OLFACTION MEDIATED HOST SELECTION IN A SPECIALIST WEEVIL USED FOR BIOLOGICAL CONTROL OF AN INVASIVE PLANT

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AUTHORIZATION TO SUBMIT THESIS

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ABSTRACT

The weevil *Mogulones crucifer* Pallas (Coleoptera: Curculionidae) was released in Canada in 1997 for the biological control of the invasive plant *Cynoglossum officinale* L. The weevil was not permitted for release in the United States due to concerns over potential non-target attack on threatened plant species in the same family as *C. officinale*. To assess the risk posed by the weevil to native Boraginaceae plant species I 1) examined the behavioral response of the weevil to olfactory cues of *C. officinale* and selected native rare and threatened confamilial plants, and 2) characterized the headspace volatile profiles of plant species (GC/MS) and conducted electrophysiological experiments (GC-EAD) to measure the antennal responses of the weevil. Results indicate that during host finding, *M. crucifer* prefers *C. officinale* over all tested plants. I found that among non-target plants *H. californica* shared greatest number of compounds with *C. officinale* whereas *A. occidentale* shared least. I also identified six electrophysiologically active compounds in *C. officinale* that potentially contribute to this discrimination. Findings suggest that the weevil is unlikely to locate and therefore colonize any of the tested rare or threatened native confamilial species.

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To my family

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INTRODUCTION

Plant invasions

The term 'biological invasion' refers to the expansion of a species into a new area beyond its natural range of distribution (van der Velde et al., 2006). Biological invasions are considered among the greatest threats to biodiversity and natural resource management (Ehrenfeld, 2010; Pimentel, 2011; Simberloff et al., 2013). The introduction of invasive plant species into novel environments occur at alarming rates (Moser et al., 2009). Anthropogenic factors such as trade and tourism are considered among the most important pathways for species introductions and spread beyond their native range (Holmes et al., 2009). Invasive plants not only compete with flora, they also reduce other parts of the resident biodiversity, degrade ecosystems and their services (Mack et al., 2000; Pimentel, 2011; Pyšek et al., 2012). The management of invasive plants has consequently become an increasingly important topic for both natural resource management and biological conservation alike (Pyšek et al., 2012; Randall, 1996). Herbicide applications have been successful to manage plant invasions, especially to eradicate isolated plant patches during early stages of invasions (Mack et al., 2000). Mechanical and cultural control means like tilling, burning and grazing can be effective too but typically only in smaller areas (Mack et al., 2000). Sustained long term and large-scale control using herbicides has, however, proven difficult and often unfeasible due to topographic constrains, high associated costs and increasing environmental concerns over the use of chemicals (DiTomaso, 2000; Mack et al., 2000; Pearson & Ortega, 2009). Continuous use of herbicides with the same mode of action may also results in evolution of herbicide resistance in numerous invasive plants (Kudsk & Streibig, 2003). These constraints

of chemical, mechanical and cultural control measures have focused attention on biological control (Culliney, 2005).

Classical biological weed control

Biological control is defined as the use of herbivores, parasites, predators, or pathogens in maintaining another organism's population density at a lower average than would occur in their absence (De Bach, 1964). Approaches of biological control are (i) conservation biological control, environmental modification to protect and enhance natural enemies (De Bach, 1964) (ii) augmentation biological control, natural enemy populations are increased through mass culture and periodic release (Orr, 2009) and (iii) classical biological control. Classical biological control of invasive plants is defined as the introduction of control organisms into a region outside of their natural range, in order to suppress populations of selected invasive plants permanently (Harley & Forno, 1992). Classical biological control has been practiced to control and manage invasive plant population for more than 100 years (Vail et al., 2001). If conducted correctly, it can be an environmentally benign, practical and economically feasible method of sustained invasive plant management (Clewley et al., 2012; Culliney, 2005; McFadyen, 1998; Pemberton, 2000). Classical biological control aims to reduce and stabilize the invasive plant’s abundance below an economic or ecological threshold level (Müller-Schärer & Schaffner, 2008).

While classical biological weed control is considered environmentally sound and cost effective by its proponents, it is not free of risks (Louda et al., 2003; Louda & Stiling, 2004; McEvoy & Coombs, 2000). There have been incidences of non-target attack by herbivorous biological control agents on native plant species, sometimes even on threatened and

endangered designated plant species, raising concerns over their environmental safety (Louda & Arnett, 2000; Louda et al., 2003; Louda et al., 2005; Messing & Wright, 2006; Suckling & Sforza, 2014). These occurrences merit attention and rigorous science to understand the extent and limits of host fidelity by putative biological control agents.

