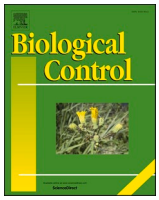
Biological Control 192 (2024) 105509

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon



Investigating the host finding behaviour of the weevil *Phytobius vestitus* for the biological control of the invasive aquatic weed *Myriophyllum aquaticum*

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HIGHLIGHTS

- *Phytobius vestitus* is a biological control candidate for *Myriophyllum aquaticum.*
- *Phytobius vestitus* responds to plant species based on olfactory cues.
- Plant chemistry and phylogeny largely determine the host selection.
- *Phytobius vestitus* is mostly attracted to olfactory cues emitted by *M. aquaticum.*
- This research warrants further investment into the potential use of *P. vestitus*.

ARTICLE INFO

Keywords:
Parrot's feather
Invasive plant
Biocontrol
Host selection
Plant chemistry

ABSTRACT

Biological control programmes involve pre-release studies that include host range tests to predict likely negative effects on native species. Behavioural responses of potential biological control agents to olfactory signals emitted by target and non-target species provide information on the host-finding and selection behaviour for safer agent selection. The weevil *Phytobius vestitus* (Coleoptera: Curculionidae) was identified as a potential biological control agent for the sustainable management of the aquatic weed *Myriophyllum aquaticum* (Haloragaceae). Using olfactometer-based bioassays, we analysed the host-finding and host-selection behaviour of *P. vestitus* across a variety of plant species closely related to *M. aquaticum* using a glass olfactometer. We further analysed the volatile organic compounds emitted by the target and non-target plant species to explain the weevil's observed preferences. We demonstrated that *P. vestitus* was able to recognise and discriminate between eight closely related Haloragaceae plant species and was significantly more attracted to the olfactory cues emitted by the target plant, *M. aquaticum*, compared to the other plant species. This study provides a first important indication of the specificity of *P. vestitus* and shows the role of plant chemistry in host selection and the possibility of refining host range assessment for potential biological control agents. However, exhaustive risk assessment for possible release into targeted new environments still requires further investigations.

1. Introduction

Biological invasion refers to the introduction, establishment, and spread of non-native or exotic species in ecosystems. These invasive species can outcompete native organisms for resources, disrupt ecological processes, and alter the structure and function of ecosystems (Blackburn et al., 2014; Mack et al., 2000; Maluleke et al., 2021; Richardson et al., 2010). The introduction of invasive species can occur through various means, including human activities such as trade, transportation, and intentional or accidental releases. Once introduced, invasive species may thrive and rapidly expand their populations, leading to the displacement or extinction of native species (Blackburn et al., 2014; Perrings et al., 2010; Richardson et al., 2010). The ecological consequences of invasion can include changes in species composition, habitat degradation, alterations in nutrient cycling and disruptions to ecosystem services. Invasive species can also have

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https://doi.org/10.1016/j.biocontrol.2024.105509
Received 7 February 2024; Received in revised form 8 April 2024; Accepted 9 April 2024
Available online 10 April 2024

economic impacts, such as reduced agricultural yields, increased management costs and damage to infrastructure (Charles and Dukes, 2007; Maluleke et al., 2021; Perrings et al., 2010; Vilà et al., 2011). Efforts to manage and control biological invasions typically involve monitoring, prevention, and mechanical or chemical measures to minimize the spread and impact of invasive species (Clout and Williams, 2009). Yet, mechanical and chemical control measures are often cost prohibitive and ineffective when the invasion is widespread and dense. Furthermore, these methods can negatively impact local biodiversity, since mechanical control may damage existing natural flora and fauna, while chemical control inevitably releases toxic chemicals into the environment (Hussner et al., 2017). Instead, classical biological control, a strategy involving the release of specialised natural enemies of the invasive organism (McFadyen, 1998), has been advocated as a cost-effective and environmentally friendly alternative for managing invasive species (Müller-Schärer and Schaffner, 2008; Schwarzländer et al., 2018).

While classical biological control of weeds has been a successful method for managing plant invasions (Winston et al., 2023), it also requires thorough risk assessments and ongoing monitoring to prevent unintended consequences. Accordingly, biological control programmes involve several steps, including, once the potential biological control agent is identified, to perform pre-release studies, such as host specificity tests to evaluate the safety of the potential biological control agent in the invaded range (Abram et al., 2021; Gaffke et al., 2021; Müller-Schärer and Schaffner, 2008; Wheeler and Schaffner, 2013). In this regard, a first step for host specificity testing is to study all the phylogenetically related species to the one to be contained, and assess their vulnerability against the biological control agent (Lefoe et al., 2022). Herbivore host range is typically phylogenetically conserved (Pearse and Altermatt, 2013) and thus the process usually involves employing the centrifugal phylogenetic testing method (Wapshere, 1974). This presupposes that the greater the genetic proximity of these non-target species, the more likely they are to be morphologically and biochemically similar to the target species (Wapshere, 1974). The host specificity of potential biological control agents is then assessed by evaluating 1) the fundamental host range through no-choice tests, and 2) the realised or ecological host range through choice tests and field host range testing in the native range (Schaffner, 2001; Hinz et al., 2014).

