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RESEARCH ARTICLE

Host plant location by chemotaxis in an aquatic beetle

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Abstract Interactions between plants and aquatic insects are poorly documented, especially for turbid freshwater ecosystems. Many Swiss lakes offer such habitats, several of which are inhabited by the leaf beetle *Macroplea appendiculata* (Panzer 1794). This donaciid beetle is the only coleopteran species known to complete its life cycle entirely under water, where it lives primarily on perfoliate pondweed (*Potamogeton perfoliatus* L.), with Eurasian watermilfoil (*Myriophyllum spicatum* L.) as an alternative host plant. Direct observations during diving trips, aquatic olfactometer bioassays, and stir bar sorptive extractions (SBSE) coupled with GC–MS analysis were used to understand how these beetles locate their patchily distributed host plants and congeners in a harsh, often swirling environment. In olfactometer assays we observed that the aquatic beetle is strongly attracted to water extracts of pondweed, whereas neither mature males nor females beetles seem to produce attractive cues. The chemical analyses revealed that perfoliate pondweed releases one dominating compound, eucalyptol. Olfactometer assays confirmed that this is a potent attractant for the beetle. We also observed attraction to phytol, which is released by the main, as well as the alternative host plant. These finding are somewhat surprising as eucalyptol has never been reported for aquatic plants and phytol is poorly soluble in water. In addition, both are frequently described as insect repellents in terrestrial ecosystems. We suggest that these terpenoids normally have a defensive function against herbivores and pathogens, but that the highly specialized leaf beetle has evolved to exploit its host's defence chemistry for optimal foraging.

Keywords Aquatic beetle · Plant-herbivore interactions · Freshwater · Macrophytes · Host location · Macroplea appendicultata · Chemical attractants

Introduction

For herbivorous insects, plants are sources of nutrients, but also meeting point for interactions with congeners and places to produce progeny. Hence, locating the appropriate plant species can be a vital component for survival. To locate and reach these vital hosts, insects have developed a myriad of strategies, including reproductive and developmental behaviours, synchronized seasonal emergence, and extremely well developed sensory abilities (Schoonhoven et al. 2005). Besides visual information, chemical emissions from plants are particularly important as cues for insects in search of their optimal hosts (Städler 1992). In terrestrial ecosystems, signals produced and released by plants are extremely diverse, from ubiquitous compounds (e.g. ethanol, hexanol, sucrose) to species specific ones (e.g. (*E*)-asarone in Apiaceae, allyl isothiocyanate in Brassicaceae, or 6-methoxy-2-benzoxazolinone in Cucurbitaceae) (Kimmerer and Kozlowski 1982; Guerin et al. 1983; Bjostad and Hibbard 1992; Bernays and Chapman 1994; Han et al. 2001; Bruce et al. 2005; Reinecke and Hilker 2014). In some cases, equivalent phenomena have been observed in aquatic environments, where many creatures need to move judiciously to locate resources (Bilton et al. 2001). For aquatic insects, visual signals play

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a reduced role (Reeves and Lorch 2011), whereas typical indicative chemicals used in terrestrial systems are expected to be ineffective in liquid, due to their low solubility in water or a quick degradation by abundant bacteria (Wetzel 2001; Insam and Seewald 2010). In contrast, the more semi-volatile or non-volatile compounds in plant exudates might be more effective in aquatic systems, where they become mobile and fully exploitable by foraging herbivores (Decho et al. 1998; Fink 2007).

In muddy waters visual stimuli are likely of no use. Therefore aquatic insects are expected to fully rely on chemical cues. This concerns numerous species, but their foraging behaviour has been poorly documented (Bronmark and Hansson 2000). Moreover, foraging in aquatic environments has been considered mainly from a prey-predator point of view (Gross and Kornijow 2002), and has led to the identifications of water soluble amino acids, sugars, and organic acids used by invertebrates to localize their preys (Carr 1988; Weissburg et al. 2002). As yet, only two systems have been studied that implicate a role for chemical mediators in plant–insect interactions in freshwater. In one case, the weevil *Euhrychiopsis lecontei* (Dietz 1896) was shown to orient towards its food resource, the Eurasian watermilfoil (*Myriophyllum spicatum*), using glycerol and uracil released by the plant (Glomski et al. 2002; Marko et al. 2005). The second study involved larvae of *Coquillettidia sp.* mosquitoes that depend on their capacity to find roots containing aerenchyma tissues rich in oxygen for complete development. The larval orientation is mediated by a blend of common low molecular weight plant exudates, which include glycerol, uracil, thymine, uridine, and thymidine (Serandour et al. 2008). These two studies certainly do not cover the full diversity of interactions of freshwater insects with their host plants.