Host range testing of weed biocontrol candidate species

To mitigate the risk of non-target attack, pre-release host specificity tests are conducted by exposing potential biological control organism to a variety of plant species (Heard, 2002; Schaffner, 2001). Selection of test plant species for host range assessments typically follows the centrifugal phylogenetic method (Wapshere, 1974), which considers closely related plant species to the target plant first and then progressively more distantly related species until the host range of the biological control candidate is well defined (Schaffner, 2001). More recently, testing of rare and endangered plant species along with economically important confamilial plants is emphasized during host range investigations (Kuhlmann et al., 2006; Schaffner, 2001). Recent development of more accurate plant taxa phylogenies using molecular techniques has improved the test plant selection for host range testing procedures (Gaskin et al., 2011; Le Roux & Wieczorek, 2009). In addition to these phylogenetically guided methods, unrelated plant species with similar morphological or chemical properties that occur sympatrically in areas invaded by the invasive are also included in host range testing (Schaffner, 2001).

The experimental host range of potential biological control organisms is identified by exposing immature or mature individuals to test plant species using different test designs, e.g. no-choice or multiple-choice tests, under differing environmental conditions ranging from

individual potted plants to open-field tests to determine their fundamental and ecological host range (Balciunas et al., 1996; Clement & Cristofaro, 1995; Schaffner, 2001; Zwölfer & Harris, 1971). Based on these methods, the "fundamental host range" is defined as the range of plant species on which a herbivore can complete its full life cycle, whereas the "ecological host range" is a subset of the fundamental host range and includes those plant species that are actually used under field conditions (Schaffner, 2001). Determination of the fundamental host range relies on no-choice testing conducted under laboratory or controlled environmental conditions where biological control organisms are exposed to a single plant species at a time (Heard, 2002). Under these experimental settings, herbivores are largely unable to exhibit their normal host selection behavior (Hinz et al., 2014; Marohasy, 1998). In contrast, determination of the ecological host range employs testing designs with more natural field cage or natural field conditions, that provides herbivores with a choice between its field host and other test plant species (Heard, 2002). As a consequence, the ecological host range of a specialist herbivore is narrower than its fundamental host range as found in multiple case studies (Blossey et al., 2001; Cristofaro et al., 2013; McFadyen et al., 2002; Paynter et al., 2004; Schaffner, 2001).

The fundamental host range of a candidate classical biological control organism is considered a safe estimate of the potential for non-target attack as the herbivore species in question does not have the physiological ability to develop on a plant species outside of their fundamental host range (Van Klinken & Edwards, 2002). It can, however, lead to overestimation of the potential for non-target attack in the field (Heard, 2000; Schaffner, 2001; van Klinken, 1999). Thus, theoretically, release decisions solely based on fundamental

host range data may result in the rejection of environmentally safe potential candidates (Dunn, 1978; Hinz et al., 2014; Marohasy, 1998; Wapshere, 1989).

Host recognition behavior

The traditional host range assessment protocol, which emphasizes the assessment of the fundamental host range, neglects the host finding and acceptance processes that are requisite for host utilization (Marohasy, 1998; Van Klinken & Raghu, 2006; Wapshere, 1989). In the field, herbivores typically live in environments with diverse plant communities. During host plant selection, insects progress through a sequence of behavioral responses, which could be summarized as: host finding, host examination and host acceptance (Bernays & Chapman, 1994; Miller & Strickler, 1984). Insects use olfactory and visual cues to locate their respective host plant(s) followed by examining the host plant based on olfactory, visual, gustatory and tactile cues, which finally lead to host acceptance when oviposition and/or sustained feeding occur. An insect herbivore must pass through this sequence of stages to identify its host plant(s) successfully (Bernays & Chapman, 1994). Thus, the examining and accepting phase, as evaluated in the typical no-choice bioassay used in fundamental host range testing, cannot occur in the field unless insects succeed during the host finding stage (Bernays & Chapman, 1994; Dethier, 1982; Miller & Strickler, 1984).