Additional tests under controlled quarantine conditions can be conducted to mechanistically understand host-finding behaviour (Fung et al., 2021; Park et al., 2018). These approaches can help to explain unexpected host selection behaviours, since insects use various sensory inputs, including olfactory signals, to detect and select their host (Brévault and Quilici, 2010; Park et al., 2018). Accordingly, the insect serving as a biological control agent may exhibit a preference for a non-target plant species that is not closely related, simply because its chemical composition bears a closer resemblance to the target species compared to a more closely related non-target species (Paynter et al., 2020; Wheeler et al., 2021). Hence, integrating chemical ecology experiments into host range testing can further help to predict behavioural responses of the potential biological control agent to target and non-target species (Anton and Cortesero, 2022; Brévault and Quilici, 2010; Fung et al., 2021; Hinz et al., 2019). Mechanistically studying behavioural responses can thus improve the ability to predict the ecological host-range under laboratory conditions (Gaffke et al., 2021; Hinz et al., 2019; Wheeler and Schaffner, 2013).

Native to South America, *Myriophyllum aquaticum* (Vell.) Verdc. (Haloragaceae), also known as parrot's feather, is a freshwater aquatic plant that has rapidly invaded many regions of the world (Orchard, 1981; Randall, 2017). In North America, the species is considered a serious weed, where it reduces biodiversity and impacts human activities in streams, irrigation channels, lakes and ponds (Wersal and Madsen, 2011). Once established, this plant is extremely difficult to eradicate. Mechanical and chemical controls have so far been unsuccessful, due to rapid vegetative propagation after mechanical action, as well as the limited allowed application of toxic chemicals that can be used (Hussner et al., 2017; Wersal and Madsen, 2011). Therefore, biological control has been suggested as a possible solution for *M. aquaticum* management (Coetzee et al., 2021). The weevil, *Phytobius vestitus* (Syn: *Parenthis vestitus*) Dietz (Coleoptera: Curculionidae), typically associated with *Myriophyllum heterophyllum,* has also been observed feeding and developing on *M. aquaticum* causing considerable damage (Diaz, Louisiana State University and Harms, US Army Corps of Engineers, pers. comm.). Accordingly, studies initiated to assess the potential use of *P. vestitus* as a biological control agent in western North America (Humair et al., 2023; Humair et al., 2024; Weyl et al., 2022) need to include an extensive risk assessment, as several native *Myriophyllum* species occur in North America (Chen et al., 2014).

The aim of this study is to investigate host finding and selection behaviour of the potential biological control agent *P. vestitus* across a variety of closely related plant species of the target *M. aquaticum*. Aiming to explain the observed weevil preferences, volatile organic compounds (VOCs) released by tested plants were analysed. Overall, this study aims to provide information on the potential use of *P. vestitus* as a biological control agent against the *M. aquaticum* invasion in western North America.

2. Materials and methods

2.1. Target and non-target species

Eight species in the family Haloragaceae, the target *M. aquaticum* and seven non-target species *Myriophyllum crispatum* Orchard, *Myriophyllum heterophyllum* Michx., *Myriophyllum hippuroides* Nutt., *Myriophyllum rubricaule* sp. nov., *Myriophyllum simulans* Orchard, *Myriophyllum verticillatum* L. and *Proserpinaca palustris* L., were used for the study. *Myriophyllum aquaticum* and *M. hippuroides* were collected in British Columbia (Canada) and transported to CABI (Delémont, Switzerland) in 2021 and 2022. The plants were maintained under controlled conditions in the quarantine facility at CABI (Delémont, Switzerland) (25 °C, 50 ± 10 % RH and 16:8 light:dark). *Myriophyllum verticillatum* was collected in 2022 and 2023 in Switzerland. *Myriophyllum heterophyllum* was collected in France in 2023, while all other species were purchased from aquatic plant shops in 2021. All tested plant species were maintained at CABI Switzerland under controlled conditions (25 °C, 50 ± 10 % RH and 16:8 light:dark), for exotic or quarantine species, and in semi controlled greenhouse conditions for naturally-growing species in Switzerland.

2.2. Potential biological control agent

Adults of *P. vestitus* originated from Baton Rouge, Louisiana (USA) and a rearing colony under controlled conditions in quarantine (25 °C, 50 ± 10 % RH and 16:8 light:dark) was established on *M. aquaticum* at CABI (Delémont, Switzerland) in May 2022. Once in Switzerland, weevils were reared on the same plant species as they were collected, i.e. *M. aquaticum.*

2.3. Host finding behaviour bioassays

To study host-finding behaviour, an in-house glass olfactometer was developed to examine insect attraction toward different target and non-target species (Figure S1). The olfactometer comprised two glass vessels, each containing water and different plant treatments, which were connected at the top via a glass Y-tube (Figure S1). All bioassays were conducted in a quarantine room at CABI under the following conditions: 25 °C, 50 ± 10 % RH and 16:8 light:dark.