Besides elucidating the poorly understood foraging behaviour of an aquatic insect, the current study on *M. appendiculata* is also of potential applied interest. Eurasian watermilfoil is an invasive pest in North America and has become a serious problem in several lakes, where it may outcompete and even eliminate native aquatic plants (Smith and Barko 1990). With its apparent host specificity and limited capacity to spread, *M. appendiculata* may be a candidate to be introduced as a biological control agent against *M. spicatum*.

Similar to their terrestrial counterparts, aquatic plants are not merely passive victims of phytophagous insects and they can be expected to produce chemical defences to ward off potential attackers. Water plant defences also have only been studied superficially (Ostrofsky and Zettler 1986; Bolser et al. 1998). In terrestrial systems various specialized insect herbivores have "hijacked" the defence compounds of their specific host plants as foraging cues and indicators of the most suitable and nutritious plant tissues (Chapman 2007; Robert et al. 2012). It is expected that aquatic insects will have similarly adapted to exploit the defences of their host plants.

In Swiss lakes, we recently discovered multiple locations that are inhabited by the aquatic leaf beetle *M. appendiculata*. It lives primarily on freshwater plants (macrophytes), such as the perfoliate pondweed (*Potamogeton perfoliatus*), and uses the Eurasian watermilfoil as an alternative host (Mohr 1985). *M. appendiculata* adults and larvae never leave the water. The beetle mates, feeds, pupates, and breaths underwater, where all life stages are consistently associated with the macrophyte hosts. In order to optimally exploit available host plants, effective host location ability can be expected. Foraging is complicated by the lakes' currents, strong thermocline effects, extremely poor visibility, and substantial distances between plants. The larvae and adults have very limited displacement capacities, but their movement shows orientation. The system seems well suited to gain new knowledge on the interactions between freshwater plant and their insects. Using direct underwater observations under natural conditions, as well as flood olfactometer bioassays, and identification of the main compounds in the host plant exudates, we addressed to answer the following questions: (1) Do the two host plant species and mature insects release organic compounds that adult beetles use to find suitable plants and congeners in turbid water? (2) If so, what are the identities, respective attractiveness, and properties of the major compounds involved?

Materials and methods

Insects and lake plants

M. appendiculata shows an entire aquatic life cycle. Both larvae and adults possess special traits that allow them to live underwater. Larvae use two specialized hooks located at the eighth tergite, which are used to suck oxygen from the aerenchyma of various root systems (Lays 2006), and the adults can rely on a hairy hydrophobic net with which they create a tinny air layer close to their body, allowing them to breathe, while avoiding the inconvenience of having to surface (Brocher 1912). The insect can pupate and complete its metamorphosis embedded in a waterproof cocoon that is attached to roots during the winter season, and from where adults emerge in spring (Owen and Menzies 1996). Both larvae and adults are apparently unable to swim and it is unclear if and how they locate their host plants. It is known that perfoliate pondweed (fam. Potamogetonaceae) is consumed by *M. appendiculata* (Forel 1904). This tall plant (up to 3 m high) produces tiny flowers close to the surface between July and August. *Myriophyllum spicatum* (fam. Haloragaceae) is another common aquatic plant in the study area and it too plays a role in the life cycle of *Macroplea* beetles, especially for larvae that fix themselves to the root system. This is not the case for the European white water lily, or nenuphar (*Nymphea alba* L., Nympheaceae), which is also abundant along the shore of the studied lakes, and this non-host plant was therefore included here as a control plant.

During the summer season (mid-June to end of August), numerous scuba diving and snorkelling explorations allowed us to locate new populations of *M. appendiculata*, and we caught around 80 individuals, mainly found as pairs *in copula*. They were obtained from the lakes of Neuchâtel (Port de Petit-Cortaillod, Swiss grid 555.468/198.870, and Pointe du Grain 554.415/197.545) and Morat (Chenevières de Guévaux 571.000/198.326, and Port de Môtier 572.935/199.587), where they were only found on perfoliate pondweeds. Several intact, complete wild aquatic plants of the three species studied were collected in 20 L buckets filled with natural sludge and lake water. Insects were temporarily placed in 50 mL Falcon tubes with a piece of host plant and transported to the laboratory, where two 100 L aquarium (one per lake) were filled with 10 cm of sludge, complete macrophytes, and original lake water, kept at 21 ± 1 °C. A 14:10 h day:night cycle was maintained during the whole experiment using two 36 W neon tubes placed above the aquaria. Two aquaria pumps allowed for proper oxygenation of this artificial biotope, and plants were replaced with wild fresh ones if required. All insects were kept in similar aquaria with plants before bioassays.