Acceptance or rejection of a particular plant during host finding depends on the responses of insects to its traits such as color, shape or odor (Bernays & Chapman, 1994). Among the various sensory cues, olfaction plays a particularly important role in plant-insect interactions, especially during host finding (Nieberding et al., 2012; Schoonhoven et al., 2005; Urru et al., 2011). The olfactory receptors on the antennae of many herbivorous insects

can detect a wide range of semio-chemicals (Bruce et al., 2005; Hansson, 2002). Plants synthesize various volatile secondary metabolic compounds, often with known ecological functions (Beyaert et al., 2010; Dudareva et al., 2013). These volatiles can be used as olfactory cues, detectable by antennal receptors and generating sensory signals which in turn result in behavioral responses (Heard, 2002). The mechanisms involved in host plant selection based on plant volatile compounds can be described by two contrasting hypotheses: 1) species-specific odor recognition, in which host plant selection is based on plant odors that are unique to the host, and 2) ratio-specific odor recognition, in which host selection is based on the particular ratio between constituent compounds, which are widely distributed among plant species (Bruce et al., 2005; Visser, 1986). Knowledge of the chemical basis of the host finding behavior could therefore greatly improve our ability to predict whether a non-target plant species that can support the development of a biological control organisms would actually be at risk of attack in the field (Heard, 2000; Schaffner, 2001; Thomas & Willis, 1998; Wheeler & Schaffner, 2013).

The rangeland invasive plant *Cynoglossum officinale* L. (Boraginaceae) and the root mining weevil *Mogulones crucifer* Pallas (Coleoptera: Curculionidae) offer an appropriate study system to test the significance of plant chemical cues during host selection. For *M. crucifer* there is preliminary data that indicate that host selection by this weevil could be mediated by the volatile organic compounds (hereafter VOCs) emitted by its host plant *C. officinale* (Andreas et al., 2008a).

Study system

Cynoglossum officinale L. (Boraginaceae)

Cynoglossum officinale is thought to have its origin in the mountainous ranges of the Caucasus and is widely distributed throughout Europe (De Jong et al., 1990). It was first recorded in North America in Montreal in the late 1800s (Macoun, 1884) and has since spread throughout the continental United States, except Texas and Oklahoma and southern Canada (Forcella & Harvey, 1988; Upadhyaya & Cranston, 1991; Upadhyaya et al., 1988; USDA-NRCS, 2016). It is a declared noxious weed in seven western states (Colorado, Idaho, Montana, Nevada, Oregon, Washington, and Wyoming) in the United States (USDA-NRCS, 2016).

Cynoglossum officinale is a monocarpic, biennial to short-lived perennial herb in the Boraginaceae family (De Jong et al., 1990; Forcella & Harvey, 1988; Upadhyaya & Cranston, 1991; Upadhyaya et al., 1988). Following vernalization, seeds germinate in spring producing rosettes with long pubescent leaves in the first year (Boorman & Fuller, 1984; De Jong et al., 1990; Upadhyaya et al., 1988). With the first frosts in fall, leaves die off and carbohydrates are stored in the taproot and the plants overwinter as hemicryptophytes (Boorman & Fuller, 1984). In the subsequent spring, *C. officinale* flowers depending on the size of rosette at the end of the previous field season (de Jong et al., 1986). Plants that exceed the minimum threshold root size for reproduction form flowering primordia. This is followed by stem elongation and then flowering and finally seed set (de Jong & Klinkhamer, 1989; de Jong et al., 1986; Klinkhamer & de Jong, 1987). The monocarpic plants usually die following seed set (De Jong et al., 1990). It has, however, been shown for its introduced range in the United States that a proportion of houndstongue plants will flower repeatedly (Williams,

2009). Bolting plants develop red to purple and sometimes brownish flowers and each flower can produce up to four flattened, tear drop-shaped, single seeded nutlets, arranged in a pyramidal pattern (Klinkhamer & de Jong, 1993). Small barbed hooks on dried nutlets facilitate dispersal via epizoochory by attaching to the fur of passing animals (DeClerck-Floate, 1997). In its native range, *C. officinale* grows as isolated plants or in clusters along roadsides, in open woodland, sand dunes and fallow lands (Boorman & Fuller, 1984; De Jong et al., 1990). In North America, the plant invades rangelands, pastures, forests, and many disturbed habitats such as railroad and highway rights of way, gravel pits and fallow lands (Cranston & Pethybridge, 1986).

Cynoglossum officinale infestations can pose serious ecological and economic problems. Ecologically, the plant competes with native forbs especially for moisture (Upadhyaya et al., 1988). Economically, *C. officinale* reduces forage production in rangelands and pastures (Jacobs & Sing, 2007). In addition, the plant contains very high concentrations of pyrrolizidine alkaloids (van Dam et al., 1995). Fresh foliage or even dried hay containing houndstongue foliage is extremely toxic to mammalian livestock that ingest it, especially horses and calves, and causes kidney and liver diseases that can lead to death of animals (Baker et al., 1991; Baker et al., 1989; Knight et al., 1984; Stegelmeier et al., 1996). In addition, large numbers of barbed nutlets can attach to livestock fur and cause skin and eye irritation in affected animals and lead to potential losses when marketing livestock (Upadhyaya & Cranston, 1991).

