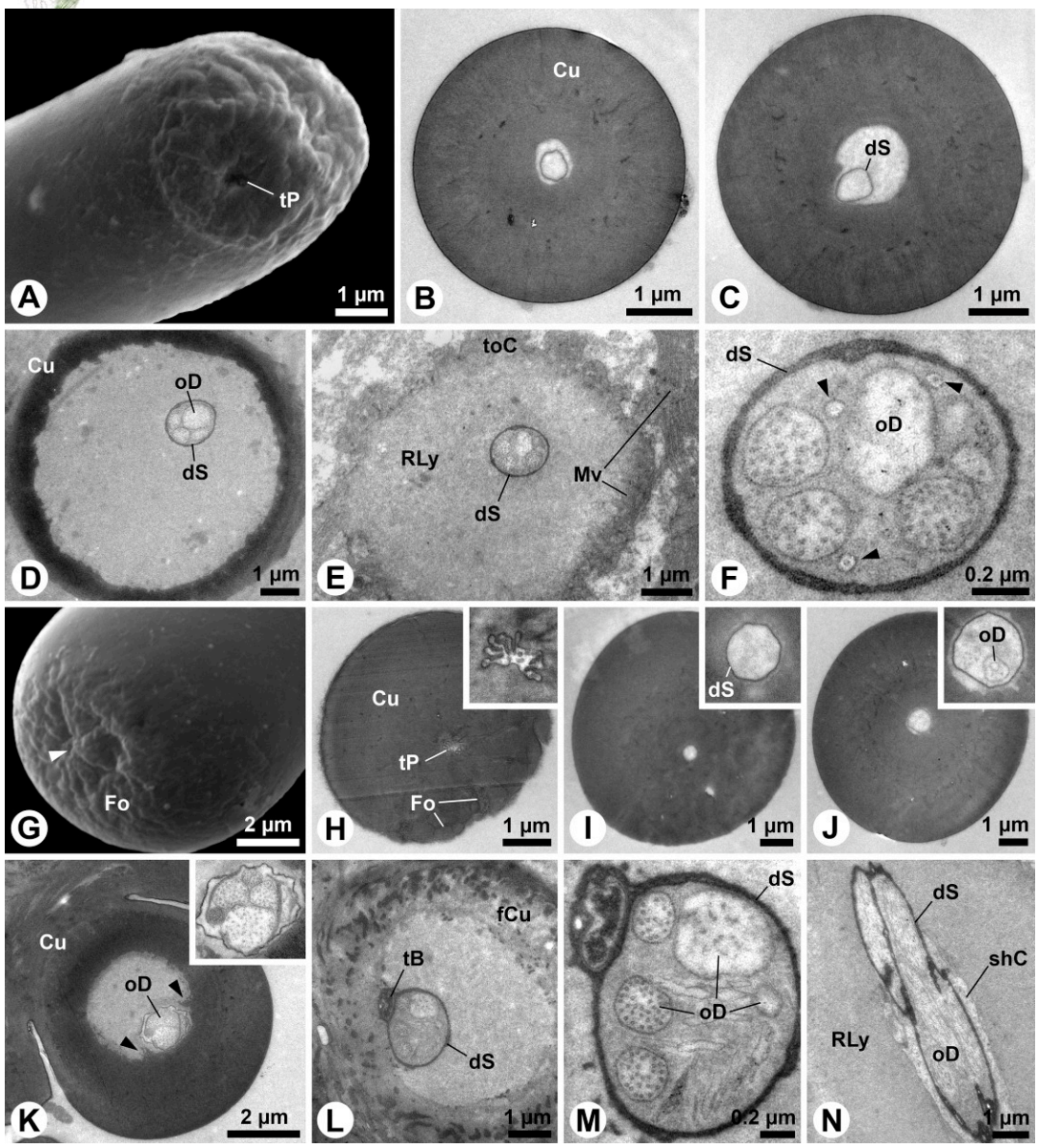


Figure 2. 6: S3 and S4 sensilla on antennae

A: SEM. Tip of the S3 sensillum. B-F: TEM. B: Transverse sections of the apical part of S3. The central lumen contains an empty dendritic sheath. C: Transverse section of the middle part of S3. The empty dendritic sheath follows a furrow along the inner surface of the shaft. D: Transverse section of the socket of S3 revealing several ensheathed outer dendritic segments. E: Below the socket, the dendrites are surrounded by the receptor lymph producing tormogen cell. F: Some dendritic profiles show microtubules. Note the small profiles (arrowheads). G: SEM. Tip of a S4 sensillum with a terminal pore (arrowhead). H: Oblique section of the pore area of S4 (inset: 3.5x magnification of the pore). I: Subterminal transverse section of the same sensillum. The narrow lumen (4x magnification see inset) contains a thin dendritic sheath but no observable dendrites. J: Transverse section of the center of the sensillum shaft. The still narrow lumen (inset: 3x magnification) houses at least one outer dendritic segment. K: Oblique transverse section of the area where the socket (top left) extends into the shaft (lower right). The dendritic sheath contacts the shaft cuticle (inset: 2x magnification of dendrites). Two cuticular lamellae (arrowheads) flank the dendritic sheath. L, M: Transverse section of the socket, containing a dendritic sheath attached to the cuticle. One dendrite terminates in a tubular body. Not all dendrites exhibit clear microtubules. N: Oblique longitudinal section of the innervation of a S4 sensillum. The dendritic sheath is adjacent to the extensions of its origin, the thecogen cell. Abbr.: Cu, cuticle; dS, dendritic sheath; fCu, flexible cuticle; Fo, fold; Mv, microvilli; oD, outer dendritic segment; RLy, receptor lymph; shC, sheath producing cell; tB, tubular body; toC, tormogen cell; tP, terminal pore.

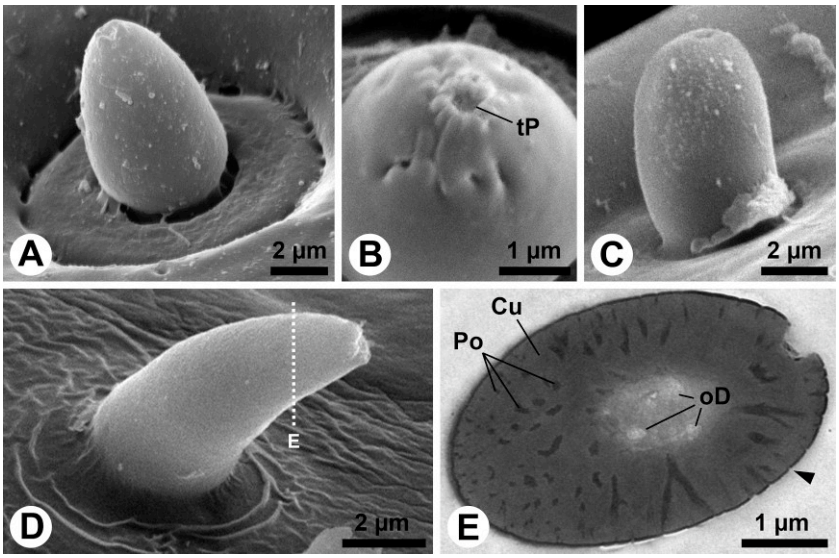


Figure 2. 7: S5, S6 and S7 sensilla on antennae

A-D: SEM. A: Lateral view on the egg-shaped S5 sensillum. Note the large, circular socket. B: Higher magnification of the tip of a S5 sensillum. Finger-like cuticular projections surround a terminal pore. C: Lateral view on a short, blunt S6 sensillum. D: S7 sensillum with a short, conical, bent shaft. Its tip seems to be damaged. The dotted line indicates the approximate cutting plane of the transverse sections shown in Figure E. E: Oblique transverse section of the S7 sensillum. The cuticle of the sensillum is penetrated by numerous pores, which connect the outside with the lumen, where outer dendritic segments are present. Note the minute pore openings (arrowhead). Abbr.: Cu, cuticle; oD, outer dendritic segment; Po, pore; tP, terminal pore.

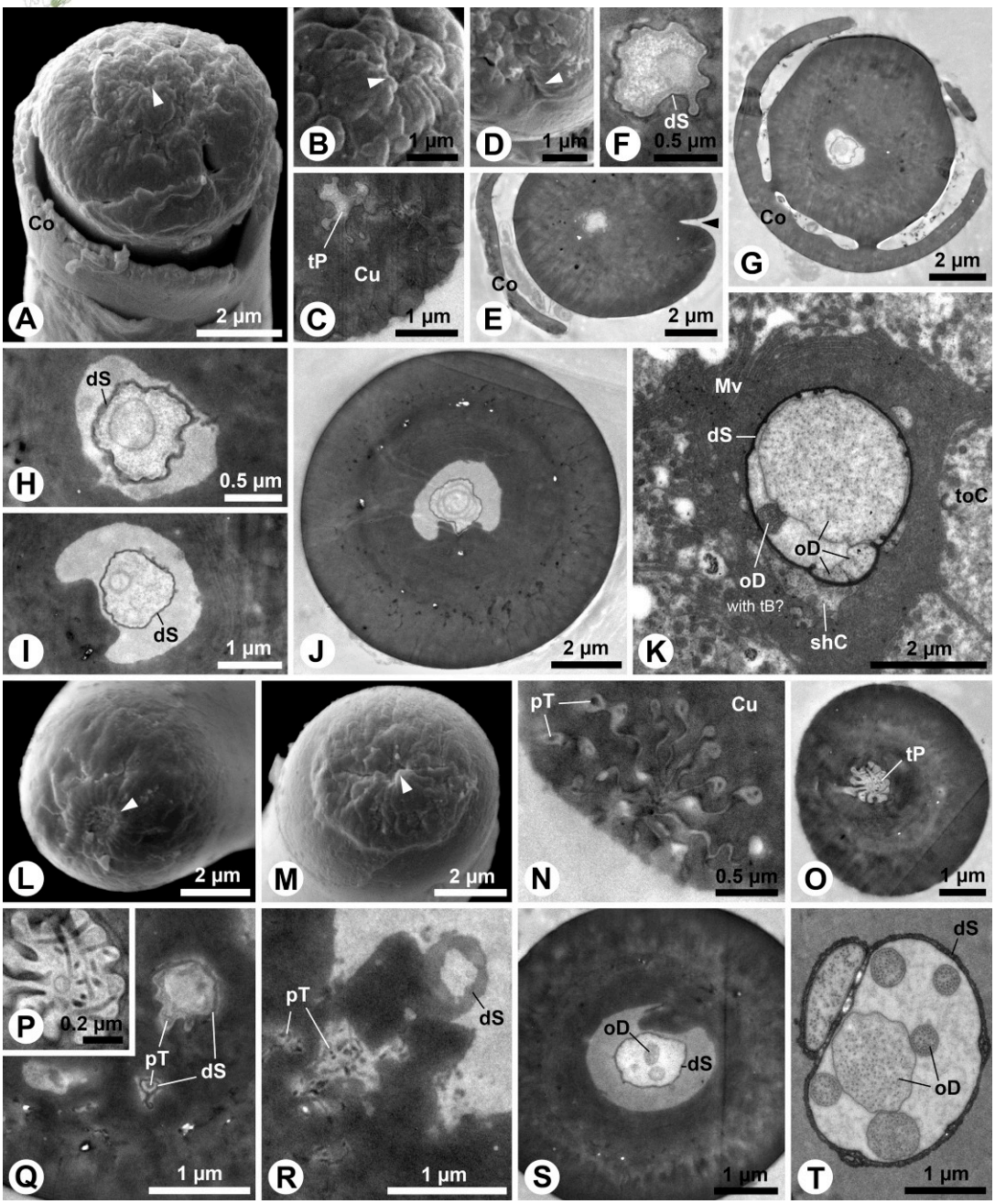


Figure 2. 8: S8 and S9 sensilla on palps

A-B: SEM. A: Tip of a S8 sensillum from the maxillary palp with inconspicuous terminal pore (arrowhead) and conspicuous opening (see Figures D, E). B: Magnification of the terminal pore (arrowhead), surrounded by cuticular protrusions. C: TEM. Oblique section of the terminal pore area. D: SEM. The conspicuous opening (arrowhead) is just a deep fold. E, F: Oblique transverse section of the S8 sensillum on the level of the fold (arrowhead). Parts of the collar are visible on the left. A dendritic sheath but no dendritic elements are observable. G, H: Further posterior section of the collar origin, revealing membranous structures in the lumen. I: The dendritic sheath in the shaft center extends along a cuticular lamella, generating a crescent lumen. J: The dendritic sheath in the S8 shaft is very closely allied to the lamella. K: Transverse section below the socket. In this specimen the dendritic sheath encloses four outer dendritic segments. One of them contains conspicuously dense arranged microtubules. L, M: SEM. L: Tip of the S9 sensillum of the maxillary palp. Finger-like protrusions surround the pore (arrowhead). M: S9 with an elevated terminal pore (arrowhead) region. N-T: TEM. N: Oblique section of the pore region with putative pore tubules adjacent to the protrusions. O, P: Lumen below the terminal pore. Magnification reveals streaks of electron-dense material. Q: Channels with a thin lining (putative dendritic sheath) below the tip of S9. R: Putative pore

tubules extend towards the central lumen. A dendritic sheath is attached to a cuticular lamella. S: Transverse section of a S9 base with ensheathed outer dendritic segments. T: Seven outer dendritic segments are present below the socket. Abbr.: Co, collar; Cu, cuticle; dS, dendritic sheath; Mv, microvilli; oD, outer dendritic segment; pT, pore tubules; shC, sheath producing cell; toC, tormogen cell; tP, terminal pore.

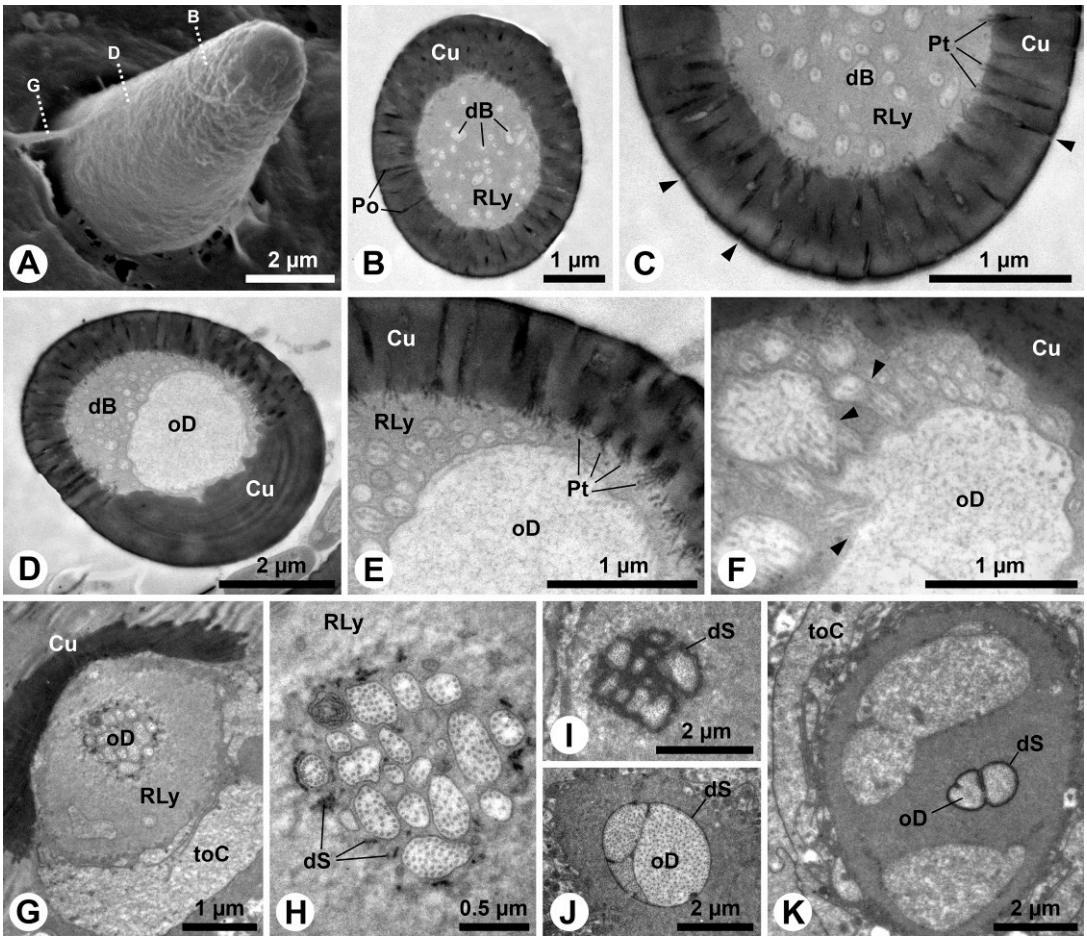


Figure 2. 9: S10 sensillum on palps

A: SEM. S10 sensillum from the maxillary palp. The surface is slightly sculptured. Dotted lines indicate approximate cutting planes of transverse sections shown in Figures B, D and G. B-K: TEM. B: Oblique transverse section of the apical part of the shaft. The cuticle is porous and the wide lumen is sparsely filled with thin dendritic branches. C: Bundles of short pore tubules are directed towards the lumen of the sensillum. The pore openings (arrowheads) on the surface of the sensillum are very small. D: Oblique transverse section of the basal part of the shaft, where the porous part of the cuticle merges in an non-porous part. Note an inflated outer dendritic segment. E: Small dendritic branches and the large inflated dendritic segment come in close contact with the pore tubles. F: Several dendritic branching points (arrowheads) are visible in this section. G: Oblique section of the socket. H: Magnification of the 18 dendritic segments shown in Figure G. Only few, loosely arranged electron-dense remnants of a dendritic sheath are present. I: This further posterior section shows 10 outer dendritic segments embedded in a matrix of dendritic sheath material. J: Four large outer dendritic segments are present below the socket. K: Finally, only two outer dendritic segments represent the entire innervation of the S10 sensillum. Abbr.: Cu, cuticle; dB, dendritic branches; dS, dendritic sheath; oD, outer dendritic segment; Po, pore; pT, pore tubules; RLy, receptor lymph; toC, tormogen cell.

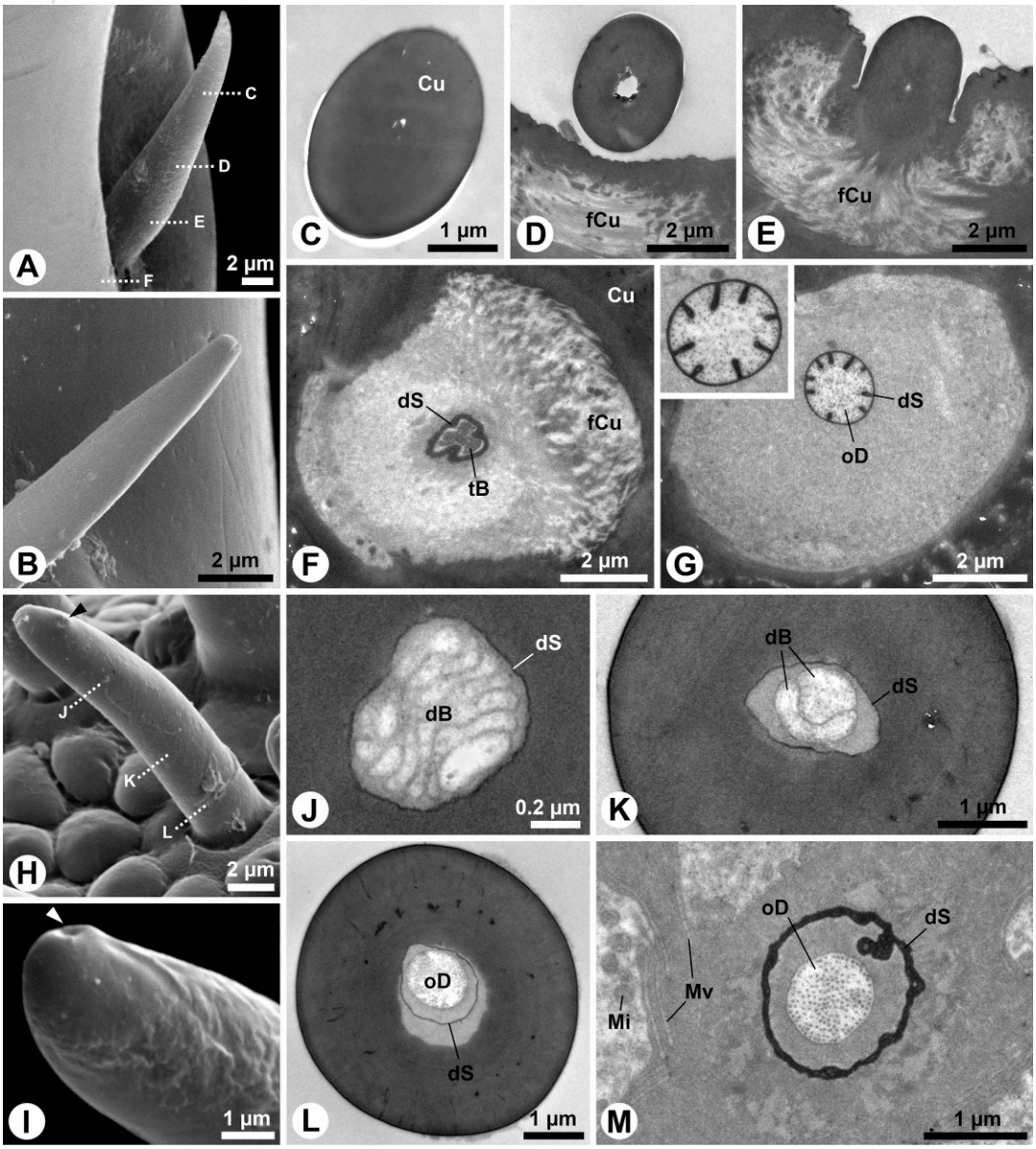
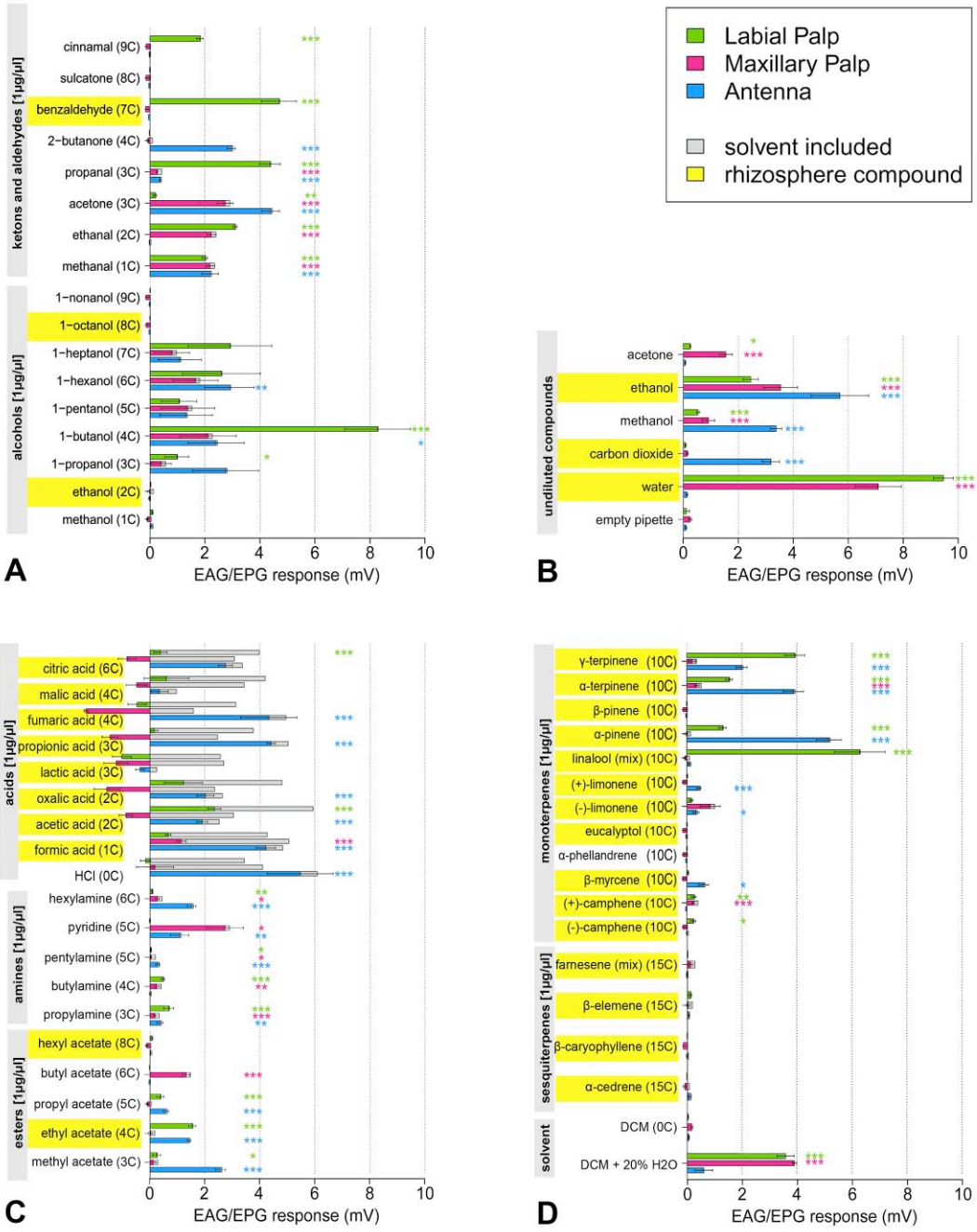


Figure 2.10: S11 and S12 sensilla on palps

A-B: SEM. A: S11 sensillum with a pointed tip on a maxillary palp. Dotted lines indicate approximate cutting planes of transverse sections shown in Figures C-F. B: S11 sensillum with a blunt tip from a different maxillary palp. C-G: TEM. C: Oblique section of the sensillum tip. Note the massive cuticle and sparse lumen. D: This section represents the middle portion of the shaft. A lumen is visible, but it is empty. E: Oblique section of the area where the shaft merges in the flexible cuticle of the socket. Note the minute lumen of the shaft. F: A little deeper inside the socket, a thick dendritic sheath with a single tubular body, attached to the flexible cuticle, becomes visible. G: Below the socket only one large ensheathed outer dendritic segment can be found. Note that the number of radial folds of the dendritic sheath changes in different section levels (see inset). H, I: SEM. H: Slightly bent S12 sensillum from the labial palp, bearing a subterminal pore opening (arrowhead). Dotted lines indicate approximate cutting planes of transverse sections shown in Figures J-L. I: The subterminal pore (arrowhead) of this S12 sensillum from a different labial palp opens much closer to the apex (cp. Figure H). J-M: TEM. J: Lamellate dendritic profiles are present in the apical part of the sensillum. K: In this section only two dendritic profiles are visible. L: Shortly above the socket only one dendrite remains inside the dendritic sheath. M: This single dendrite can also be found deeply below the sensillum socket. Abbr.: Cu, cuticle; dB, dendritic branches; dS, dendritic sheath; fCu, flexible cuticle; Mi, mitochondrion; Mv, microvilli; oD, outer dendritic segment; tB, tubular body; tP, terminal pore.