Plant exudates and insect compounds collection

Field-collected individuals of the three plant species were used. We selected fresh, fully developed plants, but without flowers. Plants presenting signs of illness, or traces of damage were excluded. The roots and rhizomes were removed and plants were thoroughly rinsed twice with pure water (Elix$^{®}$ 20, Millipore) before being submerged in 250 mL of pure water containing NaCl at 0.1 g/L in order to represent natural freshwater conditions. The plant material was soaked for 24 h at 21 ± 1 °C with the same light conditions as applied for aquariums. Afterwards, solutions were filtered through Whatman no 597 filter papers. A part of these water extracts were kept at 5 °C until chemical analysis, while the rest was used the very same day in olfactometer bioassays. Females and males, usually *in copula*, were carefully separated before placing them individually in 10 mL of H_2O Elix containing NaCl at 0.1 g/L during 24 h.

Olfactometer assays

Attraction of individual insect towards the different solutions was tested in a 6-arm olfactometer, based on Rasmann et al. (2005). This device, which was initially modified after an aboveground olfactometer (Turlings et al. 2004), is usually used to observe displacements of entomopathogenic nematodes into sand or soil. For our purposes it was slightly adapted and filled with water. It consisted of a central glass chamber (8 cm in diameter for 11 cm deep) with six equally distributed side arms of 24 mm diameter and 29 mm long, connected each to a glass pot (5 cm in diameter, 11 cm deep) in which a source of attractants could be placed. Teflon sealing tape and a Teflon connector containing an ultrafine metal screen (2'300 mesh, Small Parts, Inc.) were used to make the whole device leak-proof and to allow passive diffusion of chemicals from the solutions at around 1.5 mL/min from external pots towards central chamber (see below). First, the system was flooded with 430 mL of H_2O Elix containing NaCl at 0.1 g/L. Then 10 ml of one of the plant extracts, insect extracts, or control water were added to each external pot, which pushed solutes into the six channels leading to the central chamber. In order to determine quantities needed for proper diffusion of the solutes, preliminary tests were carried out using 9 mL of H_2O Elix containing 20 µl of food colorants Patent Blue V (E131). Ten minutes after adding the solutions, the diffusions were visible like arrowheads with points reaching the centre of the olfactometer, until all the flows finally mixed after 12–15 min. Hence, this setup guarantees 10 min observation periods before extracts mixed in the centre. No food colorants were used during bioassays. A maximum of 3 extract solutions from plants and/or insects were tested simultaneously per bioassay. Two arms releasing such extracts into the central chamber were always separated by an arm that carried a control solution (H_2O Elix containing NaCl at 0.1 g/L in all case). Experiments focused on the attractiveness of plants or congeners. Possible synergistic effects of plant exudates combined with compounds released by conspecific beetles were not tested. All positions and orientations were changed regularly, the device was surrounded by black polystyrene, and a light source was placed directly above each olfactometer to avoid any light-mediated positional biases. Individual insects were used in each assay, avoiding possible influences of congeners on each other's choices. All insects tested came from aquaria, and they were rinsed with control water for 30 s before being released in the central room of the olfactometer. The whole system was disassembled and thoroughly cleaned between each observation (rinsed with water, acetone, pentane, and placed in an oven for 2 h at 250 °C). The position of each insect in the olfactometer parts was monitored every 10 s during the 600 s assay. Preferences of males or females were observed separately. Because the sexes responded in a similar manner, and because there was no attraction between conspecifics (Table 2), the data for males and females were pooled for statistical comparisons.