Management of *Cynoglossum officinale* in North America

Both chemical and non-chemical methods of management have been practiced to manage *C. officinale* in North America (DiTomaso & Kyser, 2013). Chemical control of *C. officinale* using 2, 4 -D, chlorosulfuron, Picloram, metasulfuron has been common practice since many years despite the fact that this is a challenging and costly management practice because of the widespread and patchy distribution of the invasive plant and potential damage to native flora (Peachey et al., 2016; Upadhyaya & Cranston, 1991; Upadhyaya et al., 1988). In addition, *C. officinale* infests often habitats managed by U.S. federal agencies and on federal lands the use of herbicides is increasingly restricted (Tu et al., 2001). Mechanical control of *C. officinale* can be accomplished by tilling, cutting or hand-pulling (DiTomaso & Kyser, 2013). Tilling is probably the easiest form of mechanical control, but infestations are often found on slopes or in terrain that is difficult to till. In addition, the disturbance caused by tilling could trigger germination of dormant seeds of *C. officinale* and other exotic invasive plants, potentially increasing the problem (Klinkhamer & De Jong, 1988). Cutting *C. officinale* flowering stems 0-7 cm above the ground can be effective if done before seed set because typically the plant lacks the resources to compensate for the damage by producing another flowering stem (Dickerson & Fay, 1982). Similar to cutting, plants may be pulled at the flowering stage but great care has to be taken to cut or pull every flowering plant in an infestation (De Clerck-Floate & Schwarzländer, 2002a; Dickerson & Fay, 1982). The area-wide sustained long term control of *C. officinale* using herbicides, by mechanical means or both is financially unrealistic and pragmatically unachievable (De Clerck-Floate & Schwarzländer, 2002a). Consequently, a program to develop classical biological control organisms for *C. officinale* was initiated in 1987 in Canada and the United States. Since then,

seven insect species have been studied for their potential as a biological control organism by CABI Switzerland (Freese, 1987; Hinz et al., 2007; Hinz et al., 2005; Hinz et al., 2003; Hinz et al., 2006; Schwarzländer, 1999). These included the weevils *Mogulones crucifer* Pallas, *M. trisignatus* Gyllenhal, *M. borraginis* (Fabricius) and *Rabdorrhynchus varius* Herbst, the flea beetles *Longitarsus quadriguttatus* Pontoppidan and *L. exoletus* Linnaeus and the syrphid *Cheilosia pasquorum* Becker. During host range assessments, four of these candidate species, i.e. *C. pasquorum, L. exoletus, M. trisignatus,* and *R. varius* were not sufficiently host-specific to *C. officinale* and consequently are no longer considered for its biological control (Hinz et al., 2005; Hinz et al., 2003; Hinz et al., 2006).

Mogulones crucifer **Pallas (Coleoptera: Curculionidae)**

Mogulones crucifer (=*M. cruciger* Herbst, =*Ceutorhynchus cruciger* Herbst) is a root-mining weevil native to Eurasia (Dieckmann, 1972; Koch, 1992; Lohse, 1983; Schwarzländer, 1997). The weevil typically has one generation per year in its native range but can have a partial second generation (Schwarzländer, 1997). In brief, overwintering adults become active in the early spring and start feeding on foliage and with increasing temperatures weevils begin to mate and lay eggs in the bases of petioles of newly developed leaves near to the crown root (Schwarzländer, 1997). Larvae tunnel down the leaf petiole and mine inside the upper root and root crown of *C. officinale* plants (Schwarzländer, 1997). Early instar larvae are found in root crowns but later instar larvae will mine in the taproot and secondary roots of plants. Mature larvae leave the roots and form a cocoon out of soil particles for pupation (Schwarzländer, 1997). Adults emerge in early summer and start feeding on foliage before they aestivate throughout the summer and fall and then hibernate in the leaf litter (De

Clerck-Floate & Schwarzländer, 2002b; Schwarzländer, 1997). A proportion of weevils will, however become active again in September and continue to feed on foliage and mate and lay eggs before first frosts force them into hibernation (Schwarzlaender, 1997). *Mogulones crucifer* prefers bolting plants over rosettes, and large plants to small plants for oviposition (Prins et al., 1992; Schwarzländer, 1997). The total fecundity per female is 192.1 ± 20.4 eggs (Schwarzländer, 1997). Because of the partial second generation, larvae can be found throughout the year in the roots of *C. officinale* (Schwarzländer, 1997).