<u>Figure 2. 11</u>: Mean amplitudes for electrophysiological recordings on larval antennae and palps

Recordings were obtained from antennae (blue bars), maxillary (pink bars) and labial palps (green bars) from third instar *M. melolontha* larvae whole-body mounts (n= 15 replicates on 6 animals, 1-3 per animal). Response to respective controls (empty pipette, DCM, dist. water and DCM supplemented by 20% water) has been subtracted. The grey bars behind colored bars display gross responses without solvent correction. Asterisks indicate significantly higher responses to the tested compound than to respective solvents (Welch two sample t-tests with sqrt transformed data). Significance levels: *** at $p<0.001$; ** at $p<0.01$ and * at $p<0.05$. A: alcohols, ketones and aldehydes at a concentration of 1µg/µl in DCM. B: Undiluted compounds, stimulation with empty pipette and CO_2, C: Amines, esters at a concentration of 1µg/µl in DCM and acids at the same concentration in DCM supplemented by 20% dist. water. D: Monoterpenes, sesquiterpenes and solvents at a concentration of 1µg/µl in DCM.

2.5 Discussion

Our ultrastructural and electrophysiological studies reveal highly developed chemosensory structures in soil-dwelling *M. melolontha* larvae. Olfactory, as well as contact-chemosensory neurons, are present in sensilla on antennae, maxillary and labial palps. Morphological characteristics indicate olfactory function in three out of 17 sensillum types located on larval antennae and palps olfactory, and gustatory function for seven sensillum types. A multitude of host-derived compounds elicit physiological responses in antennae and palps. Each head appendage has its own olfactory response profile. Some responses are appendage-specific down to the level of enantiomers (Fig. 2.11D). The pore plates on the larval antenna are the most prominent chemosensory structures, both in terms of area covered as well as numbers of innervating sensory neurons. The apices of all examined head appendages are dominated by contact chemo-sensilla or multimodal mechano- and contact chemo-sensilla equipped with single terminal pores and distinct dendritic structures. The most abundant peg-like sensillum type S2, a combined contact chemo- and mechano-sensillum, occurs on antennae, maxillary and labial palps. Further contact-chemoreceptive sensilla are S3, S4, S5, S8 and S9. Larvae of *M. melolontha* have been observed pushing their heads into the sidewalls of their burrows (Schwedtfeger (1939) and personal observations), which is interpreted as probing behavior with antennal and palpal apices (Fig. 2.1A,B) predominantly tasting the surrounding matrix. Hence, the corresponding sensilla may serve to orient along gradients of water-soluble chemicals present on the matrix. In contrast, size (S7, S10) or position (S7, pore plates) of the olfactory sensilla prevent direct contact to the substrate and thus warrant stimulation through the gas phase only. Behavior and spatial arrangement of sensilla indicate that the larvae use both contact and olfactory cues present in the rhizosphere.

2.5.1 Sensillum characterization and terminology

Following Keil (Keil, 1999) the olfactory sensilla on *M. melolontha* larval head appendages are single walled sensilla basiconica, i.e. tapering pegs with wall pores (S7, S10), and sensilla placodea (pore plates). All contact chemo-

sensilla fall into different sub-categories of single walled sensilla chaetica with a pore at or close to the tip (S3, S4, S5, S8, S9). Interestingly, none of the observed sensilla displays a double cuticular wall, and all sensilla with mechano-sensory function except S1, S13 and S14 fall into the category of s. chaetica as well. Despite its untypical furcate tip, S1 appears to be a mechanosensory sensillum trichodeum. The function of the furcation (Fig. 2.1D, 5A), however, remains elusive.

2.5.2 Olfactory sensilla – multiporous single walled

Antennal pore plates are common in scarab larvae. Their abundance on the apical antennal segment may differ from one (Moron, 1991) to more than a dozen (Jerath and Unny, 1965; Ratcliffe and Chalumeau, 1980) in xylophagous and saprophagous larvae (Micó and Galante, 2003), but there are always three in rhizophagous larvae, irrespective of subfamily affiliation (Jepson, 1937; Micó *et al.*, 2001; Weissteiner, 2010, and this study). The presence of minute pores with pore tubules and subjacent branching outer dendritic segments indicate their olfactory function. Some adult scarab beetles bear small but 'larval-like' planar sensilla placoidea (Kim and Leal, 2000), while in other species these organs are superficially modified to dome-shaped (Romero-Lopez *et al.*, 2010) or sculptured s. placoidea with foldings or cavities (Renou *et al.*, 1998). The innervation pattern of adult s. placoidea, however, is in each case similar to the sensory units we found underneath the cuticle of larval pore plates (cp. Review by Meinecke, 1975 and citations therein). We therefore interpret the functional sensory units underneath the pore plates as cryptic s. placodea, homologous to the adult s. placodea, and the pore plates as multi-sensillum olfactory fields. Based on the average size of the pore plates in relation to the average distance between adjacent dendritic bundles, we estimate a number of 80-120 sensory units in each of the three large pore plates on the distal antennal segment and about 10-15 units for the small pore plate on the cuticular protrusion of the subapical antennal segment. Hence, about 300 sensory units with a total number of about 1000 sensory neurons innervate the pore plates of one larval antenna (Fig. 2.3). Regarding the number of functional sensilla and olfactory sensory

neurons (OSNs), *M. melolontha* larvae thus resemble adult insects like *Drosophila melanogaster* (Shanbhag *et al.*, 1999). Only one olfactory basiconic sensillum, innervated by a maximum of two or three OSNs is located on the tip of each palp (S10), and on the cuticular protrusion of the subapical antennal segment (S7), respectively (Table 2.1, Figs. 2.7 & 2.9). This clearly indicates that major olfactory input comes from the multi-sensillum olfactory fields on the antennae.

2.5.3 Contact chemo-sensilla – single terminal pores

The number of outer dendritic segments indicates 4 or 5 chemoreceptive neurons for most contact chemo-sensilla, except for S9a & b with 6 chemoreceptive neurons per sensillum. In contrast to sugar sensitive cells, which are commonly found in insects, pH sensitive cells have to our knowledge so far only been described in ground beetles (Merivee *et al.*, 2005). In a set of preliminary experiments we observed behavioral responses to diverse sugars and organic acids (Eilers, unpubl.). We therefore assume that sugar and pH-sensitive neurons are present in the s. chaetica. Single gustatory sensillum recordings were attempted to identify the responsive profiles of the s. chaetica. However, well established protocols (e.g. Marion-Poll and Van der Pers, 1996; Marion-Poll and Descoins, 2002) did not result in successful stimulation of taste sensilla on the palps of *M. melolontha*. The lack of response to all applied gustatory stimuli (sugars, salts, organic acids, caffeine, and aqueous dandelion root extracts) may be related to a missing fulfillment of essential homeostatic needs in the larvae, as the experiments were not performed in their natural environment, soil. External signals, which might have interfered with the gustatory recordings, are for instance the presence of light, inadequate moisture, temperature, oxygen or carbon dioxide levels, or – despite all experimental efforts - the presence of vibrations or similar mechanical disruption. An insects homeostatic sensory system operates in a narrow range and even a minor discrepancy from the preferred milieu may induce major physiological changes in the animal (Zimmer *et al.*, 2009; Vermehren-Schmaedick *et al.*, 2010).

2.5.4 Hygro- and thermoreception

Avoiding heat, drought and excess wetness is crucial for the survival of *M. melolontha* larvae (Schwerdtfeger, 1939; Ene, 1942). Only maxillary and labial palps of *M. melolontha* larvae respond to changes in air humidity in our electrophysiological experiments. Highly lamellated dendritic structures as found in the digitiform organ on the maxillary and sensillum S11 on the labial palps, are characteristic for thermo-hygroreceptors (Altner and Loftus, 1985). We therefore suggest that the digitiform organ and S11 sensillum are the responsible hygro-/thermoreceptive organs.

2.5.5 Electrophysiological responses to volatile stimuli

Out of the 52 compounds, relevant for below ground living insects or analogs of these compounds, the antenna of *M. melolontha* larvae respond to 27, the maxillary palp to 13 and the labial palp to 23 compounds. Sixteen of the tested compounds elicit similar responses in antennae and labial palps. All classes of tested volatiles aside from sesquiterpenoids elicit antennal responses, among them monoterpenes and 1-hexanol, typical plant volatiles. The antennal s. placodea most probably have an important role in the detection of these typical plant derived compounds (but see below). Furthermore, the antennae are the only head appendages responding to CO_2. Cockchafer larvae were shown to orient upwards in faint gradients of 0.001 vol%/cm within a wide range of ambient CO_2 concentrations (Hasler, 1986). Together, sensitive behavioral and robust electrophysiological responses indicate that rather multiple than a single or few neurons mediate responses to CO_2. Similar to CO_2, 2-butanone elicits electrophysiological responses on the antennae only. This compound activates CO_2 receptive OSNs also in mosquitoes (Ray *et al.*, 2011; Stopfer, 2011; Turner *et al.*, 2011). Taken together with our results this indicates that the s. placodea on the antennae are involved in CO_2 perception. Considering that CO_2 may be present as carbonic acid in moist soil, further possible candidates for larval CO_2 detection would be contact chemoreceptors present only on the antennae, such as S4, and S5. Different response profiles are characteristic to OSNs housed within single sensilla like the cryptic s. placodea found in *M. melolontha* larvae

(Hallem and Carlson, 2006; Hill *et al.*, 2009). CO_2-sensitive neurons may pair with other OSNs (Stange and Stowe, 1999). Interactions between CO_2 and other rhizosphere compounds have been demonstrated at the behavioral level (Reinecke *et al.*, 2008). Whether this is indeed reflected in co-localized OSNs for odorants and CO_2 requires single sensillum recordings for confirmation.

Exclusively labial palps respond to benzaldehyde and cinnamaldehyde, typical aromatic plant volatiles eliciting responses in antennae of a wide array of adult insects (e.g. Visser and Yan, 1995; Raguso *et al.*, 1996; Koschier *et al.*, 2000; Ruther *et al.*, 2000; Stelinski *et al.*, 2003). Butyl acetate, for instance, elicits a response in maxillary palps only, while methyl, ethyl and propyl acetate elicit responses in labial palps and antennae only. Hexylamine and 1-hexanol elicit responses in antennae, while no antennal response is detected to hexyl acetate (all C6). Similarly, butyl acetate and butylamine elicit no responses in antennae, but 1-butanol does (all C4). Some responses are even head appendage-specific when comparing enantiomers. The labial palps respond to (-)-camphene, while maxillary and labial palps respond to (+)-camphene. Antennae respond to most of the tested organic acids, labial palps respond to citric and acetic acid and maxillary palps to stimulation with formic acid (Fig. 2.11C), although stimulated with gas phase. Thus, EAG and EPG responses cannot be assigned to chemical classes or carbon chain lengths (volatility), but are head appendix specific at an individual compound base.

Following morphologic criteria, each palp bears only two OSNs. It is unlikely that electroantennographic or –palpographic signals are picked up from single neurons. Despite the prominent olfactory pore plates on the antennae this reasoning together with the wide variety of appendage-specific responses rather indicate that (i) there is no clear-cut distinction between antennae and palps with respect to olfactory function and that (ii) typical gustatory sensilla most probably have a dual function serving both olfaction and taste. Four-to-six sensory neurons are present in each s. chaeticum, a sufficient number to allow for a set of taste neurons to be combined with OSNs within one sensillum. In larvae of the sphingid hawk moth *Manduca sexta* thick walled gustatory sensilla on maxillary palps were shown to have olfactory capabilities as well. They respond to plant derived volatile substances besides their

response to salt and sugar (Städler and Hanson, 1975). Again, single sensillum recordings are required to corroborate our hypothesis in *M. melolontha*. Whether the respective sensory neurons project into the suboesophagial ganglion, the primary center for processing of gustatory information (Mitchell *et al.*, 1999) or the antennal lobe, the primary center for processing of olfactory input (Hansson and Anton, 2000) also remains to be determined.

Our findings clearly show that *M. melolontha* larvae possess intriguingly well developed chemosensory organs equivalent to those of many adult insects. Weissteiner (2010) reports that the antennal lobe, the first brain center to process olfactory input, is composed of about 70 glomeruli in the congeneric *M. hippocastani*. The number of glomeruli is indicative of the diversity of olfactory receptor proteins and thereby of OSN types (Stocker, 1994), and corresponds well to what has been found in adult model insects for olfactory research (Stocker, 2001; Grosse-Wilde *et al.*, 2011). Scarab beetles spend the majority of their lifecycle as larvae below ground, feeding on plant roots. The developmental period, in which host location in a complex matrix is a major task, may have favored the evolution of a larval chemosensory equipment comparable to adult insects.

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Chapter 3

To find or not to find a tasty meal – –an analysis of ecological factors that render plant roots attractive to a rhizophagous insect

3.1 Abstract

The factors that mediate interactions between rhizophagous insects and plant roots are poorly understood to date. Rhizophagous insects may follow gradients of pure carbon dioxide (CO_2). However, the gas is ubiquitous in soil, and might thus be an unreliable cue for root herbivores. Here, we used European cockchafer larvae (*Melolontha melolontha*) and their host plant dandelion (*Taraxacum sect. ruderalia*) to investigate how biotic factors (plant age, mycorrhizal colonization) and abiotic ones (light, substrate type) affect root CO_2 emission and attractiveness. According to our findings, the attractiveness of plant roots cannot be predicted solely by emitted CO_2. In contrast, increasing CO_2 decreased the attractiveness of roots in our assays. Distinct combinations of ecological factors could even render the host plant unattractive. In conclusion, a network of interacting biotic and abiotic factors mediates a high plasticity of root cues, which determine the attraction of a rhizophagous insect to their host.

3.2 Introduction

A tremendous number of insects spend at least a part of their lives within the dark and moist dimensions of the pedosphere. Despite an increasing number of studies of the chemo-ecological interactions in subterranean insect life in recent years (e.g. Rasmann *et al.* 2005; Robert *et al.* 2012), we are only starting to understand the complex network of factors shaping these

interactions. Root-feeding insects are well known to orient towards CO_2-releasing sources (Johnson and Gregory, 2006), but in addition to this almost ubiquitous gas belowground they also use various other root-derived compounds, such as isoflavonoids (Johnson *et al.*, 2005) or low molecular weight alcohols, esters, and aldehydes (Johnson and Gregory, 2006; Johnson and Nielsen, 2012) for host plant location. So far, only scarce knowledge is available on factors that influence host quality for root-feeders, such as plant age, mycorrhizal colonization, and aboveground light intensity. Moreover, we do not know yet how the impact of such ecological factors depends on the type of substrate.

Here, we addressed these gaps of knowledge by studying the orientation behaviour of the root-feeding larvae of the European cockchafer, *Melolontha melolontha* L.*,* towards host plant roots that were grown at different conditions. We used the preferential host of *M. melolontha*, i.e. dandelion *Taraxacum sect. ruderalia* (Kirschner, Øllgaard et Štěpánek). Dandelion roots are the most suitable food source to *M. melolontha* larvae, since survival and weight gain during their three-year larval life in a dandelion rhizosphere are best compared to in rhizospheres of other plant species (Hauss, 1975; Hauss and Schütte, 1976). Dandelion tolerates a broad range of biotic and abiotic parameters which account for a high phenotypic plasticity (Luo, 2009) that *M. melolontha* larvae need to cope with.

A recently established atlas of the sensory structures on larval head appendages of *M. melolontha* identified an intriguing diversity of chemosensory sensilla, detecting a broad range of root-derived primary and secondary metabolites (Eilers *et al.*, 2012). Nevertheless, CO_2 has for long time been postulated to be the only, or at least main attractant of *M. melolontha* larvae in soil (Klingler, 1957; Hasler, 1986). Even weak gradients of 0.001%/cm CO_2 are sufficient for the larvae to pinpoint the source belowground, but at 3%/cm CO_2 the gas eventually becomes repellent (Hasler, 1986). According to Reinecke et al. (2008) host root-derived CO_2 may be masked by root exudates that counteract its attractiveness.

The quantity and composition of root exudates vary inter- and intraspecifically in dependence of the environmental biotic and abiotic conditions (Rovira, 1969; Bertin *et al.*, 2003). Colonization with arbuscular mycorrhizal fungi (AMF) may alter plant root chemistry and architecture (Wu *et al.*, 2011) and other host plant traits markedly (Pozo *et al.*, 2010). The root – fungus association may up-regulate plant defense signaling pathways (Kuzyakov and Gavrichkova, 2010; Oddsdottir *et al.*, 2010) and root exudation (Bodker *et al.*, 1998; Vannette and Hunter, 2009). Today, most studies of AMF-colonized plants used plants that were inoculated with only one commercially available fungal species (e.g. *Glomus mossae* or *G. intraradices*) (e.g. Bodker *et al.* 1998; Wolfe *et al.* 2005), although usually multiple AMF species co-occur (Oehl *et al.*, 2003). In a biennial or perennial plant like dandelion, the intraindividual temporal variability of appearance is very strong. Hence, plasticity in root exudation due to changes in plant physiology and biochemistry with plant age are likely. Light conditions significantly affect nutrient shifts between above- and belowground plant tissue. Lowlight conditions result in decreased root-to-shoot ratios (Lichtenthaler, 1981; Olff *et al.*, 1990). In turn, root growth and release of CO_2 and sugar into the rhizosphere are enhanced with increased photosynthesis activity at increased light intensity (e.g. Kasperbauer & Hunt 1992; Nagel *et al.* 2006; Kuzyakov & Gavrichkova 2010). Finally, the chemo-physical properties of the matrix surrounding the insect and the plant root may play a decisive role for host plant recognition. The clay mineral vermiculite (Reinecke *et al.*, 2008; Vaughan *et al.*, 2011), or similar substrates (Wolfe *et al.*, 2005) are commonly used in studies of root - herbivore systems, although these types of substrate may impede chemically mediated host plant location by adsorption of host cues. Other studies employ pure white sand in comparable experiments, a substrate with very low adsorption capacities (Rasmann *et al.*, 2005; Robert *et al.*, 2012). Hence, these experiments may be misleading since they do not reflect the naturally occurring field conditions.

At this background, we evaluate the importance of plant age, association of

the plant with ecologically relevant AMF, aboveground light conditions and substrate type, as well as interactions of these factors, on larval behaviour and host finding ability in a non-invasive way. We analysed the interaction of of these factors by multifactorial statistics for which additionally CO_2 measurements (as indicator of root respiration activity), plant root biomass production, and feeding preference data were included. The observed effects on root attractiveness to *M. melolontha* larvae were so pivotal that they even could render the *per se* preferred roots of dandelion undetectable or unattractive at certain conditions or combinations. Our findings highlight the importance of biotic and abiotic conditions for the chemical composition of the rhizosphere organic fraction and suggest that root-feeding insect larvae show a very fine-tuned response to the blend of compounds released by plant roots.

3.3 Materials and Methods

3.3.1 General experimental procedure

Third instar *M. melolontha* larvae were observed while orienting in a biotest arena (Fig. 3.1) where a chemical gradient was established (a) between a stimulus compartment containing either seven- or nine- week-old dandelion plants grown under a variety of conditions (see below) and a control compartment without plants or (b) between a stimulus compartment with plants with mycorrhizal symbionts and a control compartment with plants without mycorrhizal colonization. During the experiments, the larvae had no access to roots or fungal mycelia and could only move in the bioassay compartment between stimulus and control compartments. We recorded the larval positions every 10min for 6h and analysed larval direction and stationary points (change of direction) from these data. CO_2 gradients between stimuli were assessed during the observation period (see below).

3.3.2 Insects

Third instar *M. melolontha* larvae were collected in May and September of 2009, 2010 and 2011 from a meadow in Hessenthal, Bavaria, Germany

(49°93' N, 9°26'O) and near Apeldoorn, Gelderland, the Netherlands (52°21'N, 6°12'O). The soil from Bavaria consisted of silty sandstone, pH 5.9 ($CaCl_2$), indicating a humus content of 8-15% (Kerschberger *et al.*, 2000).The larvae were kept in separate plastic beakers (Agar Scientific limited, Essex, England), filled with highly sorptive clay substrate (Klasmann-Deilmann GmbH, Geeste, Germany) and were fed ground carrots. They were kept in a dark climate chamber at 16°C and 70% humidity. Insects were used only once for behavioural assays and non-feeding insects were discarded prior experiments. The larvae were kept unfed for 1 day prior to the bioassay in the climate chamber where behavioural assays were conducted (18 °C and 70% humidity, L:D 16/8 h).

3.3.3 Plants, substrates and mycorrhiza

T. sect. *ruderalia* (dandelion) seeds (Treppens, Berlin, Germany) were surface-sterilized with (2% w/w) dichloroisocyanuric acid (DCCA, Fluka) solution as described by (Krügel *et al.*, 2002). Two different substrates were used: fine burnt sand (pH= 5.5, particle size ∅ = 0.4-0.8mm, specific surface area approx. 0.01-0.005m^2/g), and vermiculite (pH=6.92, particle size ∅ = 2-3mm, specific surface area= 60-80m^2/g, CEC = approx. 80-150meq./100g). Plants were grown for five weeks in a climate chamber with 22°C, 70% relative humidity, 6.8klx light intensity and 88 µmol·s^{-1}·m^{-2} photosynthetically active radiation (PAR), at Central European summer light conditions (L:D 16:8h). Afterwards, 10 seedlings each were planted into stimulus compartments of the biotest arenas. After being transplanted, plants were grown at 18°C, 70% humidity, L:D 16:8h and either exposed to 32.3 ± 0.48 klx = 417.29 ± 20.55µmol·s^{-1}·m^{-2} PAR (standard light treatment) or 61.16 ± 0.72 klx = 791.37 ± 31µmol·s^{-1}·m^{-2} PAR (enhanced light treatment) (see light spectra for both treatments in Fig. S1). Control compartments were filled with substrate, and both chambers were constantly moisturized with nutrient solution modified from Arnon and Hoagland (1940) see (Reinecke *et al.*, 2008). The light intensity did not bias the surface temperature of the substrate (18°C in each case). The plant-free top edge of arenas was covered with a stainless steel frame and attached synthetic cloth to prevent algal growth.

Five days before experiments started, the cloth-frame was removed, and the initially empty central compartments of the arenas were filled with substrate and passively moisturized through diffusion from outer compartments through the gauze. A first set of experiments was conducted with 7-week-old plants. Afterwards, the substrate of the central compartments was removed. The plants were left under the same conditions for another two weeks until experiments were repeated with 9-week-old plants following the same procedure. The root and shoot fresh weights of plants were measured after the second set of experiments had been terminated.

AMF spores were obtained from rhizosphere samples collected at the meadow in Bavaria where the larvae were collected. The spores were isolated and fractionated by wet sieving and decanting (Gerdemann, 1963) followed by centrifugation in 60% (w/w) sucrose solution (1 min, 2000 rpm) (Table S2). Afterwards, the spores were rinsed and stored at 4°C for maximal 24 h. Dandelion (*T.* sect. *ruderalia*), wild carrot (*Daucus carota*), ribwort plantain (*Plantago lanceolata*) and clover (*Trifolium pratense*) were used as a trap culture to propagate and homogenize obtained spores (Oehl *et al.*, 2003, cp. chapter 4, Fig. 4. 1). The seeds were surface-sterilized and grown as described above the dandelion seeds. Mycorrhization was monitored weekly via light microscopy after boiling plant root samples for 3min in 10% KOH (w/v), then for 3 min in 0.05% tryphan blue in 5% acetic acid (Vierheilig and Piche, 1998). Dandelion seedlings were inoculated with chopped roots (ca. 20g) from the trap culture at the two-cotyledon stage. Uninfested control plants were kept under identical conditions in separate climate chambers to avoid cross-infection with AMF. They were treated with respective amounts of autoclaved inoculum. After five weeks, seedlings were planted into stimulus compartments of biotest arenas. Prior to the experiments, the mesh frames between the arena compartments (Fig. 3.1B) were gently moved up and down twice a week to truncate mycorrhizal hyphae that were about to traverse the mesh.