Chemical analysis and identification of likely attractant

Organic compounds collected in the soakings of plants (n = 30), mature female and male beetles (n = 13), and plants plus beetles (n = 5) were analysed using a gas chromatograph (Agilent 7890A) coupled to a mass spectrometer detector (Agilent 5975C). All components were trapped with the stir bar sorptive extraction technique (SBSE). Magnetic bars coated with polydimethylsiloxane polymer (Twister® Gerstel GmbH, 10 mm length, 0.5 mm film thickness) were placed in 50 mL of water extracts from plants or insects during 30 min and subjected to 250 rpm agitation. Afterwards, they were dried for 2 min in 2 mL glass vials under N_2 flow (20 mL/min) at room temperature before they were desorbed in a thermal desorption unit in splitless mode. Initial temperature was 40 °C for 0.5 min, then the temperature was increased 100 °C/min to 250 °C (hold time 6 min). A cryo injection system (CIS) at −80 °C was used to focus the compounds that were released during the desorption procedure before releasing them simultaneously (12 °C/s to 280 °C, hold time 8 min). The PTV inlet was operated in the solvent vent mode, a vent pressure of 17.6 psi, a vent flow of 50 mL/min, and a purge flow of 50 mL/min. The helium carrier gas pressure was 17.6 psi (flow rate 1.1 mL/min) at constant flow mode and pushed the sample onto an Agilent HP-1MS column (60 m length × 0.25 mm i.d., and 0.25 μm film thickness). The GC oven program started at 40 °C for 5 min, then ramped to 250 °C at a rate of 3 °C/min (hold time 2 min). A 2 min post run at 280 °C was carried out. The detector transfer line temperature was set at 280 °C and the ion source and quadrupole temperatures were set at 230 and 150 °C respectively. Electron impact (EI) mode and ionization potential of 70 eV were used with a scanning over the mass range of 33–300. Controls (n = 5) and blank (n = 9) analysis were run in order to determine which of the chemicals were not released by the studied organisms. These contaminants, plasticizers, etc., were excluded from subsequent data treatment. Initial identifications of compounds were based on NIST05 mass spectral library as well as PBM Library Search (Agilent Technologies, Inc.). Identities of the compounds of interest were confirmed by comparisons of retention times and mass spectra with those of pure standards (Fluka). Without any internal standard added to the initial samples, it was not possible to determine the quantities of bioactive compounds released in the water.

Statistical data analysis

Significant differences in choices among extracts by *Macroplea* leaf beetles were assessed with log-linear models on the basis of the assumption that insects would disperse equally among the six arms in the absence of any attraction. In this study, as only one insect was present at a time in olfactometer, the model was run with the time spent in or near each arm, during the 600 s observation period. Statistical analyses were done using R (R Development Core Team 2008).

Results

Behaviour and ecology of *M. appendiculata*

At first glance, this chrysomelid beetle (subfamily Donaciinae) shows limited aquatic-life adaptations. Our observations confirm that neither larvae nor adults are able to swim efficiently, and that they never come to the surface or the shore. Located at the eighth tergite, hooks tightly fix the larvae to roots, stolons, rhizomes, or stems, and allow respiration. Once separated from the host plant, neither larvae nor pupae were able to find it again. When harassed, the adults jumped off the host plants, and then slowly sink to the lake bottom where they crawl. They waved their antennae continuously in water. From our visual inspections we deduced that leaf consumption by adults was minimal, in agreement with a low metabolism as suggested in former studies (Otto 1985; Kölsch and Krause 2011). A maximum of 7 adult insects per young plant was observed, with a majority of host plants not carrying any beetle, which implies that this species has little potential as a biocontrol agent.

In the study lakes, both adults and larvae were found on perfoliate pondweeds, between 1 and 6 m deep, whereas Eurasian watermilfoil hosted larvae only. Elsewhere, these leaf beetles have been found in various Palearctic swamps and rivers (Grillas 1988; Medvedev 2006; Nelson et al. 2007; Lou et al. 2011), but little is known about their exact ecology, behaviour or distribution, as it is very difficult to observe them in their habitat, at great depths of larger turbid freshwater lakes. How they may colonize new host plants has remained unclear. Although there is no concrete observational evidence, it has been speculated for two related aquatic species *M. mutica* and *M. japana* (Kölsch et al. 2006; Kölsch and Pedersen 2008) that they can take-off and have limited flight capacity (Mohr 1985; Mann and

Crowson 1983; Zhang et al. 2010). This behaviour has not been been observed nor studied for *M. appendiculata*. It has been proposed that postglacial colonization of European inland lakes by the beetles is passive and facilitated by vectors like waterfowl (Green and Sanchez 2006; Mende et al. 2010). Observations during our diving trips suggest that there is very little opportunity for this type of migration in our study area.