Host range testing of *Mogulones crucifer*

The experimental host range of *Mogulones crucifer* was studied between 1988 and 1996 using 32 Eurasian Boraginaceae species in 19 genera and an additional 5 native North American Boraginaceae species in the genera *Adelinia* (=*Cynoglossum*), *Amsinckia,* and *Andersonglossum* (=*Cynoglossum*). Weevils were able to complete development on a number of European Boraginaceae species tested and the native North American *Adelinia grande* (Douglas ex Lehm.) J. I. Cohen (Jordan et al., 1993; Schwarzländer, 1996). In all-choice tests, however, the weevil showed a strong preference for its field host, *C. officinale* (Jordan et al., 1993; Schwarzländer, 1996). In no-choice tests, feeding and oviposition was observed only on *Amsinckia tessalata* A. Gray and there was no weevil development on *A. carinata* A. Nelson & J.F. Macbr., *A. tessalata*, *Andersonglossum boreale* (Fernald) J. I. Cohen, and *A. occidentale* (A. Gray) J. I. Cohen (Jordan et al., 1993; Schwarzländer, 1996).

Based on these results, a petition for field release of *M. crucifer* in the United States and Canada was submitted in 1996 to the Technical Advisory Group (TAG), a federal interagency expert committee that advises the United States Department of Agriculture

Animal Plant Health Inspection Service (USDA APHIS) regarding the release of classical weed biological control organisms (De Clerck-Floate & Schwarzländer, 2002b). Subsequently, the Canadian Biocontrol Review Committee approved the release of *M. crucifer* in Canada in 1997 following a favorable recommendation by TAG but approval for release in the United States was not granted because of the concern of the United States Fish and Wildlife Service (USFWS) over the environmental safety of the weevil with regard to native threatened and endangered listed (T&E) Boraginaceae species (De Clerck-Floate & Schwarzländer, 2002b). These are *Amsinckia grandiflora* (Kleeb. ex A. Gray) Kleeb. ex Greene, *Hackelia venusta* (Piper) H. St. John, *Oreocarya crassipes* (I.M. Johnst.) Hasenstab & M.G. Simpson, *Plagiobothrys hirtus* (Greene) I.M. Johnst., and *P. strictus* (Greene) I.M. Johnst. Complementary host range tests were conducted with North American Boraginaceae between 1997 and 1999 with emphasis on plants that were not included in previous host range testing (De Clerck-Floate & Schwarzländer, 2002b). Out of 22 native North American plants tested, nine species in five genera (*Cryptantha, Hackelia, Lappula, Mertensia, and Oreocarya*) supported complete development of the weevil and there was in addition incomplete development on the T&E species *P. hirtus* (De Clerck-Floate & Schwarzländer, 2002b). However, non-target plant species experienced much lower levels of *M. crucifer* attack in comparison to *C. officinale* (De Clerck-Floate & Schwarzländer, 2002b). Additional no-choice and choice larval development test were conducted during 2001-2004. In these tests several native confamilial species including three T&E species, *H. venusta, P. hirtus, A. grandiflora* supported larval development (Andreas, 2004). Since its original release in 1997 in British Columbia and Alberta, Canada, weevil populations have since increased greatly and dispersed widely (De Clerck-Floate et al., 2005). The weevil was also found on four

sympatrically occurring confamilial plant species, *H. floribunda* (Lehm.) I.M. Johnst., *Lappula squarrosa* (Retz.) Dumort., *Lithospermum ruderale* Douglas ex Lehm. and *Oreocarya spiculifera* (Piper) Payson at *M. crucifer* release sites in Canada. The *M. crucifer* attack rates on these plant species were variable across the year but generally low (Andreas et al., 2008b). Sensitization and spillover were proposed as the probable mechanisms for this non-target attack (Andreas et al., 2008b). Sensitization occurs when an herbivore insect encounters favorable host plants, which stimulates the central nervous system resulting in increased responsiveness of the insect to related non-hosts resulting in feeding and oviposition on nearby plants that would otherwise be rejected (Heard, 2000). Spillover occurs when there are large herbivore populations and depleted target host plant populations leading to temporary utilization of unrelated or related unsuitable non-target plants in proximity to the preferred host plant (Blossey et al., 2001; Holt & Hochberg, 2001). Spillover may also cause apparent competition between the target and non-target plant species (Holt & Hochberg, 2001; Holt, 1977). In response to these host specificity data, USDA APHIS denied the release petition for the weevil in the United States and a pest alert for the weevil was issued when it was first retrieved on a *C. officinale* plant south of the Canada - United States border near Danville, Washington State in the United States (USDA, 2010). The weevil is spreading south and westward at approximately 12 km (7.5 miles) per year and its abundance is increasing rapidly (Winston, 2011). In a recent post-release monitoring study in Canada, it was shown that non-target attack by *M. crucifer* on native North American confamilial species sympatrically occurring with *C. officinale*, *Hackelia micrantha* (Eastw.) J.L. Gentry, a congener of the T&E-listed *H. venusta*, can be explained as spillover (Catton et al., 2014; 2015). These authors also conclude that plant population level non-target impacts