3.3.4 Biotest arena and experimental proceeding

Biotest arenas were custom-built, based on a design developed by Reinecke *et al.*, (2008) from pellucid Perspex mounted on aluminium frames. Pre-tests revealed that the larvae are not disturbed by red light. For this reason, red Perspex covers were attached on top of the pellucid Perspex (Fig. 3.1A). Each arena was subdivided into three compartments (stimulus, bioassay, and control compartment) of equal width (W = 20cm) by polyamide gauze (38µm mesh openings, 22% open area, Sefar Nitex®, Sefar AG, Heiden, Switzerland) mounted on removable polyvinyl chloride frames (Fig. 3.1B). Dimensions of arenas were H×W×D =30×20×3cm for plant and control compartments and H×W×D =30×20×1cm for bioassay compartments. Each arena was automatically irrigated with nutrient solution (see above) using tensiometers (Blumat, Bambach GbR, Tensio-Technik, Geisenheim), attached inside each stimulus and control compartment.

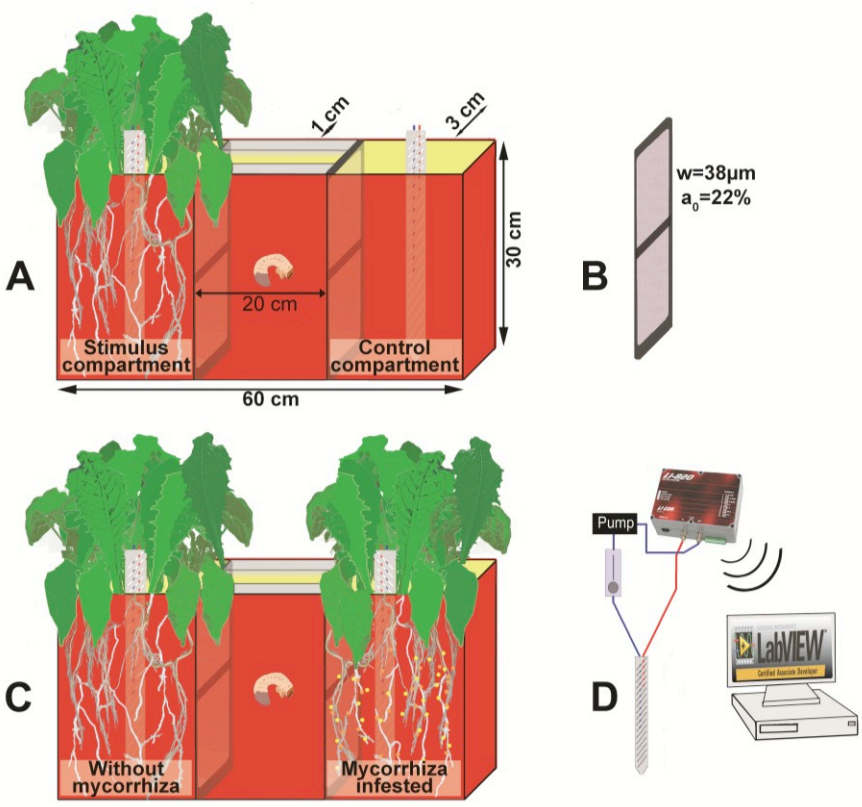


Figure 3.1: Experimental setup. (A**)** Biotest arena with plants in a stimulus compartment, adverse substrate - filled control compartment and central test compartment containing the larva. Compartments were restricted by removable gauze-frames (38µm mesh size) (**B**). Plants with arbuscular mycorrhiza (AMF) (stimulus compartment) were additionally tested against non-infested plants (control compartment) (**C**). Both external compartments contained a pipette-shaped CO_2 measurement device, from which air was pumped in a closed loop system through an infrared CO_2 analyzer controlled by LabVIEW (**D**).

All experiments were conducted between 11am and 5pm. The light- and scotophase started at 6 a.m. and 10 p.m., respectively. At the start of each experiment individual larvae were inserted into the bioassay compartments at half distance between test and control compartments (1 larva per compartment). Grids (mesh size = 3mm) on overhead transparencies were used to note the coordinates of larval head capsules. Experiments were carried out in a climate chamber at the same conditions as the plants were grown in the arena compartments (18 °C and 70% humidity, L:D 16/8 h).

3.3.6 Carbon dioxide measurement

CO_2 monitoring started 24h prior to the experiments and stopped after the experiments. Hence, the measurements were taken during two consecutive photoperiods and were performed in the centre of stimulus and control compartments using two non-dispersive infrared (NDIR) analyzers (Li-820, Licor GmbH, Bad Homburg, Germany). Analyzers were calibrated prior each experiment. Measuring units consisted of laterally slit 10ml serological pipettes (length=25cm, ID=0.9cm, 25 slots á 6mm*2mm, VWR International GmbH, Dresden, Germany), additionally cropped below to provide drainage of condensing water (Fig. 3.1D). Two polyurethane tubes (ID=2mm, OD=4mm, Jenpneumatic, Jena, Germany) were attached into the mouthpiece of pipettes with shrinkable tubing. One tube extended 5cm deep in the pipette, the other 25cm deep. The measuring units were inserted into eight control and eight stimulus compartments two days before experiments started. Air was pumped with a flow of 0.2l/min in a closed loop from the short tube in the measuring units, through respective CO_2 analyzers and flow meters, and back into the longer tube into the pipette. After measuring the CO_2 concentration of one arena for 10s, the tubes were rinsed with ambient air for 2s, and the next arena was accessed by automatically switching respective valves. The equilibrium of CO_2 was established in less than 5s (supposing a flow of 0.2l/min and a tube volume of $3.77*10^{-3}$l, the air was pumped in 1.13s from the arena to the CO_2 analyzer). The measurement was operated via LabVIEW (National Instruments).

3.3.7 Plant root consumption

Following observations in biotest arenas, one-choice feeding assays were performed in which the consumption of plant roots from biotest arenas was measured for 10 of the 16 applied treatments. After being removed from arenas, each larva was rinsed with tap water. Approx. 1g of plant root material from each arena was cut, weighed and placed together with the larva from the same arena between two pieces of moisturized filter paper (5cm diameter, 0.3ml water per paper) in 100ml plastic beakers (Agar Scientific limited, Essex, England, see Fig. S2). The pots were kept in darkness at 20°C, 70% humidity for 18h. Afterwards, the roots were weighed again to determine the respective consumption.

3.3.8 Statistical analysis

The statistic software “R” was used to perform statistical examinations and evaluations as well as to create all charts (R version 2.15.0, 2012-03-30, Team, 2012, “R” package for figure 2, 4, and 5: “plotrix”). CO_2 concentration data and larval observation data were square-root (sqrt) transformed to achieve maximal approximation to normality. Furthermore, autodependence of time-series data was controlled and displayed by producing lag plots (“R” package: “graphics”, command: “lag.plot”). Data were analyzed applying multivariate mixed-effects models, including a residual first-order autoregression structure for temporally autocorrelated larval position data (“R” package: ”nlme”, Pinheiro and Bates, 2000). The model included (1) light intensity, (2) substrate type, (3) mycorrhiza, (4) plant age, and (5) CO_2 concentration difference between compartments as fixed factors, and (6) observation time point as random factor. In addition to the full model including all 16 treatments, models with respective data subsets (e.g. 9 week-old plants without mycorrhiza, grown on vermiculite) were analyzed. Multiple comparisons of measured CO_2 concentrations, plant biomass parameters, larval stationary points (not autocorrelated) and post-experimental root consumption were conducted applying Kruskal-Wallis tests (“R” package “agricolae”, alpha = 0.05). Furthermore Mann-Kendall trend tests were applied to determine monotonic trends over time for CO_2 concentrations.

3.4 Results

The majority of plant treatments induced approach behaviour of the larvae towards the plant roots (Fig. 3.2). The larval orientation was dependent on plant age, mycorrhiza infestation, aboveground light intensity, and substrate type. Larvae stayed further away from the roots if the CO_2 emission was high (Table 3.1). The substrate type was per se the strongest predictor for larval responses to chemical cues from the dandelion rhizosphere (Table 3.1). The plant age, infestation with AMF and changes in light intensity also affected larval behaviour, however, the impact of these factors on larval orientation was context dependent (see below).

Table 3. 1: Results of the mixed effects model for larval distance to the stimulus compartment. The model includes the following fixed factors: light intensity, mycorrhiza infestation, substrate type, plant age and difference in CO_2 content between compartments. The time point was included as random factor (n= 20 insects per treatment, 16 treatments). The model includes a residual first-order autoregression structure. Significance levels: *** p<0.001; ** p<0.01,* p<0.05; . p<0.1.

	Value	Std.Error	DF	t-value	p-value	
(Intercept)	-2.639	2.045	11748	-1.290	0.197	
Light	5.695	2.217	11748	2.569	0.010	*
AMF	-9.244	3.411	11748	-2.710	0.007	**
Substrate	10.601	2.417	11748	4.385	0.000	***
Age	0.540	0.238	11748	2.265	0.024	*
CO_2	0.124	0.062	11748	2.015	0.044	*
Time	0.062	0.095	35	0.653	0.518	
Light: AMF	5.783	3.042	11748	1.901	0.057	.
Light: substrate	-9.481	2.257	11748	-4.200	0.000	***
Light: age	-0.553	0.266	11748	-2.082	0.037	*
Light: CO_2	-0.107	0.064	11748	-1.665	0.096	.
Light: time	-0.242	0.089	11748	-2.714	0.007	**
AMF: substrate	-2.797	3.133	11748	-0.893	0.372	
AMF: age	0.979	0.398	11748	2.458	0.014	*
AMF: CO_2	0.262	0.093	11748	2.827	0.005	**
AMF: time	0.643	0.123	11748	5.246	0.000	***
Substrate: age	-1.150	0.308	11748	-3.729	0.000	***
Substrate: CO_2	-0.213	0.065	11748	-3.288	0.001	**
Substrate: time	-0.118	0.100	11748	-1.171	0.242	
Age: CO_2	-0.013	0.007	11748	-1.764	0.078	.
Age: time	-0.009	0.011	11748	-0.779	0.436	
CO_2: time	-0.002	0.003	11748	-0.730	0.465	

	Value	Std.Error	DF	t-value	p-value	
Light: AMF: substrate	-0.435	1.209	11748	-0.360	0.719	
Light: AMF: age	-0.562	0.342	11748	-1.644	0.100	
Light: AMF: CO_2	-0.170	0.077	11748	-2.216	0.027	*
Light: AMF: time	-0.031	0.038	11748	-0.821	0.412	
Light: substrate: age	0.940	0.289	11748	3.246	0.001	**
Light: substrate: CO_2	0.209	0.059	11748	3.562	0.000	***
Light: substrate: time	0.077	0.041	11748	1.893	0.058	.
Light: age: CO_2	0.010	0.008	11748	1.288	0.198	
Light: age: time	0.028	0.011	11748	2.709	0.007	**
Light: CO_2: time	0.006	0.002	11748	2.456	0.014	*
AMF: substrate: age	0.690	0.388	11748	1.777	0.076	.
AMF: substrate: CO_2	-0.006	0.083	11748	-0.075	0.940	
AMF: substrate: time	-0.274	0.050	11748	-5.427	0.000	***
AMF: age: CO_2	-0.028	0.011	11748	-2.607	0.009	**
AMF: age: time	-0.067	0.014	11748	-4.713	0.000	***
AMF: CO_2: time	-0.015	0.003	11748	-4.616	0.000	***
Substrate: age: CO_2	0.022	0.008	11748	2.720	0.007	**
Substrate: age: time	0.005	0.013	11748	0.392	0.695	
Substrate: CO_2: time	0.006	0.003	11748	2.324	0.020	*
Age: CO_2: time	0.000	0.000	11748	0.870	0.384	
Light: AMF: substrate: age	-0.116	0.113	11748	-1.021	0.308	
Light: AMF: substrate: CO_2	0.040	0.020	11748	2.063	0.039	*
Light: AMF: substrate: time	-0.032	0.010	11748	-3.235	0.001	**
Light: AMF: age: CO_2	0.016	0.009	11748	1.915	0.056	.
Light: AMF: age: time	-0.002	0.005	11748	-0.507	0.612	
Light: AMF: CO_2: time	0.001	0.000	11748	2.909	0.004	**
Light: substrate: age: CO_2	-0.020	0.007	11748	-2.817	0.005	**
Light: substrate: age: time	0.008	0.005	11748	1.629	0.103	
Light: substrate: CO_2: time	-0.003	0.001	11748	-4.560	0.000	***
Light: age: CO_2: time	-0.001	0.000	11748	-2.512	0.012	*
AMF: substrate: age: CO_2	-0.009	0.010	11748	-0.834	0.404	
AMF: substrate: age: time	0.021	0.005	11748	4.003	0.000	***
AMF: substrate: CO_2: time	0.003	0.001	11748	3.788	0.000	***
AMF: age: CO_2: time	0.002	0.000	11748	4.127	0.000	***
Substrate: age: CO_2: time	-0.001	0.000	11748	-1.740	0.082	.

3.4.1 Mycorrhizal fungi

Mycorrhization altered the response of *M. melolontha* larvae to dandelion rhizospheres significantly: the larvae were more frequently observed closely to the AMF plants than to AMF-free plants (Table 3.1). Compared to all -AMF plants other than vermiculite-grown, 9-week old plants, the larvae exhibited higher numbers of stationary points (indicating changes in direction) when

they were offered the respective mycorrhiza-treated plants (Fig. 3.4A). Roots of mycorrhiza-infested, 9-week-old plants grown on sand were consumed to a significantly lower extent than roots of the respective 7-week-old plants (Fig. 3.4B). These plants had lower amounts of root biomass and shoot biomass than uninfested plants (Fig. 3.5B, C) and produced a less steep CO_2 gradient.

3.4.2 Substrate and plant age

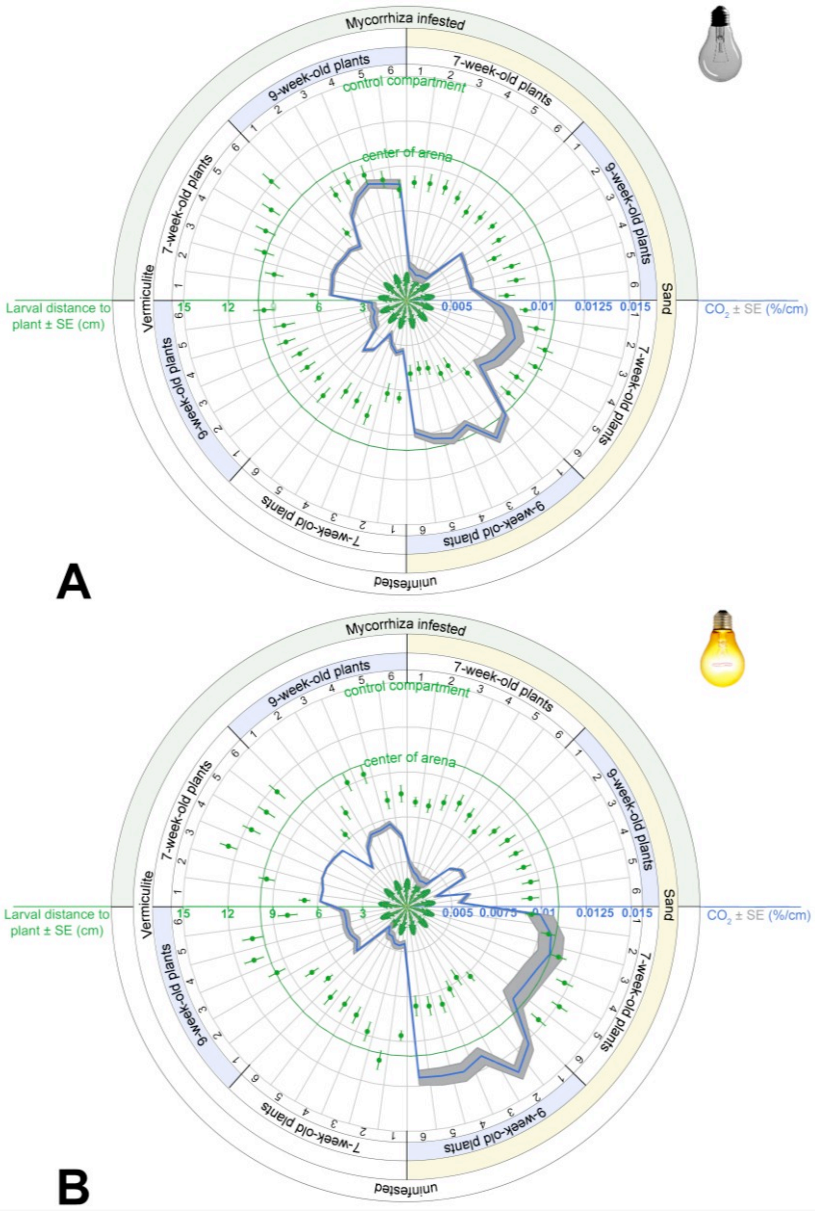
Changing the substrate from vermiculite to sand with remaining experimental conditions unchanged, led to a closer approach of the larvae to the plant roots (mixed effects model testing, Fig. 3.3, Table 3.1). This general phenomenon was not related to CO_2. While CO_2 concentrations in mycorrhiza-infested rhizospheres were higher in vermiculite compared to sand, CO_2 concentrations of uninfested rhizospheres in sand exceeded those in vermiculite (Fig. 3.5A).

Plant age had a low impact on the behaviour of the larvae at standard light conditions and without mycorrhiza (Fig. 3.2, Fig. 3.3) despite the effect of plant age on root-emitted CO_2 (7-week-old: 0.128 ± 0.001%; 9-week-old: 0.151 ± 0.001%, respective control CO_2 subtracted). However, in sand, increased plant age reduced the number of stationary points (indicating changes in direction) and *vice versa* in vermiculite (Fig. 3.4A). Roots inducing increased occurrence of stationary points were preferentially consumed. A significantly higher amount of root tissue was fed from 7-week-old plants compared to 9-week-old ones grown in sand, and reversed in vermiculite; here, the older plant roots were preferentially consumed (Fig. 3.4B). Plant age affected the distance of larvae to stimulus compartments differently in both substrates, so that the interaction of substrate and age became an important predictor of larval orientation (Table 3.1).

3.4.3 Light intensity

In mycorrhiza-free plants, root respiration was increased by enhanced light exposure of plant shoots (479 $\mu mol \cdot s^{-1} \cdot m^{-2}$: 0.145 ± 0.002% CO_2; 791 $\mu mol \cdot s^{-1} \cdot m^{-2}$: 0.162 ± 0.002% CO_2, control CO_2 subtracted). Furthermore, enhanced light intensity led to an increase of the root biomass in 9-week-old plants (479

µmol·s^{-1}·m^{-2}: 145.02 ± 1.96g;791 µmol·s^{-1}·m^{-2}: 170.84 ± 2.56g), whereas the shoot biomass in these plant treatments stayed almost constant (479 µmol·s^{-1}·m^{-2}: 47.37 ± 0.89g;791 µmol·s^{-1}·m^{-2}: 46.93 ± 0.73) (Fig. 3.5B, C). When light intensity was enhanced, the larvae stayed at a greater distance to the plant (Fig. 3.2, Table 3.1). The effect of a change in light regime on larval orientation in arenas differed among substrates (Table 3.1). Interestingly, neither the number of stationary points of larvae (changes in direction, Fig. 3.4A), nor the post-experimental root consumption (Fig. 3.4B) were affected by changes in light regime with one exception: enhanced light intensity led to higher consumption of young, sand-grown, mycorrhiza-infested plants.



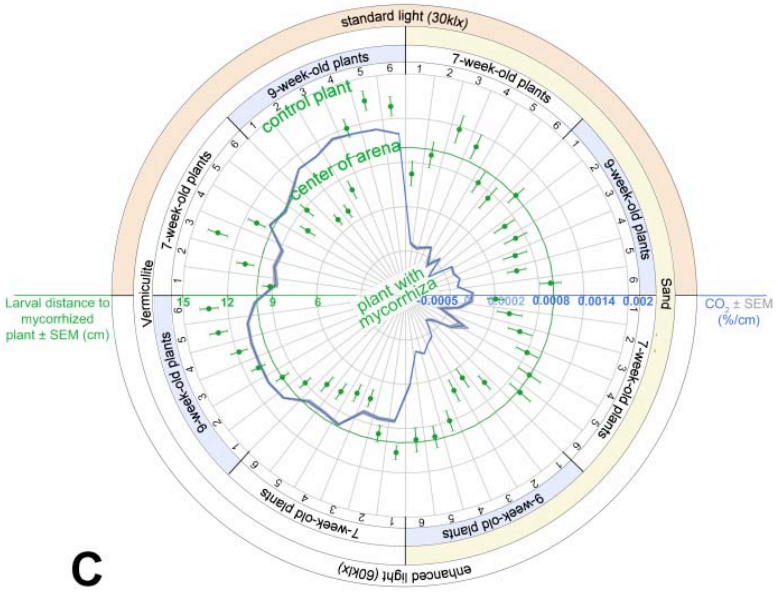


Figure 3.2: Larval orientation to host plants under standard (**A**) and enhanced (**B**) light intensity and larval orientation in arenas with AMF vs. AMF-free plants (**C**). Outer circles display experimental treatments. Green dots indicate the average larval position (± SEM; n=20 per treatment) during each hour of the experiment. The centre of graphs illustrates the stimulus compartment for A & B and the periphery illustrates the control compartment; for C the centre illustrates the AMF plant containing compartment and the periphery illustrates the AMF-free plant containing compartment. Read the graphs clockwise within each treatment from hour 1 to 6. The blue line displays CO_2 gradients between the compartments (shades: ± SEM; n=8 per treatment).

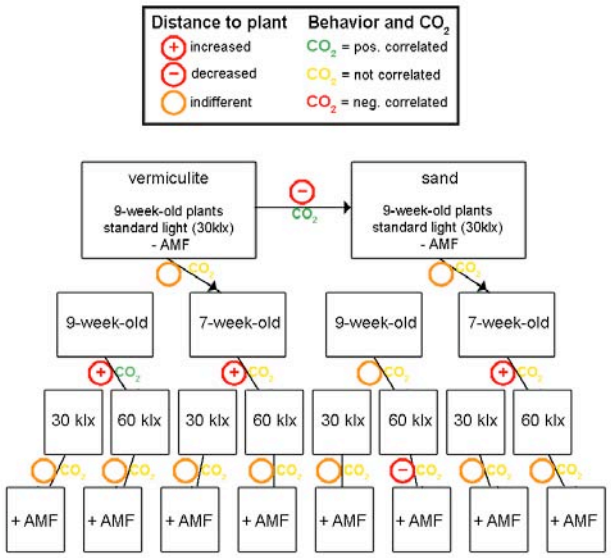


Figure 3.3: Organisational chart displaying the correlation of larval position to single factors of the applied treatments and CO_2 gradients compared by mixed effects models of respective subsets (n=20 larvae per treatment).

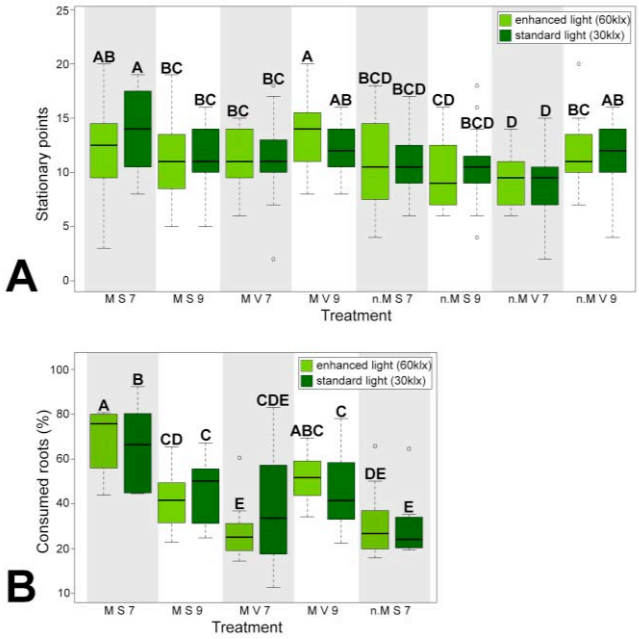


Figure 3.4: Number of stationary points (change of direction) of the larval paths through arenas (**A**) and percentage of consumed roots by larvae (**B**), after observations were terminated (n=20 per treatment). Different letters indicate significant differences ($p > 0.05$; Kruskal−Wallis test's).

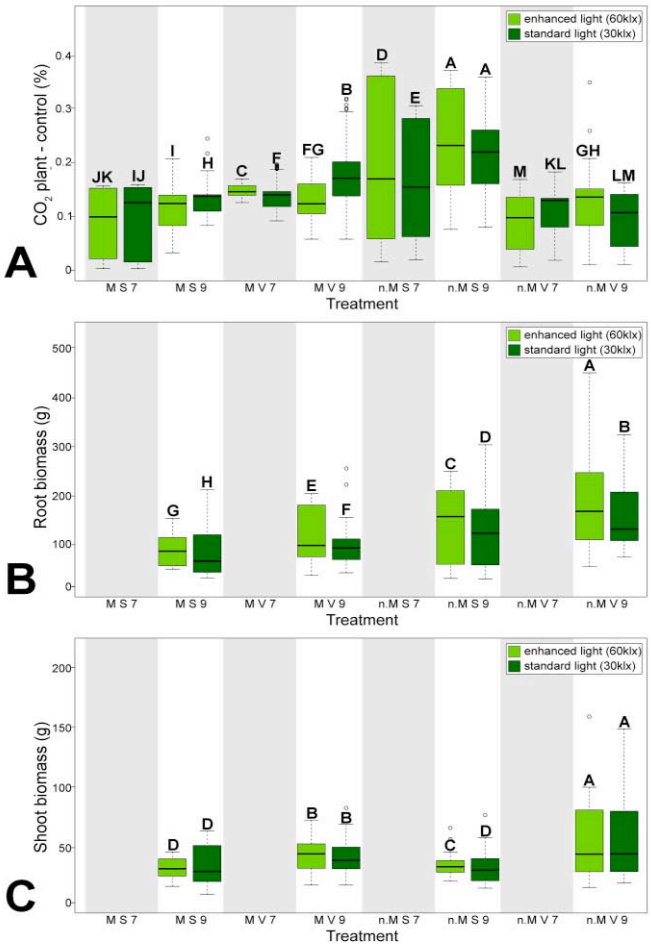


Figure 3.5: Plant stimulus compartment CO_2, control compartment CO_2 subtracted (n=8 per treatment) (**A**), root fresh weight (**B**) and shoot fresh weight (**C**) of plants (n= 20 per treatment). Different letters indicate significant differences ($p > 0.05$; Kruskal−Wallis test's).