Plants and conspecifics olfactometer assays

Eighty-six bioassays were carried out with aquatic olfactometers. The attraction of *Macroplea* beetles to the three aquatic plant species showed clear differences. Both females and males responded in the same way, and were most strongly attracted to extracts from their main host plant *P. perfoliatus* (pondweed) in comparison with all other solution types ($p < 0.001$). During standard assays of 600 s, insects spent on average a third of the available time (193 ± 24 s.) in or near the arm with the water extracts of pondweed. They spent significantly less time in the other solutions (i.e. other aquatic plants, and controls), with an overall average of 82 ± 7 s. They exhibited no significant attraction to the extracts of the alternative host plant (watermilfoil) or that of the non-host (nenuphar), which were visited as much as control solutions (Fig. 1; Table 1).

Soakings from mated females or males did not attract nor repel individuals that were released in the centre of the olfactometer. The distribution of the insects was homogenous among the six available choices in these assays. Overall, the beetles distributed themselves equally among the male extracts, female extracts, and control solutions (Fig. 2; Table 2).

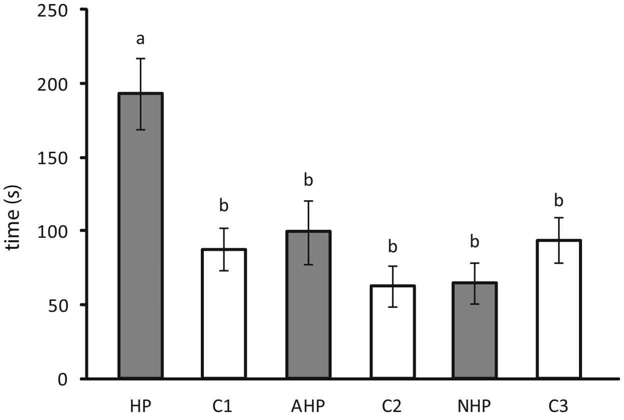

Fig. 1 Time spent by *M. appendiculata* adults in arms carrying different extracts during aquatic olfactometer bioassays (N = 54). HP, AHP, NHP, and C represent solutions from host plant (*Potamogeton perfoliatus*), alternative host plant *(Myriophyllum spicatum),* non-host plant (*Nymphaea alba*), and controls, respectively. *Graph* shows mean ± SE. *Lettering* indicates significant statistical differences ($p < 0.001$)

Table 1 Statistical comparisons of attractiveness of plant water extracts in aquatic 6-arms olfactometers for *M. appendiculata* adults (N = 54)

N = 54	PH	PHA	PNH	Control 1	Control 2	Control 3
PH		0.003	0.000	0.001	0.000	0.001
PHA	0.003		0.132	0.626	0.107	0.922
PNH	0.000	0.132		0.301	0.912	0.197
Control 1	0.001	0.626	0.301		0.253	0.792
Control 2	0.000	0.107	0.912	0.253		0.162
Control 3	0.001	0.922	0.197	0.792	0.162	

Extract from the main host plant *P. perfoliatus* induced a strong and significant attraction

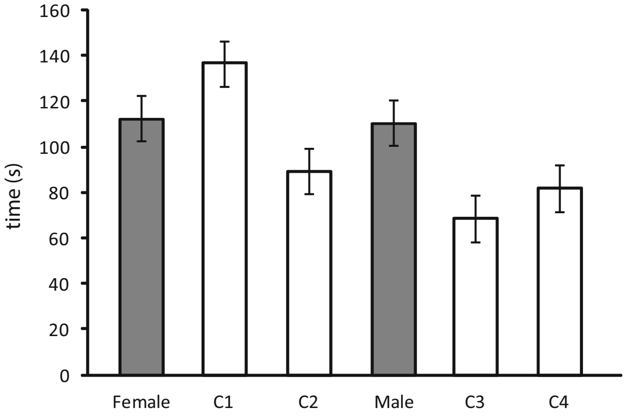

Fig. 2 Time spent by mature *M. appendiculata* adults in olfactometer arms with water extracts from conspecific females, males, or controls (C1, C2, C3, and C4), respectively (N = 15). *Graph* shows mean ± SE. No statistical differences between the six arms were observed

Chemical analysis

Generally, polydimethylsiloxane residuals, coming from the stir bar coating adsorbent, produced several major peaks in chromatograms, and only few compounds from a biological origin were detected in the 62 samples analysed. Among these organic compounds, some were present in the extracts of all the three aquatic plant species (e.g. α-copaene and butylated hydroxytoluene), one in both host plants (phytol), and one compound was specific of *P. perfoliatus*, the main host (eucalyptol). Abundances varied strongly among these relevant compounds, with eucalyptol showing clearly the highest intensity overall. The consistent presence of butylated hydroxytoluene in all three plant-exposed solutions, but not in control solutions, was unexpected because it is known as a synthetic antioxidant. However, various freshwater phytoplankton species are