are highly unlikely to occur from this spillover as *M. crucifer* was unable to maintain persistent populations on the non-target host (Catton et al., 2015). In North America, the Boraginaceae are a diverse family with species-rich genera such as *Cryptantha* (> 150 species) that are according to the most recent phylogeny closely related to *C. officinale* (Kartesz, 1999). In host range investigations *M. crucifer* has been found able to accept and develop on non-target plant species that are closer related to *C. officinale* than *H. micrantha* (Cohen, 2014). To ensure the environmental safety of *M. crucifer*, it would be essential to evaluate the risk of the weevil to native plant species for which there are conservation efforts underway (e.g. *Amsinckia grandiflora*) (Pavlik et al., 1993) or that are T&E listed and closely related to *C. officinale* (Cohen, 2014).

As is true for other herbivores considered or used as biological weed control agents, the ecological host range of *M. crucifer* is narrower than its fundamental host range (De Clerck-Floate & Schwarzländer, 2002b). In order to attempt to predict the ecological host range experimentally, it would be essential to determine the confamilial non-target plant species that weevil could encounter during host selection by studying relative attractiveness of the weevil to these plants. The assessment of the host selection behavioral responses which are mediated by various sensory cues could provide important data on the relative attractiveness of these ecologically at-risk non-target plant species and thus be useful in predicting the ecological host range (Wheeler & Schaffner, 2013). For *M. crucifer* there is preliminary data that indicate that the host selection by this weevil could be mediated by the VOCs emitted by its host plant *C. officinale* (Andreas et al., 2008a). Thus, a phytochemical analysis of emitted VOCs of *C. officinale* and selected native confamilials and a simultaneous exploration of the responses of the weevil to those plant species or their VOCs

using behavioral bioassays could provide the basis for predicting the ecological host range of *M. crucifer*.

Objective

The objective of this research is to explore and validate an approach to assessing non-target attack risks of classical biological weed control organisms that takes into account the early stages of the host selection behavior and its underlying phytochemical basis. Specifically, this research aims to address whether plant chemistry involved in the host finding behavior of *M. crucifer* can provide data on the potential for non-target attack of native representatives of the family Boraginaceae that are within the fundamental host range of the weevil. In doing so, the research could elucidate the role that host finding may have in predicting the ecological host range of a specialist herbivore considered for biological control of weeds. This research is intended to assist land managers and policy makers alike in the evaluation of the risks this weevil may or may not pose to native confamilial Boraginaceae in the United States and thus inform recently renewed discussions to potentially reconsider this weevil for release.

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Chapter 2

BEHAVIORAL RESPONSES OF *MOGULONES CRUCIFER* TO VOLATILES OF *CYNOGLOSSUM OFFICINALE* AND RARE, THREATENED AND ENDANGERED CONFAMILIAL PLANT SPECIES

Abstract

Mogulones crucifer Pallas is a root-feeding weevil that was released in Canada for the biological control of the noxious rangeland weed, *Cynoglossum officinale* L, in 1997. The weevil has since become successful in suppressing *C. officinale* populations in British Columbia and Alberta, Canada. It has, however, not been permitted for release in the United States, and instead being declared a plant pest by USDA APHIS in 2010 due to concerns about potential non-target attack on threatened or endangered (T&E) plant species in the Boraginaceae family. To describe better the ecological host range of the weevil, and consequently predict the risk of non-target attack, I examined the behavioral responses of *M. crucifer* females to olfactory cues of *C. officinale* and selected native rare or T&E confamilial plant species using laboratory host finding bioassays. The findings indicate that prior to contact with the host, both in dual and multiple-choice olfactometer bioassays, *M. crucifer* was able to discriminate and move preferentially towards volatiles from *C. officinale* relative to volatiles from all non-host plant species and the control (purified air). Weevils were either not able to distinguish non-target plant volatiles from the control (purified air) or even avoided non-target volatiles in some cases including one T&E species indicating repellence. This work provides pertinent data in determining *M. crucifer*'s ecological host

range and improves our understanding of the weevil's abilities to find, accept, and attack confamilials in North America.