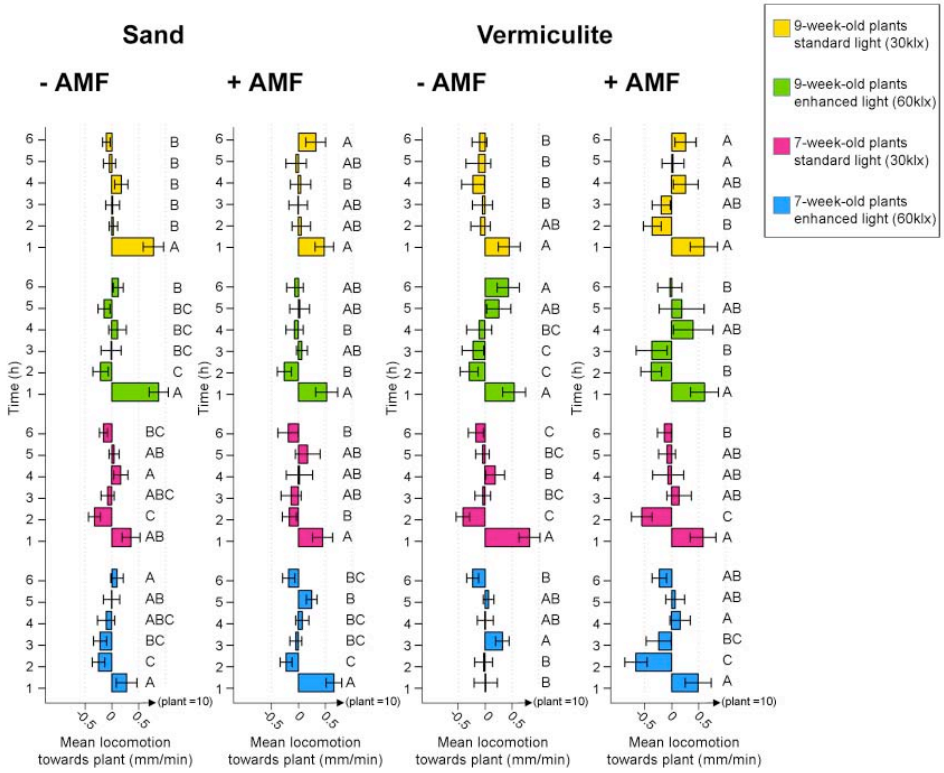


Figure 3.6: Larval locomotion towards plant stimulus compartment over time (treatment vs. substrate-filled control chamber, n= 20 per treatment, 6 observation points per hour). Letters indicate significant differences ($p > 0.05$; Kruskal−Wallis test's).

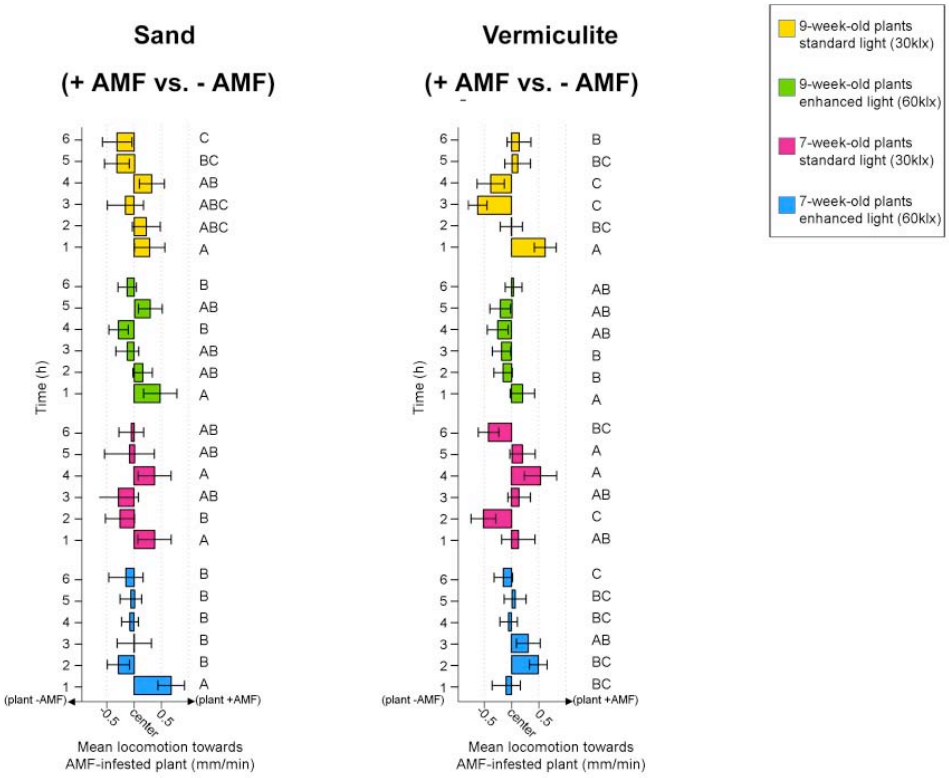


Figure 3.7: Larval locomotion towards compartments containing mycorrhiza infested plants (+AMF), measured for the different treatments over time (+ AMF plants vs. –AMF plants, n= 20 insects per treatment, 6 observation points per hour). Letters indicate significant differences ($p > 0.05$; Kruskal−Wallis test's).

3.5 Discussion

Our study showed that attractiveness of plant roots to root-feeding larvae is dependent on all biotic (mycorrhiza, plant age) and abiotic factors (light intensity, substrate) that were tested. Interestingly, the concentration of CO_2 in the rhizospheres of plants was negatively correlated with its attractiveness; the distance of larvae from roots increased with increasing CO_2 concentration (Table 3.1). This finding contrasts the results that show that CO_2 when offered per se is attractive to many soil-inhabiting arthropods, including M. melolontha (Hasler, 1986; Johnson and Gregory, 2006). The CO_2 gradients that we measured in our set-ups would in principle have been sufficient to induce attraction (0.001%/cm) and did not exceed the threshold of 3%/cm (Fig. 3.5A) at which the response to the gas has been described to turn from attractive to repellent (Hasler, 1986). Our results indicate that the attractiveness of CO_2 per se is masked by chemicals released from the plant roots, and other cues are likely applied by the larvae for host location.

3.5.1 Temporal dynamics, stationary points and root consumption

The larval position, orientation, and changes in direction (stationary points) underlie strong temporal dynamics within the timeframe of the conducted experiments. During the first hour, most larvae show distinct locomotion towards the plant (Fig 2, Fig. 3.6). Since the larvae were unable to reach the plant roots due to the restrictive mesh in the arenas, they were eventually forced to reduce their directed locomotion and started changing their direction back and forth between plant stimulus and control compartment. The frequency of stationary points was highest when mycorrhiza-infested plants were offered in the stimulus compartment, particularly in vermiculite (Table 3.1, Fig. 3.4). In nematode chemotaxis, increased turning events or pirouettes are observed if the animals reorient because they have lost track of an attractant gradient (Pierce-Shimomura *et al.*, 1999). In *M. melolontha,* however, the highest number of stationary points was observed in treatments where the larvae consumed the highest amounts of roots afterwards (Fig. 3.4A, B). Hence, the increased turning may show the increased effort of the larvae to overcome the obstacle and find a way to reach attractive compounds

released by the plant roots. We propose that the observed behaviour in *M. melolontha* larvae is fairly directional and resembles a combination of chemotaxis (directional pinpointing) and chemokinesis.

3.5.2 Mycorrhiza and plant biomass

In dandelion, infestation with mycorrhizal fungi (AMF) led to decreased biomass production of roots and shoots (Fig. 3.5A, B). These results are opposing the findings by Wu *et al.* (2011), who described an increase in root and shoot weight of *Prunus persica* (peach) due to inoculation with *G. mosseae* and *G. versiforme*. However, the authors also state that AMF-induced changes in plant traits depend on the fungal species. In our study, the decreased root surface area of AMF plants growing in sand was accompanied by decreased CO_2 emission, which indicates lower root respiration. However, in vermiculite even higher CO_2 values were measured in the rhizosphere of AMF-infested plants (Fig. 3.5A). Upon herbivore attack, plant defence-signalling pathways can be supported by AMF, thereby keeping herbivores away from plant root consumption (Oddsdottir *et al.*, 2010). Furthermore, fungal-induced excretion of repellent compounds may prevent root herbivory, or the roots might be more tolerant to herbivory, as the fungus supports the plant with nutrients (Bodker *et al.*, 1998; Vannette and Hunter, 2009). However, if nutrients are easily accessible to plants, the mycorrhizal associations may be parasitic rather than mutualistic, as the costs for the plant outweigh the benefits provided by the fungus (Bethlenfalvay, 1982; Bethlenfalvay *et al.*, 1982; Bethlenfalvay, 1983; Hendrix *et al.*, 1992; Johnson *et al.*, 1997; Brundrett, 2004). In the case of dandelion, the plant performs better without AMF. Additionally, observation of larvae in closer vicinity and increase of stationary points (turning events) in the presence of AMF plants indicate that these plants were preferred by the larvae. Hence, in addition to the weaker performance due to mycorrhizal colonization, dandelion plants associated with AMF may need to cope with a higher risk of herbivory. If both plant treatments are simultaneously provided, the larvae access the AMF-treated plant during the first hour (Fig. 3.7).

3.5.3 Substrate and plant age

In sand, the larval position and measured CO_2 followed a general temporal uniform trend (Mann-Kendall trend tests: tau = 0.044, 2-sided p-value =< 0.001 and tau = -0.553, 2-sided p-value =< 0.001, respectively). Over time, the CO_2 gradient in arenas declined and the larvae were tracked further away from the stimulus compartment. In vermiculite, however, the larval position did not follow a temporal uniform trend, albeit the CO_2 gradient in this substrate increased over time (Mann-Kendall trend tests: tau = 0.287, 2-sided p-value =< 0.001, respectively). Overall, according to the mixed-effects model, the type of substrate affected the behaviour of the larvae markedly (Fig. 3.2, Table 3.1). Although the larvae moved faster in vermiculite (V) than in sand (S) during the 6h of observation (V: 0.129 ± 0.002 mm/min; S: 0.093 ± 0.002 mm/min), the frequency by which the larvae located the stimulus compartment decreased (Fig. 3.2, Table 3.1). This may be explained by the physio-chemical properties of the substrates. Due to differences in specific surfaces, pH (Oades, 1988), but possibly also due to differences in density, aeration, and moisture, the mobility of percolating substances highly depends on substrate types. Vermiculite is a three-layer clay mineral and has a remarkable adsorption potential for various compound classes (Abate and Masini, 2007; Abollino *et al.*, 2007; Froehner *et al.*, 2009). Hence, vermiculite may adsorb important host plant cues for the larvae, thus impairing larval orientation. The CO_2 concentrations did not linearly decrease in vermiculite-filled arenas compared to sand-filled arenas (Fig. 3.5A), although the increased water-binding capacity of the clay mineral could potentially account for an increased binding of CO_2. The soil in which the larvae were collected in the field contained fractions of both sand and clay, but also organic matter. Hence, the adsorption capacity of the natural soil particles is higher compared to sand, but lower compared to vermiculite.

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Chapter 4

Novel methods for studying chemical plasticity in root exudates

4.1 Abstract

Root exudation has been studied intensively in the past and the relevance of contained infochemicals for edaphon species, such as soil-dwelling insects has been realized. However, little knowledge is available on biotic and abiotic factors, which provoke intraspecific variations of root exudates. We examined root exudation of volatile compounds and sugars in the ruderal plant dandelion (*Taraxacum sect. ruderalia*) applying solid phase extraction and aqueous extraction techniques. The root exudation patterns were examined with respect to biotic (mycorrhiza infestation and plant age) and abiotic factors (substrate type and light intensity). Furthermore, we determined recovery of various volatile compounds and sugars in sand, vermiculite and natural soil to evaluate their potential availability to edaphon species. Our results show that the release of volatile compounds (mainly sesquiterpenes) and sugars (glucose, fructose and sucrose) underlies a strong plasticity, and specific patterns of concentrations are established in the rhizosphere, depending on the applied plant treatments. The developed techniques are particularly suitable to extract and examine volatile and non-volatile root released compounds from rhizopheres with minimum disturbance to the plant roots.

4.2 Introduction

Root exudates mediate communication and interaction among plants, between plants and symbionts, destruent or consumer communities, including plant pathogens and herbivores, or on multitrophic levels (Kraffczyk *et al.*, 1984; van Tol *et al.*, 2001; Rasmann *et al.*, 2005; Bais *et al.*, 2006; Gu *et al.*, 2008; Hiltpold and Turlings, 2008; Köllner *et al.*, 2008; Kong *et al.*, 2008; Badri

and Vivanco, 2009). Hence, these compounds are of great interest as they may be used to manipulate plant-insect interactions. Volatile root derived signals have for instance been proposed as control agents for the Western corn rootworm (Hiltpold *et al.*, 2010).

Yet, little is known about how changes in root exudate composition are mediated by factors apart from herbivory, e.g. association with **a**rbuscular **m**ycorrhizal **f**ungi (AMF) or aboveground light conditions to undamaged plant roots. To our knowledge this is the first multifactorial approach evaluating changes in exudation patterns of undamaged plant roots. The quantity and composition of primary and secondary metabolites in roots or released by roots (e.g. sugars, organic acids, amino acids, terpenoids) may be affected by biotic factors such as nematode infestation (Wang and Bergeson, 1974) or AMF (Akiyama and Hayashi, 2002; Blee and Anderson, 2002; Eldhuset *et al.*, 2007; Wu *et al.*, 2011). Important abiotic factors affecting root exudation in plants are photosynthetic activity, leaf CO_2 uptake (Dilkes *et al.*, 2004), light intensity (Ferguson and Menge, 1982; Nagel *et al.*, 2006), phosphorous availability (Gransee, 2001; Hammond and White, 2008), water stress (Rogiers *et al.*, 2011), and desiccation (Katznelson *et al.*, 1954), (reviewed by Jones 2004).

Extraction and determination of root-released compounds in natural substrates involves difficulties, as concentrations are low and decomposition rates, adsorption to mineral material and quantity of non-target compounds may be high (Tang and Young, 1982; Kuzyakov and Domanski, 2000). Hence, the majority of previous studies obtain root exudate samples for chemical analysis from percolates, agar (Eldhuset *et al.*, 2007), filter paper (Wang and Bergeson, 1974), or hydroponics in which the plants were grown under hypoxic conditions (Szmigielska *et al.*, 1995; Cieslinski *et al.*, 1997; Neumann and Romheld, 1999). Another common method to obtain root-derived compounds is to remove the plants from their growth medium and dip the excavated roots in an aqueous solution (Ström *et al.*, 1994; Gransee,

2001). Finally, many studies imply exposure of plant roots to unnatural air or water-flows (Tang and Young, 1982; Weissteiner, 2010). Consequently, it remains unclear, if the detected compounds are also recovered from actual substrates or natural soil. Root exudation and recovery of compounds is markedly affected by the soil or substrate properties. In comparison to sand being a rather inert substrate, clay minerals like vermiculite may adsorb specific plant root derived compounds in large part.

The here presented sorptive extraction of compounds from substrate samples via polydimethylsiloxane (PDMS) tubing, also referred to as solid phase root zone extraction (SPRE) (Mohney *et al.*, 2009; Van Pinxteren *et al.*, 2009; Weidenhamer *et al.*, 2009), is particularly suitable for studying root exudation patterns in soil or substrate and with minimum disturbance of the rhizosphere. We optimized common SPRE techniques for GC-MS analysis of dandelion (*Taraxacum sect. ruderalia*, Kirschner, Øllgaard et Štěpánek) root exudates. The chemical plasticity of dandelion root derived volatiles and sugars was evaluated with regard to AMF infestation, plant age, light intensity, and substrate type. This study was conducted in search of chemical cues for the belowground host finding of dandelion-feeding cockchafer larvae (*Melolontha melolontha* L.), which have been shown to possess a broad chemosensory equipment, capable of detecting volatile and soluble substances (Eilers *et al.*, 2012).

4.3 Materials and methods

4.3.1 Mycorrhiza and natural soil

Rhizosphere soil was collected in the first week of October 2010 and the last week of April 2011 from a dandelion covered meadow in Hessenthal, Bavaria, Germany (49°93’ N, 9°26’O). A soil sieved (1mm mesh) subsample was used for determination of recovery rates. The pH of sieved soil (in $CaCl_2$ solution) was 5.90. Remaining root pieces were flushed out, before it was further autoclaved and baked at 200°C for 3hrs. From another subsample of field-collected soil, AMF spores were isolated and fractionated by wet sieving and

decanting (Gerdemann, 1963), followed by centrifugation in 60% (w/w) sucrose solution (1 min, 2000 rpm). Afterwards, the spores were rinsed and stored at 4°C for maximal 24 h. Dandelion (*T.* sect. *ruderalia*), wild carrot (*Daucus carota*), ribwort plantain (*Plantago lanceolata*) and clover (*Trifolium pratense*) were used as a trap culture to propagate and homogenize obtained spores (Oehl *et al.*, 2003, Fig. 4.1). The seeds were surface-sterilized with (2% w/w) dichloroisocyanuric acid (DCCA, Fluka) solution as described by (Krügel *et al.*, 2002). Seedlings were inoculated with the isolated spores at the two-cotyledon stage. Mycorrhization was monitored weekly via light microscopy after boiling plant root samples for 3min in 10% KOH (w/v), and dye in 0.05% tryphan blue in 5% acetic acid (Vierheilig and Piche, 1998).

Figure 4. 1: Mycorrhizal spores were extracted from rhizosphere soil of field-grown dandelion (**A**) and used to inoculate ribwort plantain (*Plantago lanceolata*), dandelion (*Taraxacum officinale*), clover (*Trifolium pratense*) and wild carrot (*Daucus carota*) (**B**). These plants served as a trap culture, from which an inoculum for dandelion seedlings was obtained (**C**).

4.3.2 Plants, substrates and growth durations

Dandelion seedlings were inoculated with chopped roots (ca. 20g) from the trap culture at the two-cotyledon stage (Fig. 4.1). Uninfested control plants were kept under identical conditions in separate climate chambers to avoid cross-infection with AMF. Both climate chambers were operated at 22°C and 70% r.h. under summer light conditions (L:D 16:8h) with a light intensity of 6.8klx. Control plants were treated with respective amounts of autoclaved inoculum. Ten five-week old dandelion plants were planted together into root exudates extraction chambers (Fig. 4.2), to compensate for differences among individual plants. The plants were moisturized *ad libitum* with nutrient solution modified from Arnon and Hoagland (1940) see (Reinecke *et al.*, 2008). The chambers consisted of a root compartment, perforated Teflon

discs, mounted on a stainless steel stick with screw thread, and a moisture compartment at the bottom of the silanized glass pots (Fig. 4.2). Polyester gauze, which was previously cleaned in scalding hot water, and inserted between the glass and substrate, allowed a nondestructive removal of plants from pots to insert the analytical tubing. The Teflon discs were adjusted to maintain a constant volume of 1.2l substrate in the root compartment. The materials Teflon, stainless steel and silanized glass have been chosen on account of their chemical inertness. The glass was externally covered with aluminum foil in order to prevent growth of algae. In the root exudates extraction chambers, the plants were grown another two weeks (7-week treatment) or four weeks (9-week treatment) at 18°C, 70% humidity and under summer light cycle (L:D 16:8h).

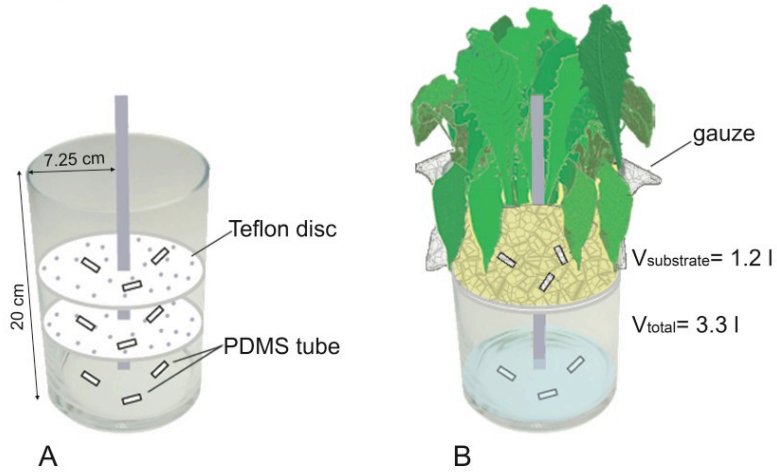

Figure 4. 2: Root exudate extraction chambers. The silanized glass pot contained two interspaced perforated Teflon discs, mounted on stainless steel (**A**). Pieces of analytical silicon tube (PDMS) were inserted into the moisture compartment at the bottom of the pot, which contained approx. 5mm, V= 80ml seeped out nutrient solution, between the discs, and inside the root compartment, before plants were inserted (**B**).

Two different substrates were compared in different experiments: fine burnt sand (pH($CaCl_2$)= 5.5, particle size ∅ = 0.4-0.8mm, specific surface area approx. 0.01-0.005m^2/g), and vermiculite (pH($CaCl_2$)=6.92, particle size ∅ = 2-3mm, specific surface area= 60-80m^2/g, CEC = approx. 80-150meq./100g). Furthermore two different light regimes were compared: half of the plants were exposed to 32.3 ± 0.48 klx = 417.29 ± 20.55µmol·s^{-1}·m^{-2} PAR and the remaining plants were exposed to 61.16 ± 0.72 klx = 791.37 ± 31µmol·s^{-1}·m^{-2}

PAR (Fig. 4.3, see light spectra for both treatments in Fig. S1). The same conditions were applied to substrate filled control pots (sand and vermiculite).

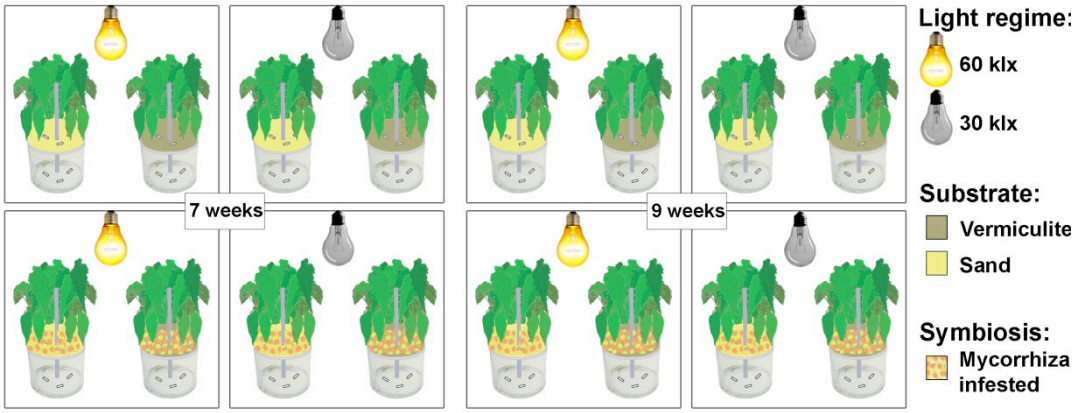

Figure 4. 3: Applied treatments to plants used for extraction and analysis of volatile and water-soluble compounds: two different growth durations (7 and 9 weeks), light regimes (32 and 61 klx equating to 417 and 791 µmol·s^{-1}·m^{-2}) and substrate types (sand and vermiculite) were compared in mycorrhiza infested and uninfested plants.

4.3.3 Sample collection and analysis of volatile compounds

Polydimethylsiloxane (PDMS) was chosen for *in situ* extraction of plant root derived volatiles in the rhizosphere. Pieces of analytical PDMS tube (inner diameter: 1.5mm, o.d.: 2.3 mm, length: 2.5mm, Reichelt Chemietechnik, Heidelberg) were cleaned for 8h in 4:1 (v/v) acetonitrile: methanol and preconditioned at 230°C for 12h under nitrogen flow in a tube conditioner (model TC 2, Gerstel). In pots of six-and-a-half-week old plants three PDMS tube pieces were inserted into each of the root compartments, on the lower perforated Teflon disc and at the bottom of each pot. For insertion of PDMS tube pieces, the plants were gently withdrawn from the pots by pulling the overlapping gauze and steel screw. The plant roots were allowed to grow through the Teflon disc and had direct contact to the tube pieces. The plants were cautiously irrigated on a daily basis, until minimum seepage from substrate was observed (approx. 5mm, equates to 80ml). After 5 days, the PDMS tube pieces were removed from pots and stored at -20°C until analysis after 1-5 weeks. When the plants were 8-and-a-half-week old, PDMS tube pieces were inserted into the remaining pots and left inside for 5 days, as described before. As external standard, 0.1µg 1-bromodecane (Sigma-

Aldrich) in 1µl dichloromethane was added to the PDMS tube pieces directly before analysis. The tube pieces were discarded after analysis. During the removal of the tube pieces, 15ml aliquots of rhizosphere substrate samples from 7- and 9-week-old plants and solution from the bottom of the pots were taken for analysis of sugars. The samples were immediately frozen in liquid nitrogen, and kept at -80°C until analysis. The relative quantities of volatile compounds collected on PDMS tube pieces were calculated from detected peak areas, corrected by external standard peaks.