Table 2 Statistical comparisons of conspecific attraction in 6-arms olfactometers for mature *M. appendiculata* (N = 15)

N = 15	Female	Male	Control 1	Control 2	Control 3	Control 4
Female		0.972	0.695	0.672	0.405	0.572
Male	0.972		0.670	0.698	0.423	0.596
Control 1	0.695	0.670		0.421	0.232	0.346
Control 2	0.672	0.698	0.421		0.672	0.885
Control 3	0.405	0.423	0.232	0.672		0.779
Control 4	0.572	0.596	0.346	0.885	0.779	

No significant attraction was observed

able to synthesize it naturally (Babu and Wu 2008), which may explain its presence in our system. Figure 3 shows typical chromatographic profiles of water extract from all aquatic plants and a control solution obtained with the SBSE method. No relative or absolute quantifications of these compounds were carried out.

The same method applied to the insect extracts did not reveal any compound that can be assigned to the beetles. It should be stressed that we did not test virgin individuals. Analyses of water extracts from plant and beetle together did not reveal any insect contributions either (chromatograms not shown here). Therefore further investigations focused on compounds emitted by plants only. Injections of pure standards with same analytical method and parameters confirmed that the tentative identifications were correct in all cases.

Olfactometer assays

Only eucalyptol (1,8-cineole) and phytol were consistently detected in the extracts of the main host plant. In order to assess if these compounds were involved in beetle attraction, pure standards were mixed individually in H_2O Elix containing NaCl at 0.1 g/L to create solutions with concentrations of 50 µM. Such a concentration is commonly used in freshwater experiments (Serandour et al. 2008). As an additional control we also tested the attractiveness of a ubiquitous plant-produced sesquiterpene, *E*-β-caryophyllene (Hiltpold and Turlings 2008).

Eucalyptol, the most abundant compound produced by the pondweed, revealed itself as the strongest attractant (solubility in water = 3.5 g/L). Phytol, a second minor compound, showed slight, but significant attraction as well (nearly insoluble in water). The beetles were not attracted to *E*-β-caryophyllene (Fig. 4). In seconds, average times spent in or near the arms with the attractants were 225.3 ± 30.5 for eucalyptol; 130 ± 31.3 for phytol; 50 ± 15.3 for *E*-β-caryophyllene, and 64.9 ± 10.8 (average for all three control arms). Statistical analyses revealed clear attraction for eucalyptol, as well as for phytol, but to a lesser extent (Fig. 4; Table 3).

Discussion

All three of the tested macrophytes are naturally present in Swiss lakes, but with an extreme patchy distribution. It was consistently difficult to find pondweed populations, sometimes with several kilometers between each patch. We found *M. appendiculata* exclusively in patches with pondweeds, which were most of the time also inhabited by watermilfoils. During dozens of hours of scuba diving we never found any sign of the leaf beetle on any of the other aquatic plants described as potential host in the literature (Kölsch and Kubiak 2011). From our observations, it is clear that random displacement of *M. appendiculata* is unlikely. The observed chemotaxis behaviour of the beetles is likely to only occur at a limited spatial scale and probably most effective within a patch of macrophyte species. However, the attraction might also facilitate distant migration within the aquatic network, allowing targeted movements towards new populations of host plants. Further observations are needed to assess these. In the olfactometer assays adults showed a strong preference for chemical emissions of *P. perfoliatus*, confirming their close association with this main host plant. The alternative host *M. spicatum* may also provide food, shelter and air, at least for part of the beetle's life cycle, especially during larval stages and as an overwintering host plant (Kölsch and Kubiak 2011). The relative importance of the two host plant species is nicely reflected in the attractiveness of the volatiles that are released by the two plants. Eucalyptol, which is the distinctive and major chemical emitted by perfoliate pondweed, was found to be highly attractive and can serve to localize this principal host plant, whereas the somewhat lesser attractiveness observed for phytol, a minor compound found in both host plant species, suggests that it could be used to locate the alternative host if required. The results from the olfactometer bioassays also corroborate that the common non-host nenuphar is not attractive to the beetle.