Introduction

Ongoing globalization will lead to increased international tourism and trade, both of which are key factors facilitating the introduction of exotic plant species (Hulme, 2009; Meyerson & Mooney, 2007; van Kleunen et al., 2015). The resulting problems to natural resource bases and ecosystem health are not being met with adequate management strategies for these plant species (Lindenmayer et al., 2015; Simberloff et al., 2005). Conventional control strategies such as mechanical control (e.g., tilling or mowing) and herbicide applications are suitable strategies in agro-ecosystems, but in often remote natural areas and on publically owned lands, these control means are economically unfeasible or increasingly regulated and limited (Culliney, 2005; Sheley et al., 2010). Classical biological control (CBC) is an alternative method for the management of invasive plants that was originally developed more than 100 years ago because management options were at a similar impasse: mechanical control became unfeasible and herbicides did not exist at the time (Goeden, 1988). Classical biological control of weeds is defined as the introduction of host-specific natural enemies into a region outside of their native range with the aim to reduce and stabilize an invasive plant's abundance below an economic or ecological threshold level (Harley & Forno, 1992; Müller-Schärer & Schaffner, 2008). While CBC is largely considered environmentally benign when conducted appropriately, cost effective, and particularly suitable for invasive plants in natural areas and low agricultural value lands by its proponents (Clewley et al., 2012; Culliney, 2005; McFadyen, 1998; Pemberton, 2000), it is not risk free (Louda et al.,

2003; Louda & Stiling, 2004; McEvoy & Coombs, 2000). There are numerous well documented examples of non-target attack on confamilial plant species co-occurring with targeted weeds by released biological control organisms (Blossey et al., 2001; Dennill et al., 1993; Louda et al., 2003; Schooler et al., 2003; Taylor et al., 2007). Most often, non-target attack is transitory and occurs when a successful biological control organism reaches outbreak densities following its release or after host plant populations decline sharply, forcing biocontrol agents onto suboptimal hosts or non-hosts (Bowers et al., 1992; Fowler et al., 2000; Lynch et al., 2002). When non-target attack is transitory, it typically impacts individual plant performance rather than non-target plant population dynamics (Crawley, 1989), but there are also examples for sustained non-target attack. Sustained non-target effects are particularly expected when the biological control agent completes its life cycle on the non-target plant, and when females can readily locate non-target plants and accept them for oviposition (Bowers et al., 1992; Thomas et al., 1987). The most notably two examples where non-target populations are negatively affected are *Rhinocyllus conicus* (Froel.) (Coleoptera: Curculionidae) which has severe effects on native thistle populations in North America (Louda, 2000; Louda & Arnett, 2000; Louda et al., 2003) and *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae), which has suppressed native *Opuntia* species in the United States (Johnson & Stiling, 1998; Solis et al., 2004). These two cases of non-target impacts and others have led to severe scrutiny of CBC of weeds in general and on the host range assessment methods and data interpretation for CBC candidate species specifically. In the United States for example, regulatory uncertainty and discussions on how to interpret host range data and apply the precautionary principle have led to a halt in approving weed organisms since 2009 (Hinz et al., 2014). This current impasse to implement an invasive

plant management strategy illustrates the need for an improved understanding and predictability of the extent and limits of how host fidelity of CBC of weed candidates is assessed and interpreted.

To assess the host range and predict the risk of potential non-target attack of CBC of weed candidate species pre-release, so-called host-specificity tests are conducted that use confamilials of the targeted weed from both the range where it is native and more importantly where it is invasive. Plants are chosen from taxa based on the degree of relatedness to the target weed (the centrifugal phylogenetic approach; Wapshere 1974), and emphasis is put on confamilials that are either rare and endangered in the introduced range or economically important (Schaffner, 2001; Wapshere, 1974). The ability of an insect to feed, lay eggs, and develop on test plant species under experimental conditions in differing no-choice and choice test designs is the basic approach to determine their fundamental and ecological host range and predict their environmental safety (Heard, 2002; Marohasy, 1998; McEvoy, 1996; McFadyen, 1998; Schaffner, 2001; Wapshere, 1974; Zwölfer & Harris, 1971). In addition, some choice tests are conducted as an open field test (Briese, 1999). Pre-release open field tests are conducted in an insect's native range and result in predictions concerning the ecological host range of a biological control organism in the introduced range, whereas post-release open field tests can be conducted in the introduced range to assess the accuracy of these predictions (Briese, 1999). The 'fundamental host range' is defined as the range of plant species on which a herbivore can complete its life cycle, whereas the 'ecological host range' is typically a subset of the fundamental host range and includes those plant species that are actually used under field conditions (Schaffner, 2001). However, laboratory-based host range determination has been viewed with skepticism (McEvoy, 1996;