4.3.4 Extraction and derivatization of sugars

Aliquots of 15ml frozen substrate (see above) were extracted twice with 15ml distilled water and sonicated for 10min in silanized glassware. The supernatant was extruded each time applying a suction filter. The yield (approx. 25ml) was lyophilized to dryness, weighted and dissolved to 2µg/µl pyridine in silianized glass vials. Phenyl-ß-D-glucoside (0.1µg, Fisher Scientific) was added as internal standard (Graham *et al.*, 2002). For derivatization, 10µl pyridine and 50µl (N, O -bis (trimethylsilyl)-trifluoroacetamide) (BSTFA) supplemented with 1% trimethylchlorosilane (TMCS) were added to 80µl sample solution and shaken for 90min at 37°C. Samples were subjected to GC-MS analysis after adding 900µl pyridine (total volume= 1ml, injection volume= 1µl). For quantification of detected sugars, calibration curves of standard compounds were obtained. Individual standard stock solutions were prepared and attenuated to final concentrations of 0.1, 0.2, 0.5, 0.8, 1, and 2µg of the respective sugar (raffinose (Fisher Scientific), melizitose (Alfa Aesar), lactose, cellobiose, trehalose, sucrose, maltose, mannose, rhamnose, (all Sigma Aldrich), glucose, fructose, galactose, arabinose, xylose (all Roth)), solved in 40µl pyridine. Standard samples were derivatized and analyzed analogous to the soil samples. Sugars were quantified comparing detected peak areas (corrected by internal standard) to the respective calibration curves obtained from standards.

4.3.5 GC-MS

Analysis of compounds adsorbed on the analytical tubes was conducted via a thermo desorption unit (TDU, Gerstel) coupled to a temperature-programmable vaporizing unit (Gerstel, KAS 4) on a GC (Agilent 7890 A) connected to a MS (Agilent 5975 C) operated in electron impact mode (70eV). Compounds were separated on a 30m x 0.25µm i.d. capillary column (HP-5MS, Agilent technologies) with 0.25µm film coating. Helium was used as carrier gas (constant flow 1ml/min). The TDU temperature was increased from 30°C to 210°C with a rate of 30°C/min and hold for 10min (1 min initial delay time, total desorption period = 18 min). Thermodesorbed compounds were trapped within the cooled injection system (KAS4) at -50°C. The GC run started with the injection system heated at a rate of 12°C/s to 220°C and hold for 5min. The GC oven program was set at 40°C for 5min, a temperature ramp of 5°C/min to 260°C kept for 7min (run time 56 min). Kovats retention time indices (KI) were calculated for each compound based on comparison with n-alkane standard compounds (C8-C20, 100ng/µl in hexane, Supelco). The following compounds were identified by comparison of KI and mass spectra to authentic reference compounds: β-elemene (Aapin Chemicals Limited), 2-ethyl-1-hexanol, methyl salicylate, 2-phenoxyethanol, α- and β-farnesene, trans-trans-farnesyl acetate (all Sigma-Aldrich), pethybrene and α-isocomene (extracted from *Petasites hybridus*, see (Saritas *et al.*, 2002), provided by Stephan H. von Reuß). The remaining compounds were tentatively identified by comparison of mass spectra and KI values with those available in the National Institute of Standards and Technology (NIST) MS library or in the MassFinder terpenoid library (Dr. Hochmuth, Hamburg, Germany).

For derivatized sugars, the same GC-MS instrument as described above was employed, except for a liquid injection port instead of the thermo desorption unit, which was kept at 240°C and operated in splitless mode. Compounds were separated on a HP1 capillary column (30m x 0.25µm i.d. with 0.25µm-film coating, Agilent technologies) with helium as carrier gas (constant flow 1ml/min). The initial oven temperature of 60°C was held for 3min, then

increased at 4°C/min to a final temperature of 300°C and held for 1min. The sugars were identified by comparison of mass spectra and retention times to those of previously measured standards.

4.3.6 Recovery of sugars and volatile compounds

For the determination of sugar recovery rates, glass vials (40ml screw top, Supelco, Sigma-Aldrich) were filled with 25ml sand, vermiculite or sieved and heated natural soil. In each case, 20ml water and 1ml of maltose, sucrose, glucose, fructose, mannose, arabinose and xylose solution, each at a concentration of 10µg/ml were added. After intense vortex agitation for 2 min, the samples were kept for 15min at 4°C, before sugars were subjected to aqueous extraction, lyophilization, derivatization and GC-MS analysis, as described above. The recovery rates of volatile compounds were established using the same vials, substrate and water volumes, but 100ng of each volatile compound. Conditioned pieces of analytical PDMS tube were inserted after agitation of vials containing the standard compounds, removed after 1hr and directly subjected to TDU-GC-MS.

4.4 Results

4.4.1 Solid phase root zone extraction of volatile compounds

The highest number of root-derived compounds and the lowest baseline was obtained from the perforated Teflon disc beneath the substrate chamber (Fig.4.4). In total, 18 plant root released compounds were detected of which 11 are identified as sesquiterpenes (Table 4.1). The amounts of detected methyl salicylate were very low, independent of plant treatment. In -AMF plants, the relative amount of 2-phenoxyethanol was higher in 9-week old plants grown under 60klx light exposure, but not in vermiculite grown plants (Fig. 4.5). In +AMF plants, less 2-phenoxyethanol was found in 9-week old plants compared to the two-week old younger plants. Panaginsene levels were constant in -AMF sand-grown plants and highest in vermiculite-grown, 9-week old plants under higher light exposure. Compound no.5 was in highest abundance in 9-week old, -AMF, vermiculite-grown plants with 60klx light exposure. The highest amounts of pethybrene were traced in sand-grown

+AMF plants. In -AMF the levels of this compound were lower in 7-week old plants with 60klx light intensity and higher with the same light and growth duration in vermiculite-grown plants. Seven-week old, vermiculite-grown plants with high light exposure released the highest levels of african-2-ene, independent of AMF. In contrast, α-isocomene was not detected in 7-week old plants –AMF under 60klx light exposure in both substrates, but was highest in the same treatments with +AMF plants. The highest amounts of β-elemene were also obtained from 7w-old plants exposed to 60klxlight. Compound no.10 was present in very low amounts with highest levels in –AMF, sand-grown, 7-week old plants with 30klx light exposure. Detected levels of cadinene were very low and the highest amounts were found in 9-week old +AMF plants, vermiculite-grown, with 30klx light exposure. In infested as well as -AMF plants, cadinene was always lowest in 7-week old plants under the lower light regime.

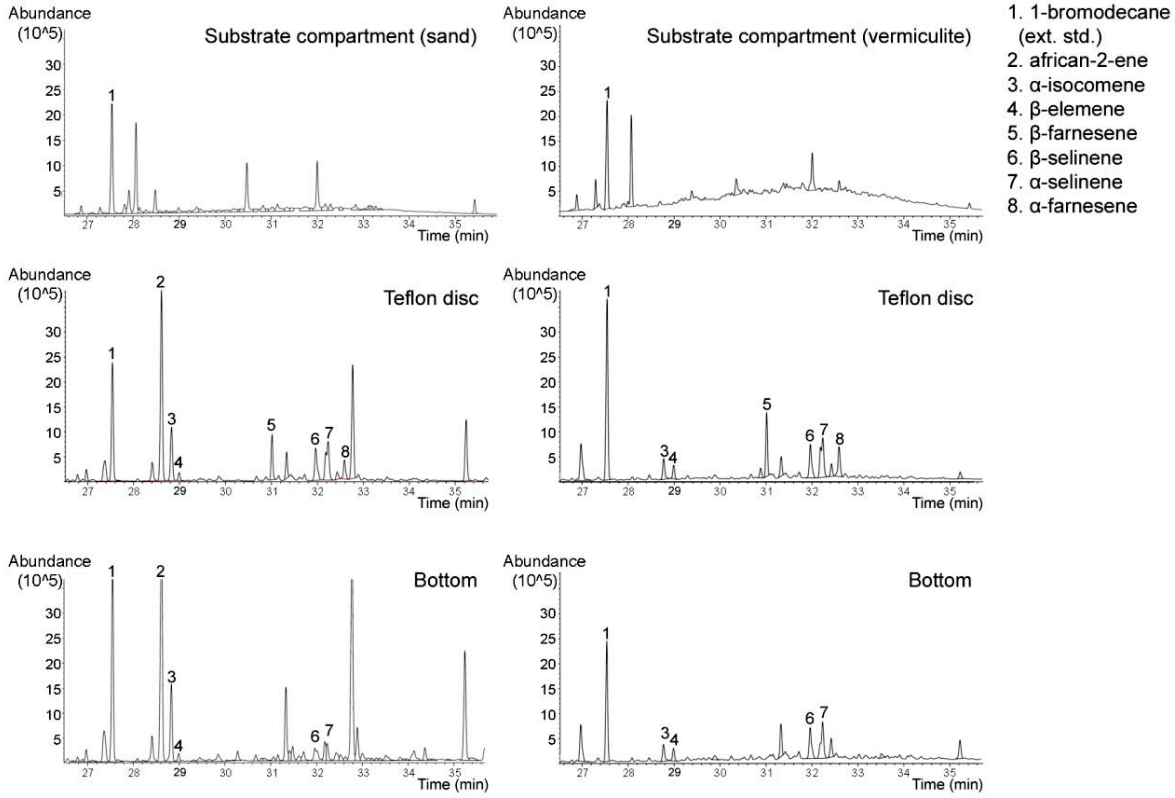

Figure 4. 4: Total ion chromatograms of volatile compounds from the root compartments, Teflon disc and bottom of the same pot, containing +AMF dandelion plants grown on sand (left) or vermiculite (right) for 7 weeks exposed to 30klx (417.29 $\mu mol \cdot s^{-1} \cdot m^{-2}$) light. Note that the ratio of target compounds to contaminations is lowest in the Teflon discs compared to root and moisture compartments.

Table 4.1: Identity of volatile compounds detected in root exudates of differently treated dandelion plants. Volatiles were collected with solid phase root zone extraction (SPRE). ♦: Compound identified by comparison to compound standard (mass spec. match >95% and KI ± 5), ●: Compound identified via NIST library (mass spec. match >95% and KI ± 5). ♣: Compound identified via Massfinder.

#	Ident. proced.	Compound	KI
1	♦	*2-ethyl-1-hexanol (likely a contamination, see chapter 4.5.2)*	1032
2	♦	methyl salicylate	1195
3	♦	2-phenoxyethanol	1221
4	♣	panaginsene	1335
5		unknown. M/z: 189(100),93(66),107(64),133(59),204(55)	1375
6	♦	pethybrene	1377
7	♣	african-2-ene	1385
8	♦	α-isocomene	1388
9	♦	β-elemene	1398
10		unknown. M/z: 147(100),105(23),148(17),91(16),119(15)	1421
11	●	(+)-cadinene	1447
12	♦	β-farnesene	1458
13		unknown. M/z: 109(100),110(65),204(43),95(35),79(35)	1463
14	●	γ-selinene	1478
15	♣●	β-selinene (eudesma-4(14),11-diene)	1485
16	♣●	α-selinene	1489
17	♦	α-farnesene	1507
18	♦	trans-trans-farnesyl acetate	1843

Root exudates of most 7-week old plants contained relatively high levels of α- and β-farnesene. The compounds no 13 and γ-selinene were present in only minor amounts, with highest levels of γ-selinene in –AMF, 7-week old, sand-grown plants treated with 60klx light intensity. Similar to the farnesene isomers, the patterns of detected α- and β-selinene among the different treatments were similar for these isomers. The highest amounts were present in root exudates of 7-week old plants exposed to 60klx light intensity. Alterations due to age and light on exudation of this compound were minimized in +AMF vermiculite-grown plants. Trans-trans-farnesylacetate was

only a trace compound in most plant samples and highest levels were observed in vermiculite-grown plants, particularly in 7-week old +AMF plants with 30klx light exposure.

The recovery rates of volatile compounds from water were highest for β-elemene and β-farnesene (Table 4.2). The tested terpenoids other than linalool were recovered from vermiculite almost as well as from water, to a lower extent from sand and poorly from soil. Moreover, hexyl acetate and butyl acetate were recovered to a great extent from vermiculite. Less than 10% of trans-trans-farnesylacetate, cinnamal, 1-hexanol, and butyl acetate were recovered from water. Compared to the amounts recovered from water, the amounts recovered from sand for trans-trans-farnesylacetate, cinnamal, and 1-hexanol were almost identical. Furthermore approx. 80% of methyl salicylate, hexyl acetate, and benzaldehyde recovered from water were recovered from sand. For soil, relatively high recovery was determined for trans-trans-farnesylacetate, cinnamal, benzaldehyde and linalool.

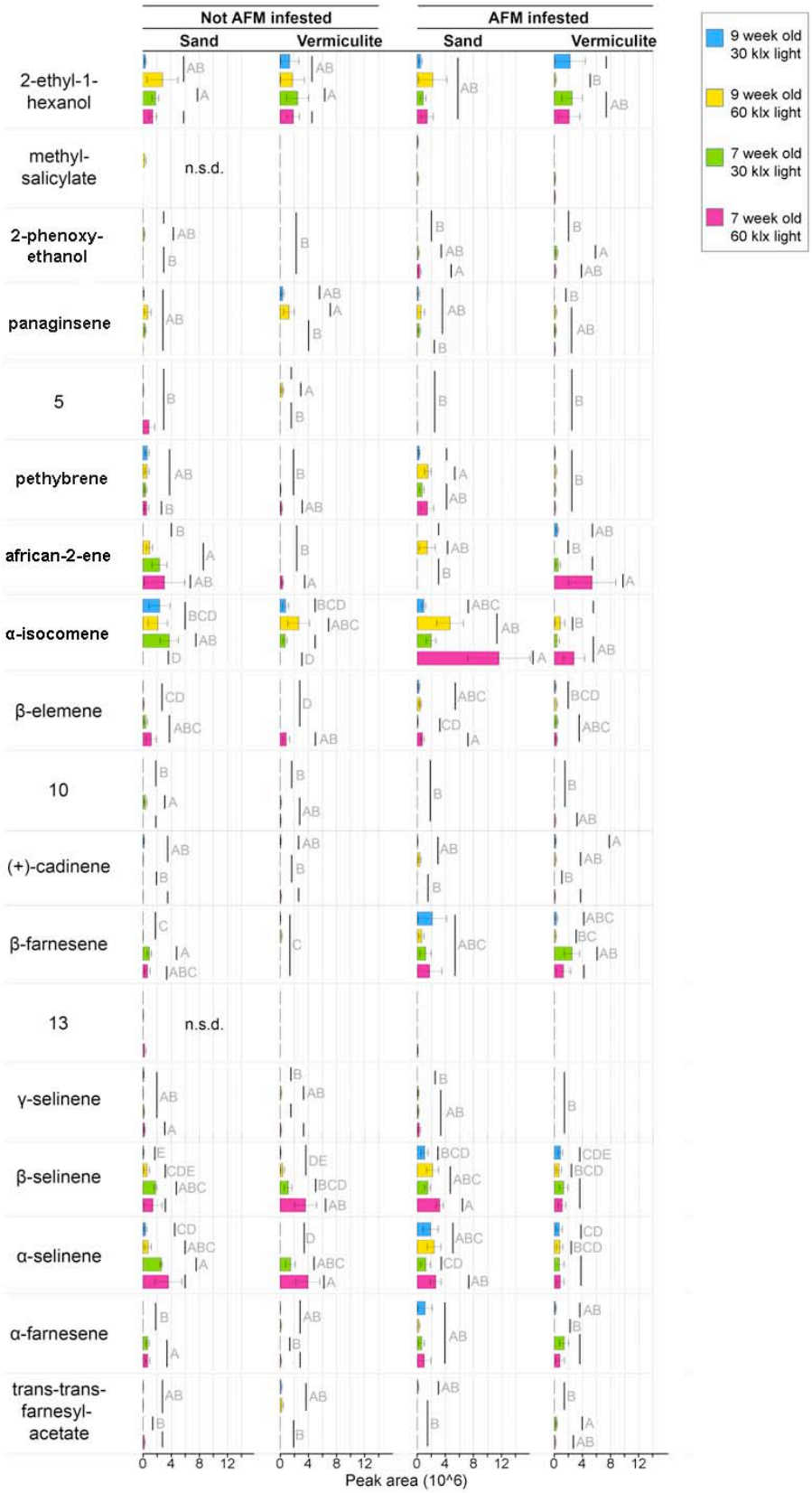

Figure 4. 5: Peak areas of volatile and compounds traced on PDMS tube pieces in Teflon disc for the 16 different plant treatments (n=6 plant pots à 10 plants per treatment). Different letters indicate significant differences ($p > 0.05$; Kruskal−Wallis test's). n.s.d.= no significant difference.

4.4.2 Sugar content and recovery

Three different sugars are detected in the substrate compartment of dandelion plants: fructose, glucose and sucrose (Fig.4.6). Apparently, the differences between samples of the same treatments are very high, accounting for high standard errors. The amounts of all three sugars are highest in mycorrhiza-uninfested (-AMF) plants grown under 60klx light intensity for 7 weeks on sand (S7H), followed by MS9H. Fructose levels are generally low, particularly for vermiculite grown plants, independent of AMF, growth duration or light regime. Glucose levels are significantly higher under high light intensity in rhizosphere samples from +AMF plants grown for 7 weeks on vermiculite (MV7H, MV7L), 7-week old –AMF plants grown on sand (S7H, S7L), and 9-week old plants on vermiculite (V9H, V9L). Similarly, 7-week old +AMF plants on sand release higher amounts of sucrose in the higher light intensity treatment (MS7H compared to MS7L). The same effect is found for nine-week old +AMF plants on vermiculite (MV9H, MV9L), -AMF plants on sand (S7H, S7L and S9H, S9L), and 9-week old -AMF plants grown on vermiculite (V9H, V9L). None of the sugars are traced in the root exudates obtained from the bottoms of the glass pots (data not shown). The recovery rates of all sugars are highest in sand and lowest in natural soil (Fig.4.7).

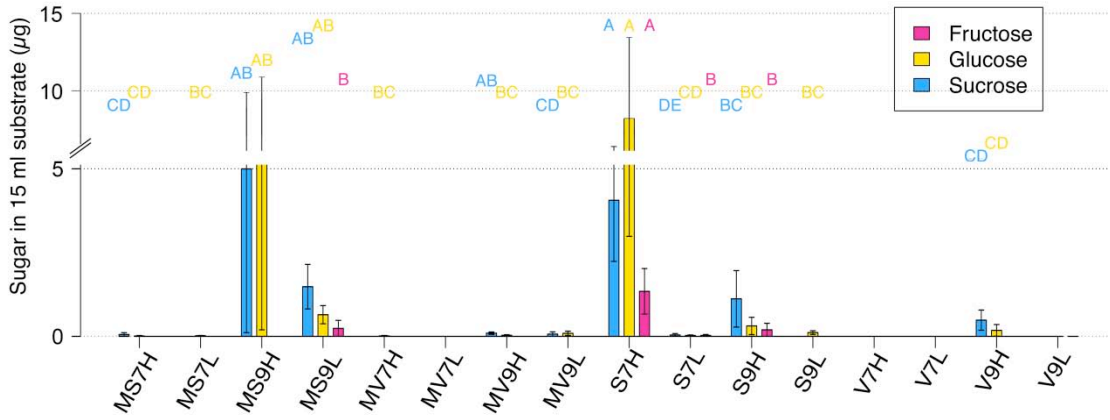

Figure 4. 6: Sugar content extracted from sand (S) and vermiculite (V) samples of mycorrhiza infested (M) or uninfested plants, grown for 7 or 9 weeks under 30klx (L) or 60klx (H) light intensity. Different letters indicate significant differences ($p > 0.05$; Kruskal−Wallis test's), error bars display the standard error (n=6 per treatment). Note that the y-axis is not continuous.

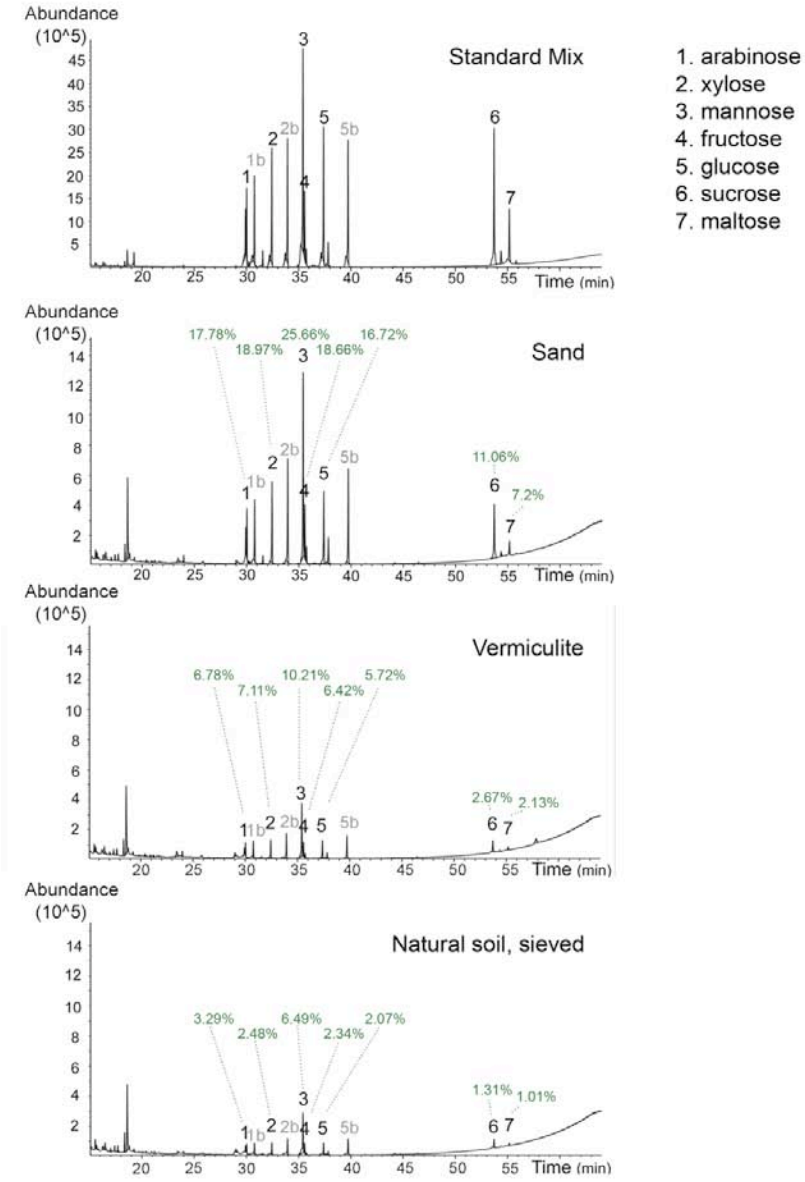

Figure 4. 7: Recovery rates of sugar standards in sand, vermiculite and autoclaved, dried and sieved natural soil compared to a standard mix in water. Trimethylsilyl derivatives of arabinose, xylose and glucose are each represented by two peaks, a phenomenon that is commonly observed in gas chromatograms of silylated monosaccharides (Medeiros and Simoneit, 2007).

Table 4.2: Percentage of compounds recovered from sand, vermiculite and autoclaved, dried and sieved natural soil, compared to compounds recovered from water (± SE, n=4). Extraction of compounds was accomplished applying SPRE on PDMS tube pieces.