The exclusive presence of *M. appendiculata* on perfoliate pondweed during our surveys in Swiss lakes nicely fit their behavioural responses in the olfactometers. Yet, it is

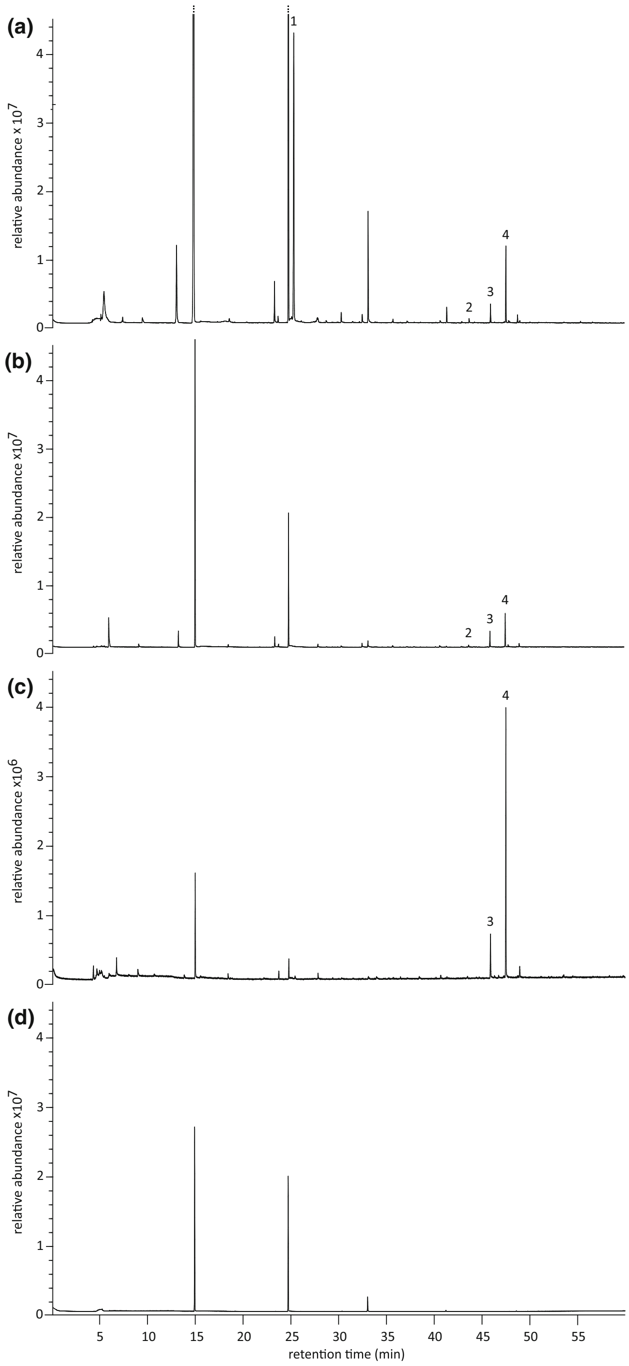

Fig. 3 Chromatographic profiles of water extracts from: **a** host plant (perfoliate pondweed), **b** alternative host plant (European watermilfoil, **c** non host plant (nenuphar), and control (water) obtained with the SBSE method. Relevant compounds are: 1 eucalyptol, 2 phytol, 3 α-copaene, 4 butylated hydroxytoluene. All other peaks are PDMS residuals or other types of impurities

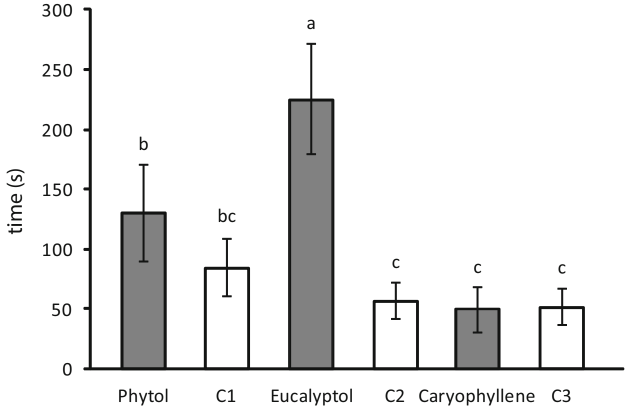

Fig. 4 Attractiveness of pure standards and water control solutions tested in olfactometer bioassays (N = 17). *Bars* in the *graph* are displayed in same order as in olfactometer arms. Each *bar* indicates the number of seconds ± SE spent by *Macroplea* beetles in each arm during a 10 min trial. *Lettering* indicates statistical differences (see Table 3 for more details)

somewhat unexpected that they responded to eucalyptol and phytol, as these compounds are frequently described as insect repellents in terrestrial ecosystems (Klocke et al. 1987; Odalo et al. 2005). These terpenoids are known to have a defensive function against both herbivores and pathogens (Morcia et al. 2012; Zengin and Baysal 2014) and they are regularly mentioned as insect feeding and oviposition repellents in the context of pest control. For instance, it has been shown that phytol repels weevil species (Ndungu et al. 1995), whereas eucalyptol is a top candidate among essential oils to deter a wide spectrum of insect taxa with implications for human health (e.g. cockroaches, mosquitoes), as well as agricultural pests (Klocke et al. 1987; Sfara et al. 2009; Alzogaray et al. 2011; Maia and Moore 2011).