Prior to the onset of the bioassays, young females (<72 h old after emergence) were isolated in individual plastic tubes and starved for a period of 24 h. All parts of the olfactometers were cleaned with 70 % ethanol to remove any residual odour. Then, the two vessels were half filled with tap water and prepared for the different no-choice and choice

tests. The no-choice tests included two set-ups (Figure S2): 1) the target species, *M. aquaticum*, in one vessel of the olfactometer, and water in the other and 2) a non-target species on one arm and water on the other side. For the choice tests, only one set-up was used: *M. aquaticum* in one vessel and non-target species on the other vessel. For both no-choice and choice tests, three stems of the target and non-target species were placed in the vessels depending on the treatment in order to have the same amount of plant material in each side. The open top parts of each vessel were then sealed with Parafilm® for 30 min, after which, the Parafilm® was removed and the two vessels were connected through the Y-tube. Once the olfactometer was assembled, one *P. vestitus* female was inserted at the beginning of the Y-tube, which was sealed with Parafilm® to avoid escaping. For 20 min, the time the insect spent in each part of the Y-tube (intersection, right arm and left arm) was recorded. In addition, the initial choice (first arm entered) and the final choice (final position) of each female was recorded. If a female did not respond within the first 60 s after the start of the test or initiated a response but remained in the centre of the Y-tube, it was deemed unresponsive and discarded from further analysis, this was typically low at about 5 % of females. For all experiments, no-choice and choice-tests, the females were divided into groups of four which were exposed to the same plant stems in the vessels, and every two tested females, the position of the vessels were swapped (for total number of replicated for each treatment see Figs. 1 and 2). After each group of four tested females, the cylinders and Y-tube were cleaned with 70 % ethanol and new fresh plant stems placed. Each female was used only once.

2.4. Volatile organic compounds analysis

Volatile organic compounds (VOCs) released from target and non-target species were analysed using a gas chromatograph (GC) (Agilent 7890B, Agilent Technologies, Santa Clara, California, United States) coupled to a mass spectrometer (MS) (Agilent 5977B) at the University of Neuchâtel. For this, fresh leaf and a portion of the stem were placed in individual glass microvials (20 mm length; 4 mm internal diameter) and then inserted into glass desorption tubes that are hermetically closed with metallic adaptors. A total of four replicates for each plant species were prepared. Tubes were then individually desorbed in a thermal desorption unit (TDU, Gerstel GmBH, Mülheim an der Ruhr, Germany) in splitless mode, with an initial 40 °C temperature kept for 0.5 min, then a ramp of 120 °C/min to reach 160 °C (hold time 4 min). A cryo-injection system (CIS, Gerstel GmBH) at −80 °C was used to trap the desorbed compounds before releasing them into the separative GC column (12 °C/sec to 270 °C, hold time 4 min). For injection, the programmable temperature vaporizer inlet (PTV inlet) was operated in the solvent vent mode, a vent pressure of 8.68 psi, and with a total flow of 43.5 mL/min. The helium carrier gas pressure was 13.29 psi (flow rate 1.0 mL/min) at constant flow mode and pushed the volatile blend onto a 30 m × 0.25 mm × 0.25 µm film thickness HP-5MS fused silica separative column (Agilent). The GC oven programme started at 40 °C for 3 min, then ramped to 270 °C at a rate of 7 °C/min (hold time 2 min). A 1 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 °C and 150 °C respectively. Electron impact (EI) mode and ionization potential of 70 eV were used with a scanning over the *m/z* 33–400 range. Blank (n = 5, empty tube in the GC–MS equipment) analyses were run to determine which of the chemicals were irrelevant for subsequent data treatment. The molecular features matrix of relative peaks' areas was assembled using the ADAP algorithm for GC–MS in MZmine 2.53 (Pluskal et al., 2010). See details in Supplementary Methods S1.

2.5. Statistical analysis

The host-finding behaviour of the weevil in terms of 1) time spent in each arm of the olfactometer, 2) initial choice and 3) final choice of the weevil in the olfactometer was analysed for no-choice (target or non-target plant *vs.* water) and choice (target *vs.* non-target plant) tests using logistic regressions with binomial distributions by means of generalized linear mixed models (GLMM) via the glmmTMB function of the package with the same name (Brooks et al., 2017) in R (v4.3.2; R Core Team, 2023). For the models 1) with the dependent variable being time expressed as proportion of time the insect spent in each arm of the Y-tube during the 20 min test duration the underlying probability distribution was taken to be binomial and overdispersion was tested (but not found to be significant) by comparing the sum of squares of the standardized residuals to the χ^2 distribution (Gelman and Hill, 2006). For models 2) and 3), the dependent variable (initial or final choice) was binary (0 or 1) and thus, also a binomial distribution was chosen. For all models, the fixed effect independent variables were plant species, treatment (i.e. plant species *vs.* water for no-choice tests, and target species *vs.* non-target species for choice tests), and their interaction. A random effect was introduced into each model to account for the possible co-dependency within the groups of beetles for which the same plant stems were used during the experiments (as described in more detail above). The fixed effects parameter significance was analysed