Compound	Peak area (%)	Recovery from moist substrates		
	water	sand	vermiculite	soil
β-elemene	100.00 ± 0	13.01 ± 3.09	24.96 ± 4.67	18.27 ± 5.69
β-farnesene	68.14 ± 4.91	44.81 ± 3.46	73.41 ± 8.54	3.37 ± 1.10
α-farnesene	46.80 ± 6.52	45.06 ± 4.21	75.59 ± 8.02	1.81 ± 0.92
methyl salicylate	15.55 ± 2.14	91.02 ± 13.12	13.63 ± 1.51	32.41 ± 2.33
farnesyl acetate	3.96 ± 1.01	77.14 ± 8.30	13.15 ± 1.59	82.44 ± 15.76
α-pinene	19.85 ± 2.03	48.16 ± 5.67	60.71 ± 4.98	28.96 ± 3.02
hexyl acetate	31.51 ± 5.54	79.20 ± 11.69	82.20 ± 11.20	29.32 ± 2.86
α-cedrene	14.24 ± 2.05	54.34 ± 6.18	32.44 ± 4.24	1.42 ± 0.62
cinnamal	6.37 ± 0.57	87.12 ± 15.68	52.45 ± 6.83	60.76 ± 7.09
linalool	18.47 ± 2.25	45.96 ± 7.85	19.33 ± 2.27	51.86 ± 6.32
butyl acetate	8.55 ± 0.74	35.76 ± 5.86	80.28 ± 10.78	36.75 ± 4.04
benzaldehyde	15.47 ± 1.38	88.32 ± 17.74	42.69 ± 6.38	87.65 ± 13.78
1-hexanol	3.70 ± 0.41	93.68 ± 14.55	55.71 ± 5.33	2.92 ± 1.28

4.5 Discussion

4.5.1 Extraction and analysis techniques

Solid phase root zone extraction (SPRE) is a useful, convenient and low priced tool for analysis of root-released volatiles. In conjunction with a growth chamber design, consisting of perforated Teflon discs in silanized glass vials, volatile and soluble compounds can be extracted without applying artificial air or water flow, or flooding of the root zone. Compared to the root and moisture chamber, extraction of compounds in the perforated Teflon disc of glass pots delivered best results in terms of detected compounds and contamination avoidance (Fig. 4.4). The root-derived substances in the root compartment may leach, degrade or be absorbed by the substrate and mucilage, leading to a high contamination of samples with non-target compounds, particularly observable in vermiculite. Although the formation rates for microbial biofilms are similar on Teflon and glass (Vanderkooij *et al.*, 1995), the likelihood of the

tubes being covered by microbial biofilms, which clog the material and prevent further uptake of target compounds, may be increased in the root compartment. However, the latter described effect cannot explain the low levels of detected plant released volatile compounds in the liquid phase at the bottom of the pots. Here, low water-solubility and high volatilization of the compounds may prevent them from entering the vicinity of the analytical tubing, which was usually superimposed by liquid. By volatile collection between the Teflon disc, the plant roots are relatively close to the substrate, in a humid atmosphere, but the matrix effect from the substrate is minimized. Interestingly, most of the plant-released volatiles are not detectable in the substrate matrix, whereas sugars were exclusively detected in the substrate chamber and not from the drainage below the rhizosphere. However, other water-soluble compounds, not investigated in this study but of great interest in root exudation research, such as flavonoids, organic or amino acids, may be primarily found in the drainage chamber. Hence, we propose that the combination of a root and a moisture-containing chamber, separated by permeable but inert material, provides best results for simultaneous determination of root-derived volatile and soluble compounds. A similar multi-chamber system has been proposed for monitoring gaseous compounds and pH from *Juncus* plants (Blossfeld *et al.*, 2011). Other than aerial plant parts, roots or tubers are releasing relatively few volatile compounds and in low doses (Karlsson *et al.*, 2009). Similarly, dandelion is releasing relatively low amounts of volatile compounds from undamaged roots, and the presented extraction procedure was adapted to the low emission. However, the durations of respective steps (extraction, thermodesorption. GC-temperature program) may be adjusted to the target plant species, treatment and compounds of interest.

4.5.2 Volatile compounds

Out of the 18 detected compounds in dandelion root exudates, 16 compounds are altered in concentration by mycorrhiza infestation, plant age and abiotic factors (substrate type, light intensity, Fig. 4.5). However, the variations in root-derived substances are not linear to certain treatments, but specific

patterns are observed:for instance some compounds are released in higher amounts at higher, others at lower light intensity etc. Although 2-ethyl-1-hexanol has not been found in controls, this compound is most likely a contaminant, e.g. originating from the sowing trays and stored in the plant roots (Nalli *et al.*, 2006). The benzenoid compound 2-phenoxyethanol is a rather seldom plant compound, occurring for instance in flowers of the genus Narcissus (Dobson *et al.*, 1997) or faba bean flowers (Griffiths *et al.*, 1999).
Methyl salicylate is a plant-signaling compound, in most cases indicating an induced plant response upon herbivore or mechanical damage of plant tissue (Deng *et al.*, 2005). Due to the low abundance of methyl salicylate in dandelion samples, we hypothesize that the injury of the plants caused by insertion or removal of PDMS tube pieces was marginal. Similarly, the release of volatile sesquiterpenes, such as α- and β-farnesene is commonly increased in damaged, stressed, or jasmonic acid treated plants (Schmelz *et al.*, 2001; Pettersson, 2007). Interestingly, the farnesene compounds are emitted from plant roots in higher amounts if plants are infested with mycorrhizal fungi. Panaginsene, a sesquiterpene hydrocarbon found in minor amounts in dandelion root exudates, was first discovered essential oils from ginseng root (Richter *et al.*, 2005). Interestingly, also other ginseng root compounds were found in dandelion root exudates: the prevalent sesquiterpenes β-elemene, β-selinene, β-farnesene, but also the rather seldom african-2-ene. African-2-ene levels were very low in mycorrhiza-uninfested vermiculite grown plants compared to the same treatments with mycorrhiza, whereas the opposite was the case for sand grown plants (higher levels without mycorrhiza). Under acidic condition, the sesquiterpene hydrocarbon pethybrene rearranges to the structurally related α-isocomene (Saritas *et al.*, 2002). This phenomenon is also found in dandelion root exudates: the *per se* slightly acidic sand (pH($CaCl_2$)= 5.5) contains higher levels of α-isocomene compared to pethybrene, but also neutral substrate vermiculite (pH($CaCl_2$)= 6.92) contains less of the latter compound.

4.5.3 Sugars

Various studies, mostly conducted on plants grown in sterile hydroponics, describe great amounts and diversities of root-released sugars (e.g. reviews by Dakora and Phillips, 2002; Badri and Vivanco, 2009; Dennis *et al.*, 2010 and references therein). In dandelion rhizosphere samples, however, only sucrose, glucose and fructose are found, and detected amounts are rather low (Fig.4.6). It has been shown in several studies, for instance in root exudates of rice (*Oryza sativa*) (Bacilio-Jiménez *et al.*, 2003), and alfalfa (*Medicago sativa*) (Hamlen *et al.*, 1972) that the amounts of root-secreted sugars decline with increasing plant age. Hence, a higher variety of sugars may be detected in exudates of dandelion plants, younger than 7 weeks. Light regime and substrate type have the highest impact on sugar concentration: low light intensity exposure and growth on vermiculite results in decreased sugar levels detected in the dandelion rhizosphere.

The substrate effect on detected sugar concentrations may partly be explained by the low recovery of sugars from vermiculite and clay mineral containing soil, compared to sand samples. However, the recovery rate of fructose in vermiculite is even higher than the rate of glucose and sucrose. Hence, the total lack of fructose and simultaneous presence of sucrose and glucose in vermiculite samples indicates substrate dependent variations in fructose root exudation. Furthermore, a higher uptake of the substance by the substrate would most likely partly be compensated by a higher exudation, given that the release of sugar occurs mostly passive, following a concentration gradient from plant tissue to soil solution (Jones and Darrah, 1993). Mycorrhizal fungi have been described before to alter glucose and sucrose levels in root tips (Blee and Anderson, 2002; Wu *et al.*, 2011). We show that the alteration in sugar secretion of mycorrhizal infested plant roots is age-dependent: in sand-grown plants, mycorrhiza infestation results in decreased sugar levels in 7-week old plants, but increased exudation in 9-week old plants. Simultaneous derivatization of different sugars of the same concentration in one sample may result in different peak areas for each

compound. The disaccharides sucrose and maltose show a lower recovery in all tested substrates, compared to the monosaccharides.

4.5.4 Root released infochemicals for edaphon species

According to our data, dandelion produces various volatile compounds (mostly sesquiterpenoids) and sugars, which could function as belowground chemical cues. According to Johnson and Nielsen (2012), short-chain-alcohols, -aldehydes and -esters are commonly observed to initiate attraction behavior, whereas long-chain hydrocarbons are mostly repellent to belowground insects. In soil, the recovery of aldehydes and esters is relatively high (Table 2.2), so that these compound classes appear to be particularly suitable infochemicals for edaphon species. Terpenoids have been described to be released from plant roots (Kai *et al.*, 2009) and to induce attraction to soil dwelling insects (Robert *et al.*, 2012). Indeed, many terpenoids could be recovered to a great extent from water (Table 2.2). However, most experiments on describing terpenoids as cues for root-feeding insects (e.g. *Diabrotica virgifera virgifera*), use pure white sand, although the natural soil for the model insect would absorb a great proportion of the compounds. Sugars are present in dandelion rhizospheres and recoverable from sand. However, these compounds may only function as chemical cues when provided without natural biota, which would decompose the carbon rich molecules. We propose that biotic and abiotic factors and particularly the properties of the substrate have an important impact on the function of compounds as chemical cues.

4.6 Acknowledgements

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Chapter 5

Behavioral responses of *Melolontha melolontha* larvae to potential chemical cues

5.1 Abstract

Root-feeding *Melolontha melolontha* L. larvae orient in gradients of carbon dioxide (CO_2). Evidence from the morphological, electrophysiological and behavioral studies presented in the previous chapters of this dissertation strongly suggests that additional cues are used by the larvae to locate their host plants. In search of chemical cues beyond CO_2, we tested the behavioral response of the larvae to gradients of volatile and non-volatile root-derived substances. Out of the 40 compounds tested, 9 components elicited a behavioral response: propionic acid, γ-terpinene, benzaldehyde, and mannose are repellent, (+)-camphene, α-pinene, acetone, 1-hexanol, and sucrose attract the larvae. These findings demonstrate that the larvae mayuse volatile and non-volatile root-derived substances of various compound classes for belowground host location in addition to CO_2. The capability to use a broad variety of cues enables the larvae to locate plant roots.

5.2 Introduction

Belowground host plant location and acceptance by rhizophagous insects are still poorly understood today. Research on the chemical ecology of subterranean insects is particularly challenging due to interactions of infochemicals with the substrate and difficulties in observing the insects. However, the lack of visual stimuli also offers the opportunity to study exclusively chemically mediated insect behavior.

Various soil-dwelling insects can pinpoint CO_2 sources belowground (Johnson and Gregory, 2006). Our model insect, larvae of the European cockchafer *Melolontha melolontha* (L., 1758) (Scarabaeidae: Melolonthinae), are attracted to even weak gradiens of 0.001%/cm CO_2 in soil (Hasler, 1986). The ubiquitous gas was supposed to be their only, or at least main attractant belowground (Klingler, 1957; Hasler, 1986). The most preferred host plant of the larvae is dandelion (*Taraxacum sect. ruderalia*) (Hauss and Schütte, 1977). Reinecke *et al.* (2008) proposed that in dandelion, root derived substances other than CO_2 are involved in host location and some of these substances can mask the attractiveness of plant root emitted CO_2 for *M. melolontha* larvae. The larvae are equipped with multiple types of olfactory and gustatory sensilla on their antennae and mouthparts, which enable them to detect multiple compounds present in the rhizosphere, such as acids, alcohols, aldehydes, amines, esters, ketones and monoterpenes (Eilers *et al.*, 2012). Many of the electrophysiologically active compounds are behaviorally active, as evidenced by the data presented in this chapter.

Plant roots release a blend of various volatile but also water-soluble compounds (e.g. Szmigielska *et al.*, 1995; Gransee and Wittenmayer, 2000; Fan *et al.*, 2001 and reviews by Rovira, 1969; Bertin *et al.*, 2003; Bais *et al.*, 2006; Dennis *et al.*, 2010). The composition of these so-called root exudates is specific for each plant species but also varies intraspecifically with cultivar, age, and biotic and abiotic stresses (Bertin *et al.*, 2003). Hence, root-excreted substances other than CO_2, for instance sugars and non-volatile organic acids may deliver specific information to root-feeders. The components are detectable in characteristic distances from their source, depending on the compound properties, mineral composition, soil chemo-physical properties (e.g. temperature and soil moisture), and biotic degradation (Kuzyakov and Domanski, 2000; Jones *et al.*, 2009).

A root-derived substance that is detected and used by an insect for host root location needs to cover a certain distance through soil, establish a distinct gradient, and protrude prevailing bioturbation and chemical and biotic

degradation. Similar to aqueous media, non-volatile and volatile compounds co-occur and establish characteristic gradients in soil, which makes the discrimination between olfactory and gustatory cues difficult (Caprio, 1977). For instance, the most commonly described belowground attractant CO_2 may be detected as an airborne stimulus, or as carbonic acid, solved in the soil solution.

The multitude of plant root derived compounds are of low volatility or nonvolatile (e.g. reducing sugars (Azaizeh *et al.*, 1995)). Together with a number of terpenoids, recoverable amounts of sugars (sucrose, fructose and glucose) were extracted and analyzed from the rhizosphere of undamaged dandelion roots (see chapter 4 of this dissertation). Here, we tested if *M. melolontha* larvae are able to use these sugars for belowground host plant location. While odor-guided behavior in soil-dwelling insects has been described before, e.g. by Robert *et al.* (2012), nonvolatile substances like sugars have primarily been described as phagostimulants in soil dwelling insects (Johnson and Gregory, 2006). In this study, electrophysiologically active volatile and non-volatile but water-soluble compounds were tested for their behavioral relevance as potential host cues.

5.3 Materials and methods

5.3.1 Insects

Third instar *M. melolontha* larvae were collected during the second week of August 2011 from a golf course near Apeldoorn, Gelderland, the Netherlands (52°21'N, 6°12'O). All larvae were kept in separate pots filled with highly sorptive clay substrate (Klasmann-Deilmann GmbH, Geeste, Germany). The pots were kept in a climate chamber under dark conditions at 14°C with 70% humidity. Only active animals were used once in behavioral assays and discarded afterwards.

5.3.2 Behavioral assay

Trials were carried out in a circular maze subdivided into six segments of equal size (total volume ≈ 750cm^3, height = 1.5cm), using fine burnt sand (particle size ∅ = 0.4-0.8mm) as substrate. The compounds were applied 20 min before the larvae were inserted, so that gradients could establish. Compounds were tested in groups of 8:

1) sugars: arabinose, fructose, glucose, maltose, mannose, sucrose, raffinose, xylose.

2) organic acids: acetic, citric, formic, fumaric, lactic, malic, oxalic, propionic acid.

3) monoterpenes: (+)- and (-)-camphene, (+)- and (-)-limonene, linalool, α-pinene, α- and γ-terpinene.

4.) Alcohols, ketones and aldehydes: acetone, benzaldehyde, 1-butanol, cinnamal, 1-hexanol, methanal, propanal, 1-propanol.

5) Esters and amines: butyl acetate, butylamine, hexylamine, ethyl acetate, methyl acetate, pentylamine, propyl acetate, propylamine.

Within each group, each compound was tested equally often (n=54) and in any of the six fields of the maze: 9-times per each of the six fields of arena, n=144 trials per group (Fig. 5.2). The position of the three control fields in the maze was opposite to the three stimulus fields, positions were arranged alternating between trials (fields A,C,E or B,D,F, see Fig. 5.1). Five minutes after moisturizing the sand it was evenly soaked and the larvae were inserted into the center of the maze. Setups with larvae were left in a dark room at 18°C, 70% humidity, and larval position was recorded after 90 minutes. If the larva entered the distal third of a field (distance > 10 cm from center), it was considered as response, in any other case (distance < 10 cm from center) the larva was considered to be non-responding.

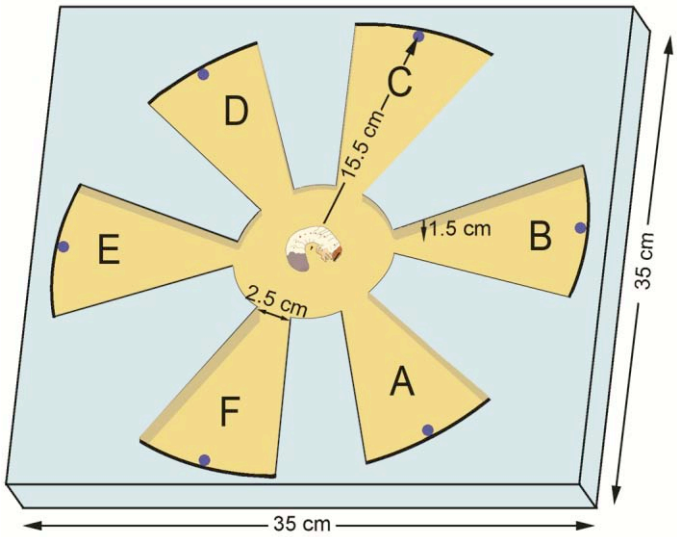

Figure 5. 1: Sand - filled six-field bioassay. Stimuli (blue dots) were applied at the end of three fields (e.g. A,C,E) and interspaced with solvent controls (e.g. B,D,F). To avoid position effects, each stimulus was tested in each field (n=54, 9 replicates per field). The larval position was noted 90 min after larva was inserted.

Trial # (n=6 per trial)	Field in maze					
	A	**B**	**C**	**D**	**E**	**F**
1	1	Control (solvent)	2	Control (solvent)	3	Control (solvent)
2	2		3		4	
3	3		4		5	
4	4		5		6	
5	5		6		7	
6	6		7		8	
7	7		8		1	
8	8		1		2	
9	1		4		7	
10	2		5		8	
11	3		6		1	
12	4		7		2	
13	Control (solvent)	5	Control (solvent)	8	Control (solvent)	3
14		6		1		4
15		7		2		5
16		8		3		6
17		1		5		2
18		2		6		3
19		3		7		4
20		4		8		5
21		5		1		6
22		6		2		7
23		7		3		8
24		8		4		1

Figure 5. 2: Semi-randomized block design for testing of stimuli in the bioassay. Each of the 8 substances per group (here displayed by numbers, highlighted with color shadings) was tested in each field of the maze (A-F), and in combination with each other substance within the group. The 24 trials were repeated 6 times, i.e. every compound was tested 54 times in 9 different substance combinations.

5.3.2 Statistical analysis

Statistical analysis were conducted applying the statistic program “R” (R version 2.15.0 (2012-03-30)) (Team, 2012). Behavioral responses to different substances in each group (e.g. acetic acid in the group organic acids) were compared to the total number of responses in this group (including all test substances and controls within this group) after a multiple comparison Kruskal-Wallis test.

5.4 Results

	Stimulus	Ctr/3:
sugar (non-responding: 13%)		
arabinose	8	7
fructose	8	6
glucose	6	7.67
maltose	12	7
mannose	4	7
sucrose	13	6.67
raffinose	8	8
xylose	9	7.67
organic acid (non-responding: 24%)		
acetic	10	6.67
citric	9	7
formic	10	5.33
fumaric	7	6.67
lactic	7	4.67
malic	5	8
oxalic	7	6
propionic	3	6.67
monoterpene (non-responding: 13%)		
(+)-camphene	12	6.33
(-)-camphene	7	8
(+)- limonene	10	8.33
(-)-limonene	6	8.33
linalool	9	6.67
α-pinene	12	8
α-terpinene	7	8.67
γ-terpinene	4	8.33
alcohol, ketone, aldehyde (non-responding: 5%)		
acetone	12	6
benzaldehyde	3	8
1-butanol	9	8
cinnamal	9	8.67
1-hexanol	17	7
methanal	9	7
propanal	8	8.33
1-propanol	10	8
ester, amine (non-responding: 13%)		
butyl acetate	7	8.67
butylamine	4	9.33
ethyl acetate	6	7.67
hexylamine	8	10.67
methyl acetate	4	10.67
pentylamine	5	10.67
propyl acetate	10	7
propylamine	7	9.33

Table 5. 1: Number of detected larvae in stimulus (Subst.) and respective control (Ctr/3) fields of the maze. Compounds that were frequented significantly more often or less often than the controls are highlighted in green or red, respectively ($p > 0.05$; Kruskal–Wallis test's, n= 54 larvae per stimulus and n=144 trials per group, including 432 controls).

Out of the 32 volatile electro-physiologically active components tested, 7 components elicited a behavioral response of the *M. melolontha* larvae, which was significantly different to the respective controls: maze fields with propionic acid, γ-terpinene and benzaldehyde were attended less frequently than the controls, and (+)-camphene, α-pinene, acetone, and 1-hexanol were attended more frequently than the controls (Table 5.1). Furthermore, mannose was significantly less attractive and sucrose significantly more attractive than the controls. The number of non-responding larvae was 5-24%, and differed depending on the set of offered compounds.

5.5 Discussion

According to our findings, the complex chemosensory system of *M. melolontha* larvae (Eilers *et al.*, 2012) enables them to orient in gradients of volatile and non-volatile substances of diverse compound classes, i.e. sugars, monoterpenes, an organic acid, an aromatic aldehyde, a ketone, and a leaf alcohol. To our knowledge, this the first study showing that belowground insects are able to follow a gradient of a sugar (sucrose) and avoid gradients of another sugar (mannose). It has been known for soil-dwelling scarabaeid larvae *Sericesthis geminata* that these insects are equipped with a relatively complex sense of taste which enables them to discriminate between different sugars (Wensler and Dudzinski, 1971, 1972). Furthermore, gustatory host location and discrimination by the use of isoflavonoids has been demonstrated in the larval clover root weevil (Johnson *et al.*, 2005). However, both studies presented the stimuli (isoflavonoids, sugars) as a food source on moist filter paper, and the larvae were kept in substrate-free chambers. In this context the gustatory cues functioned as phagostimulants, rather than as cues that can be traced over certain distances through the matrix. Sugars are the most direct products of photosynthesis and may thus be reliable chemical cues which provide information to root-feeders about the location and current carbon assimilation of a plant. Hence, *M. melolontha* larvae may use sugars to evaluate the profitability of a resource from a distance. The larvae were attracted to sucrose, which is present in root exudates of the preferred host plant dandelion (see chapter 4). The repellent sugar mannose, however, is not known to occur in rhizospheres of *M. melolontha* hosts, but was for instance found in root exudates of rice (*Oryza sativa*) (Bacilio-Jiménez *et al.*, 2003), and alfalfa (*Medicago sativa*) (Hamlen *et al.*, 1972) seedlings; whereas exudation of this sugar stopped in both cases after a short growth duration. However, in natural soils sugars are particularly prone to be decomposed by microorganisms to CO_2 or be adsorbed on clay minerals and soil organic matter (Kuzyakov and Domanski, 2000). Hence, the fate of these compounds in natural soils remains to be determined, particularly in greater distances

from plant roots, in order to ascertain their relevance as chemical cues belowground.

Root exudates may contain virtually any molecule which also occurs in aboveground parts of plants, except those that are tightly linked to photosynthesis, for instance chlorophyll (Uren, 2000; Bertin *et al.*, 2003). Correspondingly, the behaviorally active compounds belong to diverse compound classes of substances commonly found in root exudates. However, 1- hexanol is a member of the so-called green leaf volatiles, a set of compounds abundantly released from plant tissue following mechanical damage, but emitted at very low rates from intact plant tissues (Gatehouse, 2002). Assuming the same pattern in roots, *M. melolontha* larvae might use this compound to identify roots that have been selected by conspecific or other rhizophagous insects.

Compounds tested in this series of experiments were selected based on their general occurrence in rhizospheres and their electrophysiological activity when stimulating antennae or palps of *M. melolontha* larvae via the gas phase (Eilers *et al.*, 2012). However, of the behaviorally active compounds only sucrose was identified in dandelion rhizospheres. Other behaviorally active compounds, such as α-pinene and (+)-camphene may be released by other host plants of the polyphagous larvae. Interestingly, (-)-camphene was not behaviorally active; (+)-camphene is detected by the maxillary and labial plaps of the larvae and the enantiomer, (-)-camphene elicited only a weak response on the labial palps (Eilers *et al.*, 2012).

As root exudates are constituted by a multitude of compounds, of which only 40 were individually tested in this study, the identified behaviorally active substances presumably only represent a small subset of the compounds which the larvae are able to detect and use for behavioral decisions in natural rhizospheres. Some of the compounds that were shown to be physiologically active in *M. melolontha* larvae (Eilers *et al.*, 2012) may fail to elicit behavioral responses by themselves. However, root-derived substances are present as blends in natural rhizospheres and chemical backgrounds are often crucial for

insect host location (e.g.Bruce *et al.*, 2005; Schroeder and Hilker, 2008). Hence, some of the not behaviorally active compounds still contribute to the belowground orientation of the larvae by interacting with other compounds in natural blends.

Our results show that the larvae use their highly developed chemosensory system (Eilers *et al.*, 2012) to orient within gradients of highly diverse chemicals. When offered chemical stimuli from dandelion rhizospheres, both behavioral responses of the larvae (chapter 3) and chemical composition of the rhizosphere (chapter 4) depend on ecologically relevant factors such as mycorrhiza, plant age, light, and substrate type. Taken together, these results suggest that *M. melolontha* larvae are able to retrieve tremendously detailed information about the species and physiological states of their surrounding plants. Weissteiner (2010) shows that the larvae possess a complex antennal lobe, indicating their capacity to process complex chemosensory input. At this background it appears promising for future projects to investigate how naturally occurring blends interact in shaping this voracious herbivores choice among suitable hosts as well as between host and non-host plant roots.

5.6 Acknowledgements

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Chapter 6

General discussion

In summary, the results presented in this dissertation show that *M. melolontha* larvae can locate and differentiate between roots of one host plant species grown at different biotic and abiotic conditions. In the past carbon dioxide (CO_2) has been shown to be an important chemical cue, which may be masked by other root-derived compounds. The results presented here support the masking hypothesis and show that the larval orientation is accomplished by the use of chemical stimuli other than CO_2. Specific sets of biotic and abiotic treatments applied to the host plant may render its roots unattractive or untraceable, even though the root-emitted CO_2 alone would induce attraction. The larval set of chemo-sensory organs allows for the detection of volatile and nonvolatile plant root-derived compounds of various chemical classes. The electrophysiological experiments conducted indicate that typical contact chemo-sensilla possess a dual gustatory and olfactory function. Gradients of both, gustatory and volatile rhizosphere substances induce taste- and odor-guided behavior.

In the following sections the findings of this study are interpreted with respect to previous studies on root exudation, insect sensory organs, and belowground orientation. Future perspectives are discussed.

6.1 Smelling through soil

The inhomogeneous distribution of mineral, gaseous, and liquid constituents and edaphon species in natural soil, as well as degradation and bioturbation by the edaphon, may discombobulate gradients of plant root derived chemicals. However, as locomotion in soil is costly, root herbivores cannot afford strong deviations from their path towards a host. Hence, the need for reliable host cues is particularly strong in soil-dwelling organisms. Root-

released **v**olatile **o**rganic **c**ompounds (VOCs) may encode specific information about the plant and diffuse over relatively long distances belowground (Asensio *et al.*, 2008; Kai, 2008; Wenke *et al.*, 2010).