We did not specifically determine the exact origin of eucalyptol and phytol in the study system, but *M. spicatum* poses secretory trichomes in young leaves and stems (Godmaire and Nalewajko 1990). These trichomes are likely to serve a defensive purpose and are a probable source of phytol. In all cases, it is evident that *M. appendiculata* is not affected by these defences and has adapted to exploit them as a host location cues. The beetle has undoubtedly a terrestrial origin, and the switch to an aquatic milieu must have required many adaptations (Kleinschmidt and Kölsch 2011). Knowing the evolutionary history of this species suggests that some of its current behaviours still rely on cues from its ancestral environment, as shown in other aquatic weevils (Solarz and Newman 1996). For instance, antennal olfaction is also the key mode of perception in the beetles' aquatic environment. *M. appendiculata* waves its antenna continuously in water, with alternating movements between the antennae, evidently to receive chemical information.

Lack of attraction between the sexes was unexpected. In some other aquatic insect species, males are known to produce and release organic, but insoluble compounds,

Table 3 Statistical pair comparisons in attractiveness of various pure solutions in aquatic 6-arms olfactometer for *M. appendiculata* adults (N = 17)

N = 17	Eucalyptol	Phytol	Caryophyllene	Control 1	Control 2	Control 3
Eucalyptol		**0.042**	**0.001**	**0.006**	**0.001**	**0.001**
Phytol	**0.042**		**0.038**	0.271	**0.048**	**0.045**
Caryophyllene	**0.001**	**0.038**		0.275	0.803	0.932
Control 1	**0.006**	0.271	0.275		0.391	0.311
Control 2	**0.001**	**0.048**	0.803	0.391		0.870
Control 3	**0.001**	**0.045**	0.932	0.311	0.870	

Bold *p* values indicate significant differences between two solutions (see also Fig. 4). Overall, eucalyptol induced the strongest attraction

which act as surface semiochemicals (Cheng and Roussis 1998). Nevertheless, what is efficient for riffle bugs gliding at the surface is not applicable to fully aquatic beetles. As we worked only with mature and already mated adults, we think that further experiments with virgin individuals are necessary to exclude mid and long-distance attraction by the beetles. In terrestrial systems, beetles are known to emit sex pheromones, but they also use plant-produced volatiles to aggregate (Dickens 2006). The specificity of the host plant choice may be sufficient to ensure encounters between the sexes. Close-range chemical communication may then be used to further facilitate mate finding. We observed that some pairs that appear to be in a mating position started to mate truly when they came in contact with pondweed water extracts. It may well be that the presence of a suitable host plant can induce the release of sex pheromones, as is the case for other insects (Lextrait et al. 1995). It is worth investigating this further. The same is true for the beetle's oviposition strategy. We were puzzled to observe that in the aquaria females seemed to randomly lay their eggs while crawling on the bottom, and not directly on a host plant. It remains to be determined if this oviposition is stimulated by host plant-produced chemicals.

In the studied areas, observations under natural conditions suggested that *M. appendiculata* population densities and feeding rates are too small to have significant effect on Eurasian watermilfoil populations. Leaf damage by the insect was minimal, and aquatic snails were responsible for the main damages that we observed on the leaves. We did not assess the effect of the beetles and their larvae on the belowground plant parts and it remains possible that overwintering larvae have an important impact on these structures and could negatively affect future growth.

Host plant finding by aquatic beetles has not previously been studied in detail. The biology of *M. appendiculata* and closely related beetles is rather unique. Despite their classic leaf beetle appearance, they are the only beetles known to be entirely aquatic. This will have required some exceptional adaptations. Here we show that one of those adaptations is an ability to detect and exploit host plant-derived compounds to orient themselves in their aquatic environment, allowing them to locate a vital resource and complete their life cycle.

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Compliance with ethical standards

Conflict of interest Submission of the manuscript has been approved by all relevant authors and institutions, and all persons entitled to authorship have been so named.

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