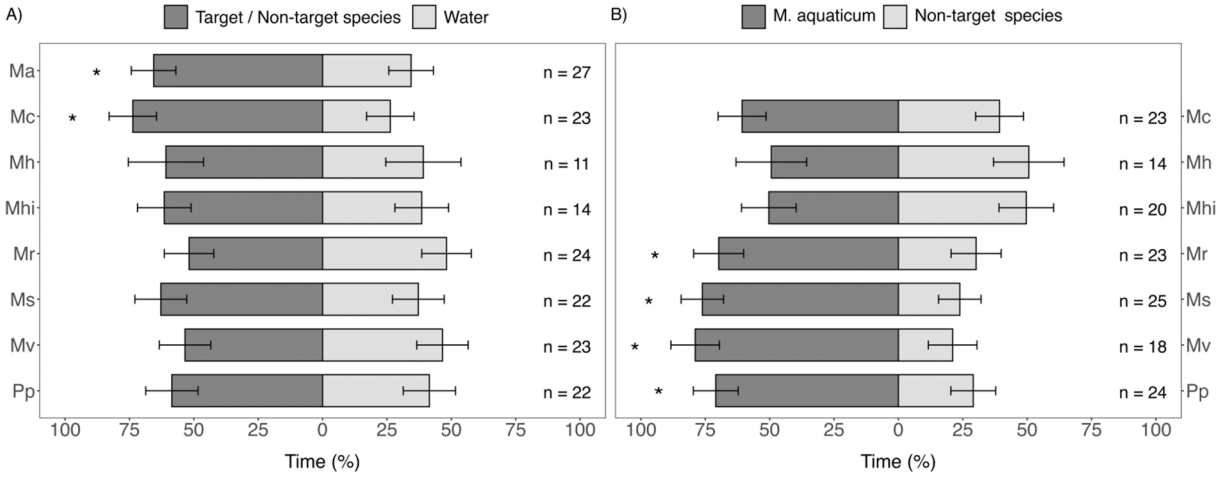

Fig. 1. Proportion of time spent (mean ± SE) by *Phytobius vestitus* adult females in each arm of the Y-tube olfactometer under A) no-choice: target/non-target *vs.* water and B) choice: target *vs.* non-target. The * indicates significant differences for each set-up ($P < 0.05$), while n = the number of replicates included in the analysis. Plant species: Ma = *Myriophyllum aquaticum*, Mc = *Myriophyllum crispatum*, Mh = *Myriophyllum heterophyllum*, Mhi = *Myriophyllum hippuroides*, Mr = *Myriophyllum rubricaule*, Ms = *Myriophyllum simulans*, Mv = *Myriophyllum verticillatum*, Pp = *Proserpinaca palustris*.