Odor-guided behavior in soil-dwelling insects has been described in other contexts, e.g. by (Hibbard and Bjostad, 1988; Johnson and Gregory, 2006; Nordenhem and Nordlander, 1994; Thomas *et al.*, 2008). *M. melolontha* larvae are also able to detect VOCs from various chemical classes (chapter 2). More than 30 different compounds (acids, alcohols, aldehydes, amines, esters, ketones and monoterpenes) elicit electrophysiological responses in antennae and palps. Out of these volatile components, 7 components elicit a behavioral response: propionic acid, γ-terpinene and benzaldehyde are repellent, (+)-camphene, α-pinene, acetone and 1-hexanol attract the larvae. Interestingly, the most attractive cue found so far, 1-hexanol (chapter 5), is a **g**reen **l**eaf **v**olatile (GLV) alcohol which has been described to be attractive to adult cockchafers as well (Reinecke *et al.*, 2002b). As root exudates may contain virtually any molecule which also occurs in aboveground parts of plants, except those that are tightly linked to photosynthesis, for instance chlorophyll (Uren, 2000; Bertin *et al.*, 2003), members of the behaviorally active compound classes acids, alcohols, aldehydes, ketones and terpenoids are also present in root exudates (Dennis *et al.*, 2010), but little is known about their composition. Hence, the exudation of components such as 1-hexanol from plant roots and their persistence in soil should be examined to demonstrate a role of these compounds as chemical cues for root herbivores.

6.2 Tasting through soil: in search of non-volatile cues

The rhizosphere of undamaged dandelion roots contains recoverable amounts of sucrose, glucose and fructose as determined in chapter 4 of this dissertation. Indeed, the majority of plant root-derived compounds are of low volatility or entirely nonvolatile (cp. chapter 1, introduction); and more than two thirds of a typical plant root exudate consist of reducing sugars such as glucose and fructose (Azaizeh *et al.*, 1995). The presence of water and vapor

allows for the use of these host-derived water-soluble or semi-volatile chemical cues in addition to VOCs, further compensating for the lack of visual cues. We found that *M. melolontha* larvae are able to follow gradients of sucrose through substrate and pinpoint the source; however, not all tested sugars were attractive (see chapter 5). The taste-guided behavior is accomplished by the employment of contact chemo-sensilla, which are present in the periphery of all larval head appendages. Their high number indicates the important role of gustation for the time the immature beetle spends belowground. In the past, sugars have primarily been described as phagostimulants for root herbivores (Johnson and Gregory, 2006), and gustatory orientation has mainly been described in vertebrates like catfish, which use amino acids for orientation in water (Caprio, 1977). Here, sucrose acts as gustatory cue for a root herbivore, as it is released by dandelion roots and *M. melolontha* larvae follow gradients of this sugar and locate its source belowground. As the most direct products of photosynthesis, sugars may be a reliable and quickly updated source of information to soil-dwelling organisms about a plant's location and its current carbon assimilation. The carbon-rich compounds from root exudates are, however, prone to be decomposed by microorganisms to CO_2 or adsorbed on clay minerals and soil organic matter (Kuzyakov and Domanski, 2000). Hence, the actual relevance of these compounds in natural soil, dependent on natural compositions of soil constituents including micro- and macroorganisms, remains to be determined, particularly in greater distances from plant roots.

Organic acids are also major constituents of root exudates; but the chemical analysis of these compounds from natural plant rhizospheres is associated with complications. Carbonic acids may for instance be volatile (e.g. acetic acid, formic acid) or transform to their conjugate bases (e.g. citrate, oxalate). This variation in solubility and volatility of rhizosphere carbonic acids renders respective extraction and sample preparation for mixes of these substances particularly difficult. Amounts of organic acids released by plant roots follow concentration gradients from the roots to the substrate (Jones and Darrah, 1993; Ström *et al.*, 1994; Jones, 1998; Jones and Brassington, 1998). Recoverable amounts of organic acids thus depend on the adsorption

capacity of the surrounding matrix of the root. Furthermore, organic acids are released from plant roots in order to mobilize nutrients bound to the substrate (Jones, 1998; Jones and Brassington, 1998). Clay minerals such as vermiculite may not only adsorb organic acids but also certain nutrients (Panuccio *et al.*, 2008), thereby facilitating a considerably enhanced acid excretion by the plant roots to mobilize these nutrients again. Due to difficulties to recover the compounds from natural substrates, samples for chemical analysis of root-derived organic acids are commonly extracted from agar (Eldhuset *et al.*, 2007), filter paper (Wang and Bergeson, 1974), or hydroponics (Szmigielska *et al.*, 1995; Cieslinski *et al.*, 1997; Neumann and Romheld, 1999), which do not reflect soil conditions. Another common method to obtain root-derived compounds is to remove the plants from their growth medium and dip the excavated roots in an aqueous solution (Ström *et al.*, 1994).

We attempted to analyze organic acids from the undisturbed rhizosphere of dandelion via aqueous extraction and HPLC and GC-MS (including lyophilization and chemical derivatization) analysis. Authentic reference standards of acids and their conjugate bases (oxalate, citrate, malate, tartrate, ascorbate, and fumarate) were successfully recovered from all tested substrates (sand, vermiculite, natural soil) and analyzed However, the established methodology was not applicable for plant-released carbonic acids. The main difficulty was to separate the acids from the matrix and purify them with low loss. So far, the attempts did not yield satisfying results and require optimization of the extraction and analysis techniques to fully assess the biological role of this compound class in soil herbivore-root interactions.

6.3 Blends of smell and taste

Non-volatile and volatile compounds are intermingled in soil and may co-occur in similar gradients. Hence, similar to in aqueous medium, it may be difficult to determine whether a cue is olfactory or gustatory (Caprio, 1977). For

instance, the most commonly described cue belowground, carbon dioxide (CO_2), may be present in soil as carbonic acid if dissolved in soil solution.

According to their morphology, ca. 75% and 87% of the sensilla on maxillary and labial palps of *M. melolontha* larvae, respectively, are contact-chemosensilla (Fig.6.1). However, upon electrophysiological stimulation via the gas phase, the antennae responded to 25, maxillary palps to 13 and labial palps to 23 out of 52 tested compounds, respectively (cp. chapter 2). The multitude of responses determined on both palps probably cannot be attributed to the few basiconic sensilla alone. Hence, these findings indicate a dual gustatory and olfactory function of the contact chemosensilla, at least on both palps of the larva. Single sensillum recordings are required to confirm an olfactory function of morphologically categorized gustatory sensilla. To determine the role of plant root-derived olfactory and gustatory cues, it would be interesting to test the attractiveness of both the volatile (e.g. presented on alginate beads) and the water-solved fraction, separately.

Overall, seven of the 32 electrophysiologically active volatile compounds (chapter 2) as well as two of the 8 tested root-derived sugars elicited a behavioral response (chapter 5). Representatives of all compound classes identified in the root exudates were among the behaviorally active compounds (see chapter 1, Fig. 1.5). It is well known that the ratio of attractive and repellent compounds in a blend is decisive for host plant acceptance or rejection by insects (Schoonhoven *et al.*, 2005). *M. melolontha* larvae are able to trace CO_2, sugars, organic acids, terpenoids, ketones, alcohols, aldehydes, and possibly other compounds like amino acids or flavonoids through the substrate. Taken together, the results from this study, i.e. the perspicacious response behavior of the larvae to dandelion roots, the morphological findings, electrophysiological data, and the behavioral response of the larvae to diverse reference compounds indicate that they are able to reveal tremendously detailed information about the species and physiological states of their surrounding plants my means of decoding blends of various organic compounds.

We could show that the larvae are able to discriminate between conspecific individuals of their preferred host species, which are exposed to different growth conditions (light intensity, substrate type, plant age and association with **a**rbuscular **m**ycorrhizal **f**ungi (AMF)), (chapter 3). However, neither CO_2, nor root-derived sugars or volatile compounds (mainly sesquiterpenoids) alone would predict larval decision (chapter 3 and 4); these compounds obviously need to be perceived as blend.

The blend perception of volatile and non-volatile compounds most probably governs larval orientation and choice behavior. Further research is required to determine the role of individual compounds in blends determining the larval behavior.

6.4 Distribution and redundancy of sensory organs

In most aerial insects, the antennae are responsible for detection of the majority of compounds that enable orientation. But in soil a partition of chemosensory functions to more than one head appendage may be advantageous to ensure a greater redundancy, considering the high risk of injury due to the rock fragments of the matrix itself, in addition to conspecifics and predators. In addition, the last larval stage during which most root material is consumed is the longest, and lasts approximately one year. Thus, the animal cannot afford to lose the ability to locate host plants and it can also not overcome injuries by molting, or in this case, early pupation.

In *M. melolontha*, the majority of peg- or cone-like sensilla is located in a cluster on the tips of the antennae, maxillary and labial palps (Fig. 6.1). This location appears optimized for probing the surrounding substrate, but at the same time leaves the sensilla relatively unprotected. The labial palps are the shortest appendages and thus least vulnerable to potential injuries. Previously, these appendages were not considered to play a major role in the detection of behavior-driving chemical compounds. However, benzaldehyde, which elicits a behavioral response in *M. melolontha* larvae (chapter 5) is exclusively detected by the labial palps (chapter 2). Apart from benzaldehyde,

most electrophysiological responses were recorded on at least two of the appendages. This is consistent with the fact that most peg-like sensilla types occur on more than one of the head appendages. Hence, the loss of these sensilla on one head appendage may be compensated for – at least to a certain degree by occurrence on another head appendage.

In contrast to the hypothesis of functional redundancy in *M. melolontha* sensilla, the olfactory antennal pore plates, which constitute the largest chemosensory surface, are exclusively located on the antennae. These plates are probably the only organs involved in CO_2 detection. Also, the larvae possess only one pair of digitiform organs, involved in thermo-/hygroreception. However, due to the lateral position and smoothness of both the plate and the digitiform organs, they are less exposed than the tip sensilla. As detailed studies on the sensory equipment of other soil-dwelling insects and particularly on larvae are scarce, the enhanced functional redundancy of sensory organs has not been previously described. It would, however, be interesting to determine the distribution and redundancy of sensory organs in other soil-inhabiting insects to examine if the redundancy is indeed a particular adaption to an insect´s life in soil.

The distribution of sensilla on the head appendages of *M. melolontha* may be optimized to ensure the detection of compounds via the gas phase on the most peripheral appendage, the antenna. These compounds, for instance CO_2, may travel over greater distances in soil and serve for host location. After the larva has reached a close vicinity to the rhizosphere, the detection of compounds by the less exposed sensilla on the palps may become more important. These sensilla may be involved in the evaluation of host plant quality.

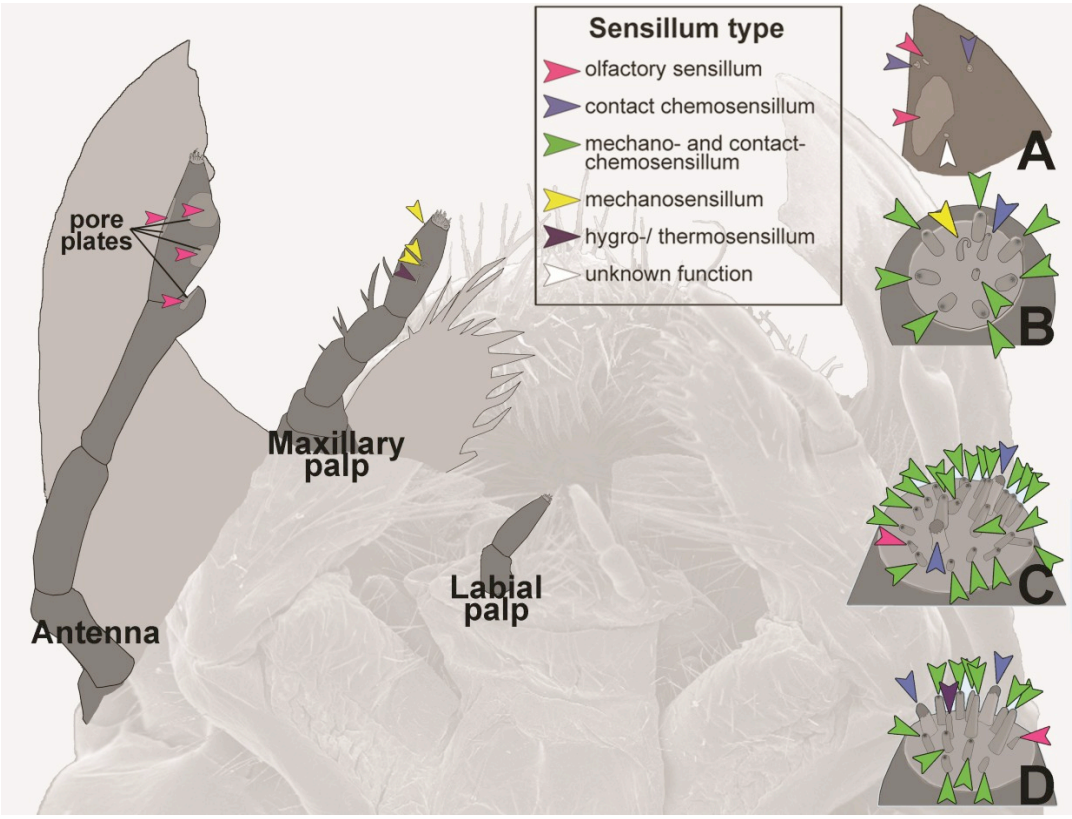

Figure 6. 1: Functional map of sensory organs on head appendages of *M. melolontha* third instar larvae, on the cuticular protrusion of the post-apical antennal segment (A), and the crone-like tip of the antenna (B), maxillary palp (C) and labial palp (D). Reconstructed from SEM and TEM images (see chapter 2)

6.5 Hygro-thermo- and mechano- sensation

Host finding is not the only challenge in the immature life stages of *M. melolontha,* and the larval sensory organs are not exclusively chemoreceptive. Because the larval soft cuticle provides virtually no protection against drought and heat, the information obtained by the hygro-/thermoreceptive digitiform organs on maxillary palps and hygro-/thermoreceptive sensilla on the labial palps may also play a decisive role in the behavior of the larvae. Furthermore, various mechanosensilla and bimodal chemo- and mechanosensitive sensilla are present on all *M. melolontha* larval head appendages. The larvae are cannibalistic (Ene, 1942) and are additionally endangered by vertebrate predators, such as moles, and wild boars and crows, which attack from aboveground (Huiting *et al.*, 2006 and personal observations). As the larvae are “in the dark”, they may rely on their

acoustic sense to sense approaching enemies by the vibrations and sound waves they generate. In elaterid larvae, digitiform organs were found to respond to vibrations (Zacharuk *et al.*, 1977). However, mechanical sensitivity of the digitiform organ in *M. melolontha* can be excluded, as the required tubular body-forming dendrites (Altner, 1977) were not observed and the lamellated dendritic termination indicates hygro-/thermoreceptive function. Yet, adjacent to the digitiform organs on *M. melolontha* maxillary palps, a row of flat pits and bent furrows is observed, which do contain tubular bodies and could be classified as mechanoreceptive. These organs are likely candidates for the detection of vibrations. Auditory organs (for sound production and detection) are in adult insects mainly involved in courtship and mating behavior (e.g. male premating stridulation) and have been discovered in many insects including Scarabaeidae (Altner, 1977; Mini and Prabhu, 1990; Hirschberger and Rohrseitz, 1995). But also Coleoptera larvae use sound; it has been proposed that acoustic signals generated by soil-and bark-inhabiting Cerambycid larvae are used to avoid competition and cannibalism in their dark habitat (Lüders, 1958). A potential role of sound and vibration perception in avoidance of intra- and interspecific predators and belowground orientation of *M. melolontha* larvae remains to be determined.

In addition to the senses of smell, taste, and possibly sound, the sense of touch may play a greater role in soil-dwelling insects than in visually more advanced aerial insects. Applying either naturally or artificially produced waves to the substrate, which for instance mimic natural enemies of the cockchafer larvae, may reveal the importance of this sense to the behavior of the larvae. However, tactile orientation may be difficult to examine in *M. melolontha* larvae, owing to the blending of mechanical and chemical senses in many sensilla.

6.6 Conclusions

The findings presented in this dissertation show that immature cockchafers possess a sophisticated chemosensory system (chapter 2), which enables them to detect a broad range of host derived chemical stimuli and discriminate among hosts (chapter 3). A multitude of volatile and soluble compounds are released from plant roots in different concentrations, depending on the plants' condition (Dennis *et al.* 2010, chapter 4). Although attraction and repellence can be induced by single olfactory and taste cues in *M. melolontha* larvae (chapter 5), as well as other soil dwelling insects (Robert *et al.*, 2012), the number of relevant cues for host location in natural soil may need to be higher in soil-dwelling compared to aerial insects, due to a nonlinear obliteration of compounds by soil biota and adsorption to the substrate. It is likely that larval host location is achieved by combined odor- and taste-guided behavior and the use of respective blends. Belowground orientation may additionally be supported by tactile information. Our findings and considerations may serve as groundwork for future research on the interaction of soil-dwelling herbivores and their host plants. Understanding the ecology of these interactions is vital for the development of successful agricultural applications such as integrated pest management strategies for the control of root herbivores.

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Chapter 7

Summary - Zusammenfasssung

7.1 German Summary – Zusammenfassung

Larven des Feldmaikäfers (*Melolontha melolontha* L.) sind durch ihren Fraß an Wurzeln verschiedener Feldfrüchte und Obstbäume wichtige landwirtschaftliche Schädlinge in Europa. Die von den Larven am meisten präferierte Wirtspflanze ist allerdings die Ruderalpflanze Löwenzahn (*Taraxacum sect. ruderalia*). Wie auch von anderen bodenlebenden Insekten bekannt ist, können *M. melolontha* Larven von Wurzeln produziertes Kohlendioxyd (CO_2) nutzen, um ihre Wirtspflanzen unterirdisch zu orten. Allerdings ist dieses Gas allgegenwärtig im Boden und liefert Wurzelherbivoren wie *M. melolontha* relativ unspezifische Informationen. Die Anwesenheit von Geruchs- und Geschmackssinnesorganen, den sogenannten Sensillen, indiziert die Relevanz dieser Sinne für die Orientierung des Insekts. Aus der Morphologie der Sensillen lässt sich zumeist deren Funktion ableiten. Obschon eine Vielzahl an Studien publiziert ist, die mikroskopische Aufnahmen und Beschreibungen von Sensillen bodenlebender Insekten beinhalten, fehlen zumeist Detailinformationen, die Schlüsse auf deren Funktion zulassen. Aufgrund der Schwierigkeiten, die mit chemischer Analytik von Substanzen und Beobachtung von Organismen im Boden einhergehen, ist bislang wenig bekannt über Abgabe und Wahrnehmung chemischer Informationen, die zwischen Pflanzenwurzeln und bodenlebenden Insekten ausgetauscht werden. In vielen vorangegangenen Studien wurden zumeist stark vereinfachte Modelle verwendet, die in ihrer Art und Einheitlichkeit von den in der Natur von Pflanzen und assoziierten Wurzelherbivoren vorherrschenden Bedingungen abweichen. Somit waren grundlegende Fragen hinsichtlich der sensorischen Ausstattung bodenbewohnender Insekten, der Verfügbarkeit chemischer Stimuli sowie der Wirtpflanzenortung und -wahl nicht nur für Maikäferlarven unbeantwortet.

Im Rahmen dieser Dissertation wurde mittels elektronenmikroskopischer Methoden eine Bestandsaufnahme der sensorischen Ausstattung der Antennen, Maxillarpalpen und Labialpalpen von *M. melolontha* Larven gefertigt (Kapitel 2). Die chemo-sensorische Funktion der Kopfanhänge wurde elektrophysiologisch untersucht. In einer Vielzahl von durchgeführten Verhaltenstests wurde die Wirkung verschiedener Behandlungen (symbiotische Beziehung zu arbuskulären Mycorrhizapilzen (**a**rbuscular **m**ycorrhizal **f**ungi (AMF), Alter der Pflanzen, Lichtintensität und Art des Substrats) auf die Wirtsfindung der Larven zu Löwenzahnwurzeln untersucht (Kapitel 3). Mit Hilfe von wässriger Extraktion und Festphasenextraktion von flüchtigen und nicht-flüchtigen Verbindungen mittels Gaschromatographie-Massenspektrometrie (GC-MS) wurde bestimmt, welche Auswirkungen die genannten Bedingungen auf die Zusammensetzung potentieller Infochemikalien in der Rhizosphere von Löwenzahn haben (Kapitel 4). Mittels elektrophysiologischer Untersuchungen und Verhaltenstests wurde zudem untersucht, ob und welche wurzelbürtigen Substanzen von den Antennen und Palpen der Engerlinge detektiert werden können (Kapitel 2) und ob diese Stoffe Verhaltensantworten auslösen (Kapitel 5).

Die Ergebnisse dieser Dissertation zeigen, dass *M. melolontha* Larven über ein komplexes chemo-sensorisches System verfügen. Mit der Vielzahl an olfaktorischen und gustatorischen Sensillen auf den Antennen und Palpen detektieren Engerlinge Substanzen verschiedenster Stoffklassen (Aldehyde, Alkohole, Amine, Ester, Ketone, organische Säuren, Terpenoide, Zucker), von denen die meisten bekanntermaßen von Pflanzenwurzeln abgegeben werden. Olfaktorische Porenplatten sind die flächenmäßig umfangreichsten Sensillen der Antennen. Sie detektieren eine Vielzahl an Duftstoffen, die von Pflanzenwurzeln abgegeben werden und dienen mit hoher Wahrscheinlichkeit auch der Wahrnehmung von CO_2. Weitere chemo-sensorische Sensillen der Larven sind in großer Zahl auf den Palpen ansässig.

Diese Verteilung der Sensillen könnte mit dem umgebenden Substrat zusammenhängen, welches zum einen die Nutzung nicht-flüchtiger

wurzelbürtiger Verbindungen ermöglicht, zum anderen jedoch auch die Abnutzung der Sensillen erhöht. Letzteres könnte der Grund für die hohe funktionelle Redundanz der Sensillen sein: Die meisten Sensillentypen befinden sich auf mehr als einem der Kopfanhänge und die meisten Duftstoffe werden ebenfalls an mehreren der Kopfanhänge detektiert.

M. melolontha Larven sind in der Lage, innerhalb einer Pflanzenart zwischen Pflanzen unterschiedlicher Behandlung zu unterscheiden. Änderungen der biotischen (AMF, Pflanzenalter) und abiotischen (Lichtintensität und Substratbeschaffenheit) Wachstumsbedingungen verändern die Attraktivität der Löwenzahnwurzeln für die Larven. Bei bestimmten Kombinationen dieser Bedingungen können die normalerweise stark präferierten Löwenzahnwurzeln unattraktiv bzw. für die Larven nicht auffindbar sein. Diese Beobachtungen lassen sich nicht anhand der gemessenen Konzentrationen des von der Wurzel abgegebenen CO_2 erklären. Somit deuten diese Befunde darauf hin, dass weitere wurzelbürtige Substanzen für die Wirtsfindung der Larven benötigt werden. Entsprechende Substanzen könnten an sich attraktiv oder abstoßend wirken, oder die Attraktivität des CO_2 für die Larven maskieren. Es wurden für die oben genannten Anzuchtbedingungen spezifische Muster flüchtiger Verbindungen wie Sesquiterpene und wasserlöslicher Verbindungen wie Zucker über unverletzte Löwenzahnwurzeln gemessen.

Einige Aldehyde, Alkohole, Ketone, organische Säuren, Terpenoide und Zucker aus Wurzelexudaten sind verhaltensaktiv und könnten von den Larven als Botenstoffe zur Wirtsfindung im Boden verwendet werden. Von den 32 getesteten flüchtigen Verbindungen sind (+)-Camphen, α-Pinen, Aceton und 1-Hexanol attraktiv, wohingegen Propionsäure, γ-Terpinen und Benzaldehyd abstoßend auf die Larven wirken. Darüber hinaus bewegen sich die Larven in einem Gradienten aus Saccharose zur Quelle, wohingegen Mannose gemieden wird.

Entgegen der bislang weitverbreitenden Annahme, *M. melolontha* Larven orientierten sich ausschließlich anhand von CO_2-Gradienten, konnte hier

gezeigt werden, dass weitere Infochemikalien aus zahlreichen Stoffklassen wahrgenommen werden und das Verhalten modifizieren. Die chemosensorische Ausstattung der Larven hat sich als überraschend komplex erwiesen und ist die Grundlage für eingehende Präferenzen dieses polyphagen Wurzelherbivoren. Die Befunde lassen die Entwicklung von landwirtschaftlich anwendbaren Techniken möglich erscheinen, mit deren Hilfe Engerlinge von den wertvollsten Kulturpflanzen fern gehalten werden könnten. Um die hier präsentierten Grundlagen der Anwendung zugänglich zu machen, ist allerdings weitere Forschung erforderlich, die weitere bodenlebende Mikroorganismen neben Mykorrhizapilzen und weitere abiotische Bedingungen, wie beispielsweise Phosphat- und Stickstoffversorgung der Pflanzen und schließlich verschiedene Wirts- und Nicht-Wirtspflanzen unter Feldbedingungen in Verhaltenstests einbeziehen sollte.