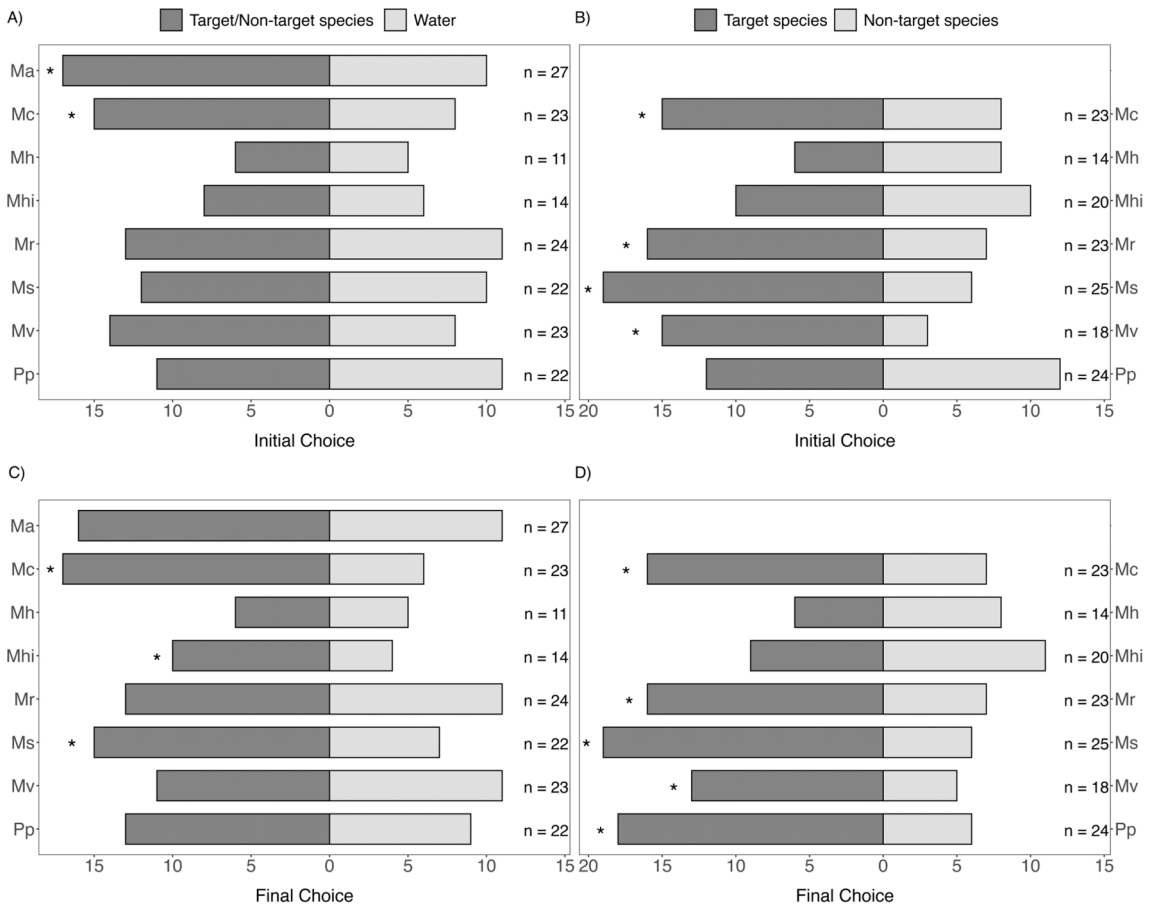

Fig. 2. Initial (A, B) and final (C, D) choice (sum) of the weevil *Phytobius vestitus* in each arm of the Y-tube olfactometer under no-choice (A, C), i.e. target/non-target *vs.* water, and choice (B, D), i.e. target *vs.* non target, conditions. The * indicates significant differences for each set-up ($P < 0.05$), while n = the number of replicates included in the analysis. Plant species: Ma = *Myriophyllum aquaticum*, Mc = *Myriophyllum crispatum*, Mh = *Myriophyllum heterophyllum*, Mhi = *Myriophyllum hippuroides*, Mr = *Myriophyllum rubricaule*, Ms = *Myriophyllum simulans*, Mv = *Myriophyllum verticillatum*, Pp = *Proserpinaca palustris*.

using type II Wald chi-square tests and planned comparisons of the treatment effects were done for each plant species based on z-ratio tests because treatment effects between plant species are irrelevant for this study.

For the VOCs, the effect of species of their diversity and abundance was first estimated by performing a non-metric multidimensional scaling (NMDS), and a subsequent permutational analysis of variance (PERMANOVA) with species as fixed factor and the VOCs matrix as response variable (library *vegan* in R, Oksanen et al., 2013). For both the NMDS and the PERMANOVA, the Bray-Curtis distance metrics was used. Next, the congruence of VOCs production in relation to the phylogenetic relatedness of the species tested was measured using a Mantel test (function *mantel* in the package *vegan*) on the VOCs distance matrix (Bray-Curtis distance), and the cophenetic distance matrix of the phylogenetic tree (Figure S3). The phylogenetic tree of the species tested was pruned from a plant megatree as described in Jin and Qian (2022), and using the packages *phytools* (Revell, 2012), and *ape* (Paradis et al., 2004).

3. Results

3.1. Proportion of time spent

In no-choice tests, only treatment (plant *vs.* water) significantly affected the time spent by *Phytobius vestitus* females in the different olfactometer arms, but not plant species nor the interaction term of plant

Table 1
Results table for assessing the effect of plant species, treatment (target or non-target plant *vs.* water for no-choice tests and target *vs.* non-target plant for choice tests), and their interaction on the proportion of time spent by the weevil *Phytobius vestitus* on each arm of the olfactometer and the first and final choice made by the weevil during no-choice and choice tests. Significant results are highlighted in bold.

Tested plant species	Proportion of time			Initial choice			Final choice		
	χ^2	df	P	χ^2	df	P	χ^2	df	P
No-choice tests									
Plant species	0.000	7	1.000	0.000	7	1.000	0.000	7	1.000
Treatment	14.998	1	**< 0.001**	8.445	1	**0.004**	14.759	1	**< 0.001**
Plant species × Treatment	6.407	7	0.493	3.857	7	0.796	8.986	7	2.254
Choice tests									
Plant species	0.000	6	1.000	0.000	6	1.000	0.000	6	1.000
Treatment	25.728	1	**< 0.001**	15.412	1	**< 0.001**	25.266	1	**< 0.001**
Plant species × Treatment	13.729	6	**0.033**	21.003	6	**0.002**	18.586	6	**0.005**

species × treatment (Table 1). Generally, weevils spent significantly more time in the arm leading to target or non-target plants (61 % of their time), when compared to water (39 %) and planned comparisons of means revealed that this effect was significant for *M. aquaticum* and *M. crispatum*, in which cases weevils spent 66 % and 74 % of the time in the arm leading to the plant, respectively (Fig. 1A). However, for all the other tested plant species, there was no difference between the time spent in each arm of the Y-tube (Fig. 1A). In choice tests, treatment and the interaction plant species × treatment but not plant species alone significantly affected the proportion of time spent by the beetle in each olfactometer arm (Table 1). Planned comparisons showed that when given a choice between *M. aquaticum* and the non-target species, the females spent 52 %, 40 %, 58 % and 42 % more time in the arms with the target species than in the arms with the non-target species *M. rubricaule, M. simulans*, *M. verticillatum* and *P. palustris*, respectively (Fig. 1B). Between *M. aquaticum* and the three remaining species, *M. crispatum, M. heterophyllum* and *M. hippuroides*, no preference was shown (Fig. 1B).

3.2. Initial choice

In the no-choice test, only treatment (plant *vs.* water) and not plant species nor the interaction of plant species and treatment had a significant influence on the initial choice by the weevil (Table 1). Planned comparisons showed that for most plants, the weevil made no difference in the initial choice of the arm, except for the target *M. aquaticum* and the non-target *M. crispatum*, to which the weevils were significantly more frequently attracted, when compared to water (Fig. 2A). When given a choice between the target, *M. aquaticum*, and the non-target species, the initial choice of the olfactometer arm was significantly influenced by the single effect of treatment and the interaction of plant species × treatment (Table 1). As demonstrated by the planned comparisons, the initial choice of the weevil fell significantly more often to the target plant, when compared to the four non-target plant species: *M. crispatum*, *M. rubricaule*, *M. simulans*, and *M. verticillatum* (Fig. 2B). However, no significant difference in the initial choice was found between the target plant and the other non-target species: *M. heterophyllum*, *M. hippuroides*, and *P. palustris* (Fig. 2B).

3.3. Final choice

Under no-choice conditions, the final choice of the beetle in the olfactometer was only significantly influenced by the treatment (plant *vs.* water) but not by plant species or the plant species × treatment interaction (Table 1). According to planned comparisons, the weevil was found significantly more frequently in the olfactometer arm with odours from *M. crispatum*, *M. hippuroides* and *M. simulans*, when compared to the arm with water, while there was no significant difference for the other plant species (Fig. 2C). When given a choice between the target *M. aquaticum* and the non-target plants, the weevil's final choice was significantly impacted by treatment and the plant species × treatment interaction (Table 1). Planned comparisons revealed that the weevil was found significantly more frequently in the arm of the target species, when compared to the non-target species *M. crispatum*, *M. rubricaule*, *M. simulans*, *M. verticillatum*, and *P. palustris.* However, there was no difference in final choice between the target species and *M. heterophyllum* or *M. hippuroides* (Fig. 2D).

3.4. Volatile organic compounds

Of the species tested, reliable data was obtained from all species except *M. heterophyllum* and *M. hippuroides* and thus these species were excluded from further analyses. Across all remaining species, a total of 156 VOCs were found, belonging to different classes of compounds (green leaf volatiles, terpenes, etc.). In this study, no formal identification was carried out and all VOCs' identity remained unconfirmed. Overall, the different plant species emitted different blends of VOCs (PERMANOVA, species effect; $F_{5,21} = 6.502$, $p < 0.001$, Fig. 3). A tanglegram based on VOCs shows the chemical distance between the tested plants, and reveals that the emission of *M. aquaticum* resulted in high intra-specific variation but closest to that of *M. crispatum*, while *M. simulans* has the most distinctive profile and *P. palustris* the most diverse blend of VOCs (Fig. 3 and Figure S3). Yet, across all plant species assessed, the VOCs released by each species did not match phylogenetic relatedness (Mantel test, $r = 0.011$, $p = 0.289$, Fig. 3).

4. Discussion

Based on the results of the current study, the weevil *P. vestitus* showed distinct responses towards the Haloragaceae plant species tested, with a clear attraction for the olfactory cues emitted by the target plant *M. aquaticum*. Studying the perception and behavioural responses to olfactory signals emitted by target and non-target species provide important insights into host-finding and selection behaviour, allowing for safe and effective agent selection (Brévault and Quilici, 2010; Fung et al., 2021; Gaffke et al., 2021; Hinz et al., 2019; Wheeler and Schaffner, 2013).

When studying behavioural responses of insect herbivores to the olfactory cues of different plant species, attractiveness is usually measured through choice (initial and final) as well as the time spent by the insect in the olfactometer arm connected to a particular volatile source (e.g., Andreas et al., 2008; Fung et al., 2021; Röder et al., 2017; Subedi et al., 2023). For instance, the studies by Subedi et al. (2023) and Fung et al. (2021), examined the behavioural responses of *Ceutorhynchus rusticus* Gyllenhal (Coleoptera: Curculionidae) and *Mogulones crucifer* Pallas (Coleoptera: Curculionidae) to olfactory and visual cues from their hosts, respectively. Their results indicated that both species were more attracted by their target plant under no-choice conditions, spending a greater amount of time in the olfactometer arm with cues from the target species (Fung et al., 2021; Subedi et al., 2023).

In the current study, *P. vestitus* spent significantly more time in the olfactometer arms connected to the target species *M. aquaticum,* and *M. crispatum* in no-choice experiments. Under choice conditions the weevil largely showed a preference for *M. aquaticum*, except when exposed to the olfactory cues of *M. heterophyllum* or *M. hippuroides*, which triggered an equal choice. *Myriophyllum heterophyllum* is considered as the native host of *P. vestitus* in eastern North America (Diaz and Harms, pers. comm.), explaining to some extent the weevil's attraction observed in our study, and despite its original population developed on *M. aquaticum* from Louisiana (Humair et al., 2023; Humair et al., 2024; Weyl et al., 2022). Similarly, the weevil was also equally attracted to *M. hippuroides,* most likely because the close phylogenetic relatedness of *M. hippuroides* to *M. heterophyllum* (Chen et al., 2014). Interestingly, *P. vestitus* was not significantly attracted to *M. heterophyllum* and *M. hippuroides* in the no-choice tests. As insect host-choice behaviour may be linked with plants' phylogenetic relatedness (Briese and Walker, 2002; Waphsere, 1974), it can be hypothesized that *P. vestitus* attraction for *M. hippuroides* in choice tests may rely on such mechanism, despite this plant never being recorded as a possible host due its western north American distribution.