7.2 English summary

Larvae of the common cockchafer (*M. melolontha* L.) are important agricultural pests in Europe, due to their feeding activities on roots of different crops and fruit trees. However, the most preferred host plant of the larvae is the ruderal plant dandelion (*Taraxacum sect. ruderalia*). As also known for other soil-dwelling insects, *M. melolontha* larvae are able to use plant root emitted carbon dioxide (CO_2) to locate their host plants belowground. However, this gas is ubiquitous in soil and thus delivers relatively unspecific information to root herbivores like *M. melolontha*. The presence of olfactory and gustatory sensory organs (sensilla) indicates the importance of these senses for the orientation of the respective insect. The morphology of sensilla usually allows their assignment to functions. Although a multitude of studies that provide microscopic images and descriptions of sensilla on soil-dwelling insects is published to date, detailed information, which would allow the determination of functions of the sensilla, is scarce. Due to the difficulties involved in chemical analysis of substances and observation of organisms in soil, little knowledge is available on the emission and perception of chemical information that is exchanged between plant roots and soil-dwelling insects. Previous studies mostly applied simplified models, which, due to their constitution and uniformity, differ from the conditions present in the natural environment of plants and their root herbivores. Thus, elementary questions regarding the sensory equipment of soil inhabiting insects, belowground availability of chemical stimuli, and host location remained unanswered, not only for cockchafer larvae.

In this dissertation, an inventory of the sensory equipment of antennae, maxillary and labial palps of *M. melolontha* larvae was established by means of electron microscopy (chapter 2). The chemosensory function of head appendages was examined by electrophysiology. In a multitude of behavioral assays, the impact of various plant treatments (symbiotic relationship to **a**rbuscular **m**ycorrhizal **f**ungi (AMF), plant age, light intensity and substrate type) on larval host location to dandelion roots was investigated (chapter 3). By means of solid phase extraction of volatile and non-volatile compounds

and analysis with gas chromatography-mass spectrometry (GC-MS), the impact of abovementioned conditions on the composition of potential infochemicals in the rhizosphere of dandelion was determined (chapter 4). Furthermore, electrophysiological and behavioral experiments were applied to reveal, if and which root-derived substances are detected by antennae and palps of the larvae (chapter 2) and if these substances induce behavioral responses (chapter 5).

The results of this dissertation show that *M. melolontha* larvae possess a complex chemosensory system. The multitude of olfactory and gustatory sensilla on antennae and palps enable the larvae to detect substances of many different compound classes (aldehydes, alcohols, amines, esters, ketones, organic acids, terpenoids, sugars), of which most are released by plant roots. Olfactory pore plates are the largest sensilla on the antennae. They detect a multitude of odorants released by plant roots, and they are probably also responsible for the detection of CO_2. Further chemosensory sensilla are present in great number on both palps.

The distribution of sensilla may be adapted to contact with the surrounding substrate, which allows the use of non-volatile root-derived compounds but also increases the abrasion of sensilla. The latter effect may explain an increased functional redundancy of sensilla: most sensilla are present on more than one head appendage and most odorants are also detected on more than one head appendage.

M. melolontha larvae are able to discriminate between differentially treated plants of the same species. Changes in biotic (AMF, plant age) and abiotic (light intensity, substrate type) growth conditions alter the attractiveness of dandelion roots to the larvae. Certain combinations of these factors may even render the usually preferred host plant unattractive or untraceable to the larvae. These observations are not predictable by the measured concentrations of root emitted CO_2. Thus, the data suggest that further root-derived substances are required for larval host location. These substances may be attractive or repellent *per se* or mask the attractiveness of CO_2.

Abovementioned plant growth conditions also accounted for specific patterns of volatile (e.g. sesquiterpenes) and non-volatile (e.g. sugars) root-derived components, measured in rhizospheres of undamaged dandelion.

Several root-derived aldehydes, ketones, organic acids, terpenoids and sugars are behaviorally active and may function as chemical cues for the larvae. Out of the 32 volatile compounds tested, (+)-camphene, α-pinene, acetone, and 1-hexanol attract the larvae, whereas propionic acid, γ-terpinene and benzaldehyde are repellent. Furthermore, the larvae follow gradients of sucrose to its source, whereas mannose is avoided.

In contrast to the prevalent assumption that *M. melolontha* larvae would exclusively orient in CO_2 – gradients, the findings presented here show that further infochemicals from various compound classes are perceived and alter the larval behavior. The chemosensory equipment of larvae was found to be surprisingly complex and is the basis for the distinct preferences of the polyphagous root herbivore. In summary, the presented results indicate that the development of targeted agricultural measures, which may have a potential to distract cockchafer larvae from most valuable crops, is possible. However, the application of the groundwork presented here requires an expansion of research by additionally including soil microbiota other than mycorrhizal fungi, including further abiotic variables such as phosphorous and nitrogen supply of the plant, and finally testing a variety of host and non-host plants under field conditions in choice assays.

Chapter 8

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Chapter 9

Supplementary information

Table.S1: Sensory organs on antennae (A), galea (G), maxillary (M) and labial palps (L) of species belonging to different Coleopteran and Lepidopteran families and subfamilies.
Abbreviations: #?, unknown number; A, Antenna; ap, apical; BC, basiconic; CF, campaniform; CH, chaetica; CP, present in cuticular protrusion on postapical antennal segment; CR, chemoreceptor; di, distal; Do, digitiform organ; dor, dorsal; Fo, foliphagous; G, Galea; GR, contact-chemoreceptor (gustatory); Her, herbivorous (foliage, blossoms, seeds or stem); HR, hygroreceptor; L, labial palps; lat, lateral; LM, light or sterio microscopy; M, maxillary palps; MR, mechanoreceptor; NP, aporous; OR, olfactory receptor; PP, sensory pore plate; Pred, predatory; Rhz, rhizophagous; Sa, saprophagous/ detritus feeder; SC, styloconic; Sca, scavenger; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TR, thermoreceptor; Xy, xylophagous or saproxylophagous; UP, uniporous; ven, ventral; WP, wall pores/multiporous.

Affiliation	Develop. stage	Structure	Location, abundance	Species	Method	Hypothe-sized function	Diet	Origin	Reference
Coleoptera: Carabidae	**3rd instar (final)**	**PP**	**A:** 1CP	*Ophonus ardosiacus* L.	SEM, TEM	OR	Her	Italy	(Giglio *et al.*, 2008)
	2nd instar		not present	*Pterostichus melanarius* I.	SEM	/	Pred (slugs)	Great Britain	(Thomas *et al.*, 2008)
		Setae	**A:** 4 trichoid, 4 basiconic			n.a.			
		Do	not present			/			
	1st to 3rd instar	**Do**	**M:** 1-28 (UP) **L:** 0-50 (UP)	22 species in 16 genera belonging to 10 tribes	SEM, TEM	MR, CR or HR	n.a.	Italy, Slovenia	(Giglio *et al.*, 2003)
		Setae	**M:** 1-12 **L:** 7-61			n.a.			
Coleoptera: Chrysomelidae	**Adult**	**PP**	not present	*Phyllotreta cruciferae* G., *Psylloides punctulata* M., *Epitrix cucumeris* H., *Psylloides affinis* P.	SEM	/	Her	Canada	(Ritcey and McIver, 1990)
		Setae	**A:** >200			n.a.			
	Larva, unknown instar		**G:** >2	*Entomosceiis americana* B.	SEM, TEM	MR and GR		USA	(Mitchell *et al.*, 1979)
	3rd instar (final)		**G:** >2	*Leptinotarsa decemlineata* S.		n.a.		Canada	(Sen and Mitchell, 1987)
		Setae	**M:** 16 **L:** 11			GR		Moscow	(Farazmand and Chaika, 2008)
		Do	**M:** 1			MR			
Coleoptera: Curculionidae	**3rd instar (final)**	**Do**	**M:** 1	*Ceutoryhnchus obstrictus* M.	SEM	n.a.	Her	Europe, North America	(Dosdall and McFarlane, 2004)
		Setae	**A:** 6 **M:** 12						
		PP	**A:** 1						
Coleoptera: Dermestidae	**Adult**	**Do**	**M:** 11	*Dermestes maeulatus* D.G.	SEM, TEM	TR/HR or CO_2	Sca	Germany	(Honomichl and Guse, 1981)

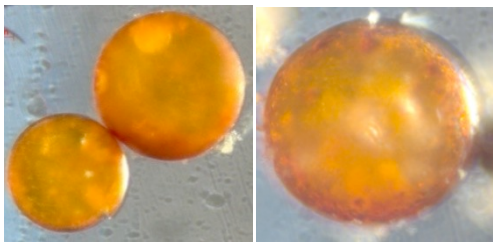
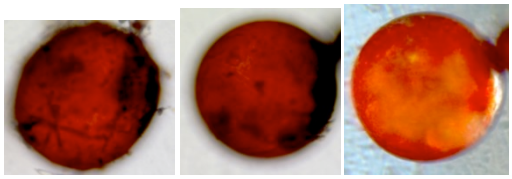
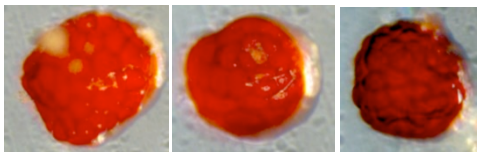
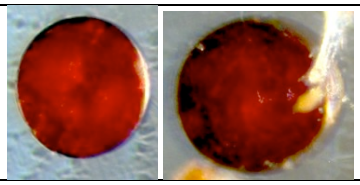
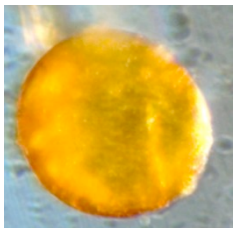
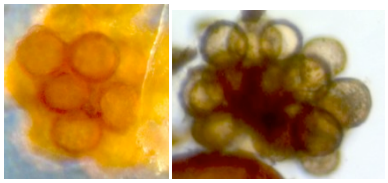
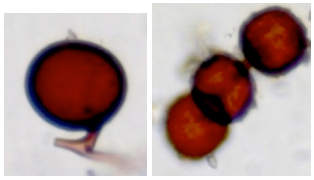
Affiliation	Develop. stage	Structure	Location, abundance	Species	Method	Hypothe-sized function	Diet	Origin	Reference
Coleoptera: Dynastidae	**Adult**	**PP**	**A:** >45,000	*Oryctes rhinoceros* L.	SEM, TEM	OR	Her	Indonesia	(Renou *et al.*, 1998)
Coleoptera: Dytiscidae	**Adult**	**Do**	**M:** 11	*Agabus bipustulatus* L.	SEM, TEM	TR	Her? Aquatic	n.a.	(Guse and Honomichl, 1980)
Coleoptera: Elateridae	**Larva, unknown instar**	**Do**	**M:** 1	*Agriotes lineatus obscurus* L. *Limonius californicus* M.	SEM	n.a.	n.a.	Canada	(Doane and Klingler, 1978)
		Setae	**M:** ca. 60 **L:** ca. 14						
	Final instar	**PP**	**A:** 1ven, 1dor, 1CP	*Aeolus cinctus* C.	LM?		Pred (termites)	Brazil	(Casari, 2006)
		Do	**M:** 1						
Coleoptera: Hydrophilidae	**Adult**	**Do**	**M:** 4-6	*Hydrobius fuscipes* L.	SEM, TEM	TR	Her? Aquatic	n.a.	(Guse and Honomichl, 1980)
Coleoptera: Scarabaeidae: Aphodiinae	**3rd instar (final)**	**PP**	**A:** 1dor,1ven	*Ataenius opatrinus H., Ataenius picinus H., Ataenius simulator* H., *Ataenius. platensis* B., *Ataenius strigicauda* B.	LM	n.a.	Sa, Rhz	Uruguay	(Verdú and Galante, 1999)
Cetoniinae			**A:** 3di, 2lat	*Ichnestoma stobbiai* H.			Sa	South Africa	(Deschodt *et al.*, 2009)
			A: 10-11	*Heterorrhina smaragdin* V.			Sa, Xy	Nigeria	(Jerath and Unny, 1965)
			A: 13-17	*Platygenia barbata* A.					
			A: 7-8	*Gnathocera trivittata* S.					
			A: 4	*Grammopyga cincticollis* H., *Clastocnemis quadrimaculatus* A.					
			A: 5	*Pachnoda marginella* F.					
			A: 2ven, 3dor, 1CP	*Coelocorynus desfontainei* A., and *C. opacicauda* A.				Cameroon	(Sipek *et al.*, 2009)

Affiliation	Develop. stage	Structure	Location, abundance	Species	Method	Hypothe-sized function	Diet	Origin	Reference
Coleoptera: Scarabaeidae: Cetoniinae	**3rd instar (final)**	**PP**	**A:** 3dor,3ven	*Hoplopyga singularis* G.P., *Hologymnetis cinerea* G.P.	LM	n.a.	Sa	Brazil, Mexico	(Micó *et al.*, 2001a)
			A: 4dor	*Tropinota squalida* S.				Spain	
			A: 4dor, 1CP	*Oxythyrea funesta* P.					
			A: 4dor	*Aethiessa floralis* F.					
Dynastinae			**A:** 7dor, ?#ven, 1CP	*Oryctes nasicornis* L.			Sa, Xy	Russia	(Grebennikov and Scholtz, 2004)
			A: 4-5	*Megasoma sleeperi* H			Xy	USA	(Van Dam *et al.*, 2006)
			A: 12dor, 15ven	*Strategus syphax* F.			unknown	Caribbean	(Ratcliffe and Chalumeau, 1980)
Melolonthinae			**A:** 3, 1CP	*Serica brunnea* L.			Rhz	Great Britain	(Jepson, 1937)
		Setae	**A:** CP: 4						
Orphninae		**PP**	**A:** 1dor	*Aegidium cribratum* B.			Sa, Xy	Mexico	(Moron, 1991)
Rutelinae			**A:** 1dor,2ven	*Anisoplia baetica* E., *Anisoplia depressa* E., *Anisoplia remota* R., *Anthoplia floricola F.*			Rhz	Europe, Africa	(Micó *et al.*, 2001b)
			A: 2 dor	*Paraheterosternus luedeckei* B.			Xy	Mexico	(Moron and Nogueira, 2000)
			A: 6dor,7ven	*Chasmodia collaris* B.				USA	(Jameson and Moron, 2001)
			A: 4dor,3ven	*Chasmodia cincticollis* B.					
		Setae	**A:** 3-4 **M:** approx. 20 **L:** approx. 13	*Phyllopertha horticola*	SEM, TEM		Rhz	Europe, Asia	(Alekseev *et al.*, 2006)
		PP	**A:** >2			OR			

Affiliation	Develop. stage	Structure	Location, abundance	Species	Method	Hypothe-sized function	Diet	Origin	Reference
	Adult		**A:** 200	*Popillia japonica* N.			Fo	Japan	(Kim and Leal, 2000)
Coleoptera: Scarabaeidae: Scarabaeinae	**3rd instar (final)**	**Setae**	**A:** 8	*Cotinis nitida*	SEM	n.a.	n.a.	n.a.	(Baker, 2005)
Coleoptera: Tenebrionidae	**Final instars**	**Setae**	**A:** 9	*Tribolium confusum* *Tribolium castaneum*	SEM, TEM	n.a.	Her (flour)	n.a.	(Behan and Ryan, 1978)
		PP	**A:** 1						
	Adult	**Do**	**M:** 14	*Tenebrio molitor* L.		TR/HR- or CO_2	Her (flour), Pred	Germany	(Honomichl and Guse, 1981)
Lepidoptera: Lymantriidae	**5th instar (final)**	**Setae**	**M:** 8 **G:** 2 UP, 3 NP	*Lymantria dispar* L.	SEM, TEM	n.a.	Her	USA	(Shields, 2009)
Lepidoptera: Noctuidae	**5th instar (final)**	**Setae**	**M:** 7 **L:** 2	*Heliothis virescens* F	SEM	n.a.	Her	n.a.	(Baker *et al.*, 1986)
		PP	**M:** 2						
		Do	**M:** 1						
		Setae	**M:** 9 **L:** 2	*Heliothis zea* B					
		PP	**M:** 2						
		Do	**M:** 1						
		Setae	**G:** 2 SC UP, 3 BC WP	*Spodoptera frugiperda* S., *Choristoneura fumiferana* C., *Lymantria dispar* L., *Prunus virginiana* L.	SEM, TEM	SC: GR BC: OR		Canada	(Shields, 1996)
			G: 2 SC UP, 3 BC WP, 1 CF UP	*Malacosoma lutescens* N.D., *Trichoplusia ni* H., *Mamestra configurata* W.		SC: GR BC: OR			
			M: 8 UP **L:** 2 NP	*Euxoa messoria* H.		UP: CR NP: MP	n.a.	n.a.	(Devitt and Smith, 1982)

Affiliation	Develop. stage	Structure	Location, abundance	Species	Method	Hypothe-sized function	Diet	Origin	Reference
Lepidoptera: Noctuidae	**5th instar (final)**	**Do**	**M:** 1	*Euxoa messoria* H.	SEM, TEM	MR	n.a.	n.a.	(Devitt and Smith, 1982)
		PP	**M:** 3			CR			
	3rd to 5th instar		**M:** 2	*Helicoverpa armigera* H.		CO_2			(Keil, 1996)
		Do	**M:** 1			TR, CO_2			
		Setae	**M:** 3 BC WP 5 BC UP 1 CF NP			WP: OR UP: GR NP: MR			
Lepidoptera: Pyralidae	**4th instar**	**Setae**	**A:** 2 CH NP, 1 SC NP, 3 BC WP **M:** 8 BC UP **L:** 1 CH NP 1 SC NP **G:** 2 SC UP 2 BC NP 1 CP 3 CH NP	*Homoeosoma nebulella* D.S.	SEM	WP: OR NP: TR	Her	France	(Faucheux, 1995)
		Do	**M:** 1			TR			
		PP	**M:** 1			n.a.			
Lepidoptera: Yponomeutidae	**5th instar (final)**	**Do**	**M:** 1	*Yponomeuta cagnagellus*	SEM	n.a.	Her	Nether-lands	(Roessingh *et al.*, 2007)
		Setae	**M:** 1						

Table.S2: Most abundant AMF spore types extracted from soil (accounting for approx. 95% of isolated spores):

Color and appearance	Diameter	Photographs
Yellow to light orange	280-375 µm	
Orange to orange-brown, with „navel“	290-330 µm	
Bright red to brownish-red, corrugated surface *(Glomus sinuosum?)*	150 – 200 µm	
Orange-brown	170-190 µm	
Golden yellow	180 µm	
Juvenile: light yellow; mature: grayish-brown, occurs in composites	40- 45 µm	
Dark brown	50-75 µm	

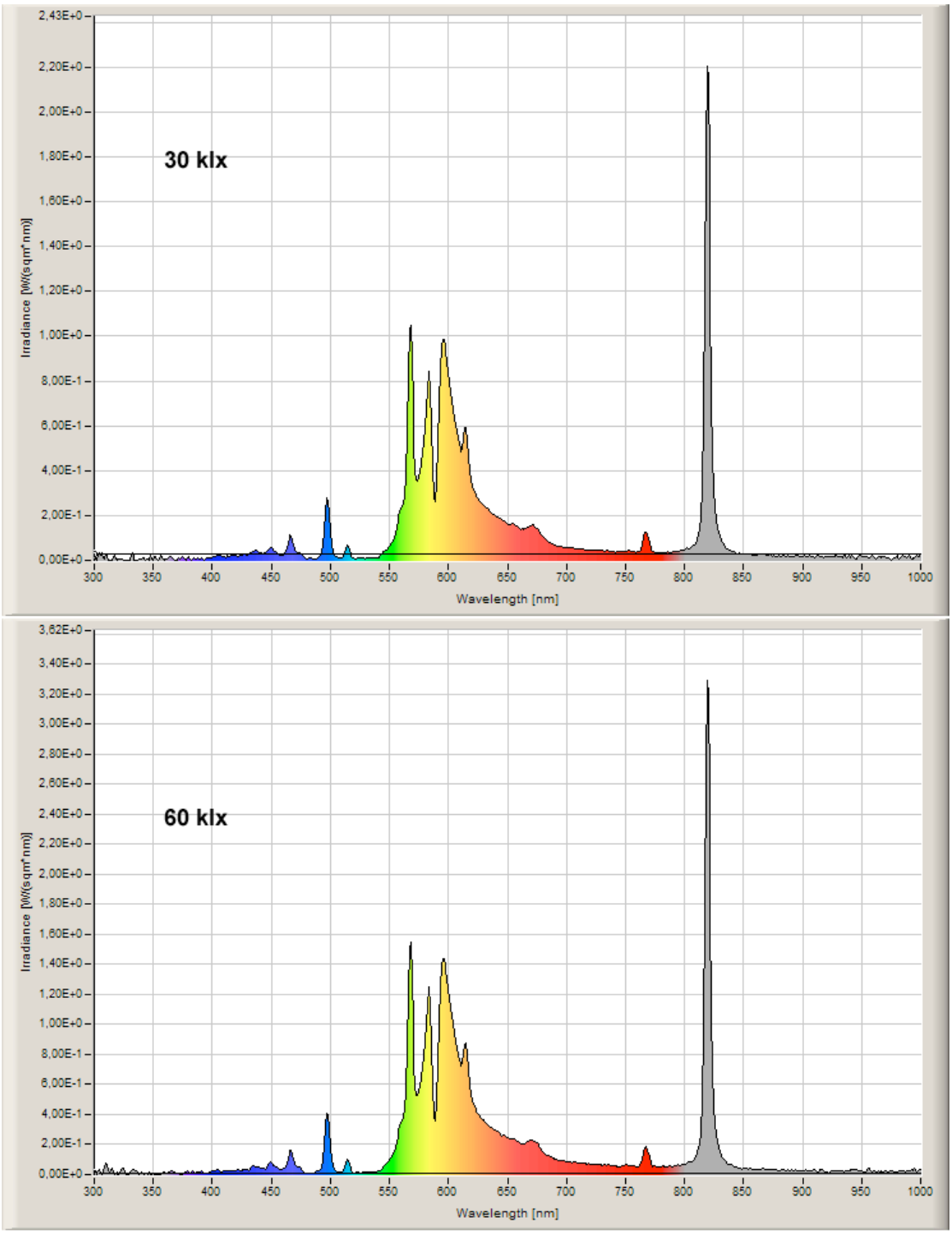


Figure S1: Light spectra for the standard light treatment: 32.3 ± 0.48 klx = 417.29 ± 20.55µmol·s^{-1}·m^{-2} PAR (top) and enhanced light treatment: 61.16 ± 0.72 klx = 791.37 ± 31µmol·s^{-1}·m^{-2} PAR, applied to dandelion plants in behavioral assays (chapter 3) and analysis of root exudates (chapter 4). Note that the spectra are identical and only the intensity is enhanced.

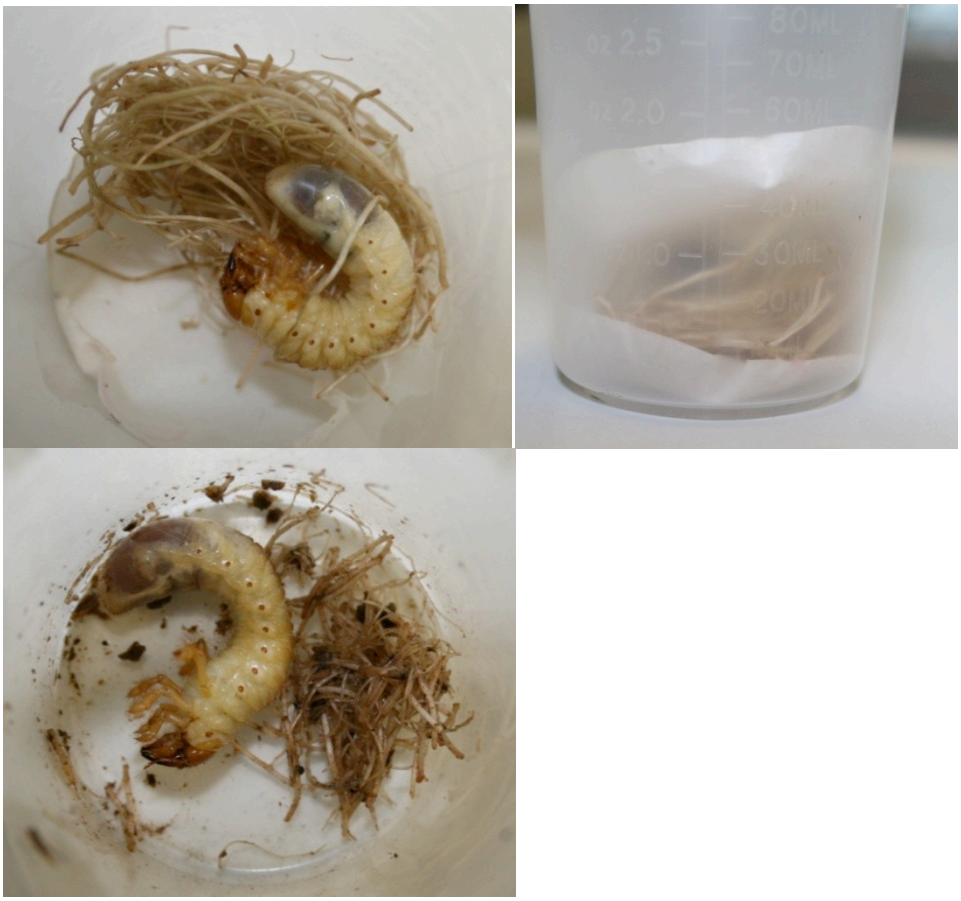


Figure S2: Food choice assay following observation of larvae. Larvae in plastic pots prior root consumption and after 24hrs (top left and bottom), and pot with roots and filter papers during root consumption experiment (top right).

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10. Lebenslauf

Der Lebenslauf ist in der Online-Version dieser Dissertation aus Gründen des Datenschutzes nicht enthalten.

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11. Erklärung

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(Elisabeth J. Eilers)

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