However, not only phylogeny can explain attraction of an insect to plants, but also the plant chemistry (i.e. volatile organic compounds) (Wheeler et al., 2021). Since herbivorous insects are attracted or repelled by plant species mainly through olfactory signals, the attraction of non-target species may be explained by similarities in plant volatile profiles (Paynter et al., 2020) as is the case in our study where *M. aquaticum* and *M. crispatum* have similar volatile profiles, albeit, *M. aquaticum* had the most within species variation. These olfactory signals are used by insects in the host finding and selection process (Brévault and Quilici, 2010; Park et al., 2018). The study by Röder et al. (2017), for example, showed how similarity in the volatile profiles of some plant species can contribute to host attraction. Röder et al. (2017) used aquatic olfactometer-based assays to understand how *Macroplea*

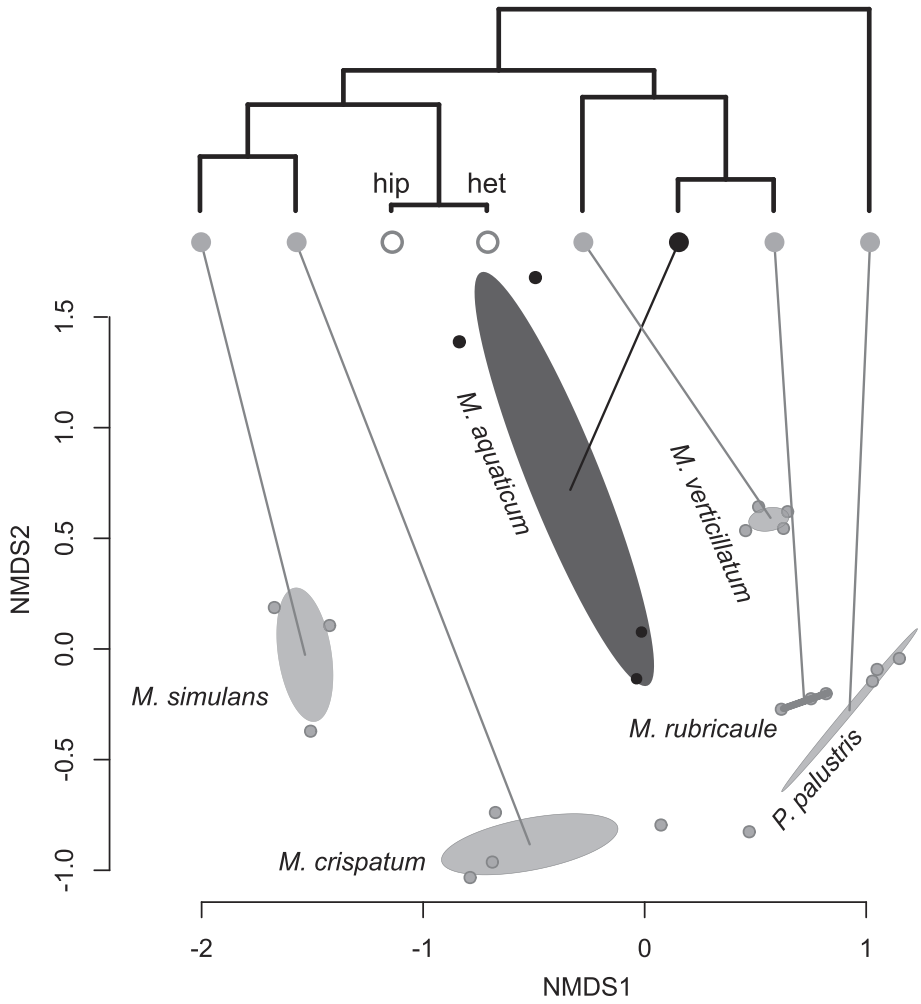

Fig. 3. Non-metric multidimensional scaling (NMDS) ordination of the volatile organic compounds (VOCs) produced by six plant species tested; the target species (*Myriophyllum aquaticum* in black), and the non-target species (*Myriophyllum crispatum*, *Myriophyllum rubricaule*, *Myriophyllum simulans*, *Myriophyllum verticillatum* and *Proserpinaca palustris* in grey). Dots represent individual replicates, and ellipses represent 95% confidence intervals around the dots. The species *M. hippuroides* (hip), and *M. heterophyllum* (het) were not tested for VOCs emissions. The phylogenetic relationship of all species tested, as retrieved from Jin and Qian (2022), is represented above the NMDS graph.

appendiculata Panzer (Coleoptera, Chrysomelidae) locates its primary host *Potamogeton perfoliatus* L. and the alternative host *Myriophyllum spicatum* L. It was observed that the aquatic beetle was strongly attracted to the main host, *P. perfoliatus*. Moreover, through the analysis of the emitted volatile organic compounds, they showed that the attraction was mediated by the dominant releasing substance, eucalyptol. Although in this study it was not possible to identify the molecules responsible for the attraction, under choice tests the weevil showed a preference for the target plant, *M. aquaticum*, over *M. crispatum*. Thus in order to obtain a full complement of host finding behaviour it is important to expose the insect to a choice between the target and non-target species.

Under no-choice conditions, *P. vestitus* appears to respond to most of the species poorly, whereas under choice conditions the same insect was attracted towards the target species *M. aquaticum*. Interestingly, both *M. heterophyllum* and *M. hippuroides* (closed phylogenetically related species) were not significantly more chosen in the no-choice tests compared to the other species. Why this result was obtained remains unclear, but it could be linked to the production of specific VOCs blends. Unfortunately, the VOCs data from *M. heterophyllum* and *M. hippuroides* were unreliable and requires further study. In addition, as with the results obtained from the proportion of time analysis, *M. heterophyllum* and *M. hippuroides* were not significantly preferred over *M. aquaticum* either as a first or final choice by the weevil under choice conditions. These results could be partially due by the use of a static olfactometer to conduct the tests, despite other studies using such device and successfully testing plant-herbivore interactions (e.g. Rasmann et al., 2014; Weyl and Hill, 2012). In our case, the weevil may need to enter the Y-tube arm to obtain the full complement of olfactory signals and thus final choice may be a better measure of weevil preference.

Further studies may consider using olfactometers with 4- or 6-arms aiming to simultaneously test for multiple hosts and relative preference for various plants odours (Turlings et al., 2004). Since olfactory discrimination is mediated by specific single or mixed volatile compounds or released by plants (Park et al., 2018), it is possible that *M. aquaticum* emits a unique pattern of molecules that attracts the weevil when subjected to a choice. That said, the preferential attraction of *P. vestitus* toward *M. aquaticum* could have been partially influenced by the fact that the insect colony was developed on this plant species, however, the fundamental mechanisms would need to be teased apart in future studies. Nonetheless, identifying behaviourally active molecules that trigger an attractive response toward the target plant could provide insights into host-finding and selection (Fung et al., 2021; Hinz et al., 2019). Further studies with *P. vestitus* may consider fractionating the mixture of volatiles collected from target and non-target plants and then using electroantennography (EAD) to determine compounds that

activate the antennae (Gaffke et al., 2021). Overall, in a biocontrol context, knowledge of compounds that act as behavioural cues for host selection is a fundamental step in understanding complex plant-insect interactions and can be useful for defining potential plant species at risk with similar volatile profiles (Brévault and Quilici, 2010; Fung et al., 2021; Hinz et al., 2019; Wheeler and Schaffner, 2013).

In sum, this study provides an important initial assessment of the specificity of *P. vestitus* towards a broad panel of plants found in its natural habitats. Understanding how insects in general respond to olfactory cues of target and non-target species can contribute to pre-release assessments and provide more information on its possible use for biological control programmes (Fung et al., 2021; Hinz et al., 2019). Further study of the volatile complex emitted by each plant species may help identify the compounds responsible for attracting *P. vestitus* to different plant species. In general, the results of this study suggest that further investment into the potential use of *P. vestitus* for parrot's feather management are warranted. However, additional studies on the life cycle and development in controlled (i.e. traditional no-choice and choice tests) and natural (i.e. open field tests) conditions, will be important to assess the potential risk of the weevil to cause unwanted non-target effects if released in a novel environment.

CRediT authorship contribution statement

A. Pessina: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **L. Humair:** Writing – review & editing, Methodology, Conceptualization. **R. Naderi:** Writing – review & editing, Investigation. **G. Röder:** Writing – review & editing, Formal analysis. **M.L. Seehausen:** Writing – review & editing, Formal analysis. **S. Rasmann:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization. **P. Weyl:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank Dr. Megann Harlow, Dr. Nathan Harms (US Army Corps of Engineers) and Prof. Rodrigo Diaz (Louisiana State University) for providing us the population of *Phytobius vestitus* and for giving us important information on the weevil. We would like to thank Dr. David Ensing and Valerie Marshall (Agriculture and Agri-Food Canada) for providing us with the Canadian populations of *Myriophyllum aquaticum* and *Myriophyllum hippuroides*. This research was funded by the Province of British Columbia through the Ministry of Forests, the Washington Department of Ecology, the Washington Department of Agriculture and a Swiss National Science Foundation grant (310030_204811) to SR. RN was funded by the Swiss National Science Foundation [grant number IZSEZ0_218528 to SR]. Contributions LH, LS and PW were supported by CABI as an international intergovernmental not-for-profit organization, and gratefully acknowledge the generous support received from our many donors, sponsors and partners. In particular, we thank our Member Countries for their vital financial and strategic contributions.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2024.105509.

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