Wapshere, 1989). Under experimental settings, especially using no-choice conditions, herbivores are largely unable to exhibit their normal host selection behavior (Marohasy, 1998). Consequently, both results of no-choice and choice tests, if conducted under confined small-scale conditions are flawed in accurately describing the ecological host ranges. Unlike laboratory conditions, herbivorous insects are able to use visual, olfactory and other sensory cues alone and in combination during pre-alighting or the host finding stage under natural conditions, to identify their appropriate host (Heard, 2000). Consequently, the assessment of the behavioral responses during host selection and the sensory cues involved could provide important information on the relative attractiveness of non-target plant species and greatly improve pre-release predictions of the biological control agent's ecological host range (Wheeler & Schaffner, 2013).

The root-mining weevil *Mogulones crucifer* Pallas (= *Ceutorhynchus cruciger* Herbst, *Mogulones cruciger* Herbst, Coleoptera: Curculionidae) probably exemplifies the current debate about CBC of weeds better than any other biocontrol organism. It was petitioned for release in North America in 1996 and released in Canada in 1997 to control the invasive rangeland weed *Cynoglossum officinale* L. (Boraginaceae). Since its release, this weevil has been very successful in Canada in reducing or controlling *C. officinale* populations and dispersing to nearby populations (Clerck-Floate & Wikeem, 2009; De Clerck-Floate et al., 2005). In the United States, however, the release of the weevil was not approved due to concerns by the United States Fish and Wildlife Service about potential non-target attack on a federally listed threatened and endangered confamilial species, *Oreocarya crassipes* (I.M. Johnst.) Hasenstab & M.G. Simpson (USFWS, 1997). The petition for release was denied in 2002 due to additional host-specificity data demonstrating a broader

fundamental host range of the weevil (De Clerck-Floate & Schwarzländer, 2002). And in 2010, when *M. crucifer* was first discovered on *C. officinale* plants in northern Washington State in the United States, it was declared a Plant Pest (USDA, 2010).

Pre-release and post-release host-specificity testing conducted between 1987 and 2004 has shown that the weevil has a broad fundamental host range across various genera including the native North American listed threatened and endangered (T&E) species *Amsinckia grandiflora* (Kleeb. ex A. Gray) Kleeb. ex Greene, *Hackelia venusta* (Piper) H. St. John, *Plagiobothrys hirtus* (Greene) I.M. Johnston. Furthermore it was able to develop on 16 out of 29 native North American Boraginaceae species tested (Andreas, 2004; De Clerck-Floate & Schwarzländer, 2002b; Jordan et al., 1993; Schwarzländer, 1996). In choice tests, *M. crucifer* typically and strongly preferred its co-evolved field host *C. officinale* (Andreas, 2004; De Clerck-Floate & Schwarzländer, 2002b; Jordan et al., 1993; Schwarzländer, 1996). In addition, the weevil attacked sporadically the native confamilial non-target species *Hackelia micrantha* (Eastw.) J.L. Gentry, *Lithospermum ruderale* Douglas ex Lehm., and *Oreocarya spiculifera* Piper (=*Cryptantha spiculifera* (Piper) Payson) at release sites in Canada (Andreas et al., 2008b; Catton et al., 2014; De Clerck-Floate & Schwarzländer, 2002b). However, *M. crucifer* attack rates on these plant species were variable across years and release sites, were generally low, and target and non-target utilization were positively correlated (Andreas et al., 2008b; Catton et al., 2015), suggesting that non-target attack is more likely to occur when non-targets grow in proximity to *C. officinale* populations with large weevil populations and sharply decrease with increasing distance from the nearest *C. officinale* plant. Finally, in a recent post-release monitoring study using a single native North American confamilial species sympatrically occurring with *C. officinale* in Canada, *H.*

micrantha, it was shown that non-target herbivory was limited to spillover, making population level impacts highly unlikely (Catton et al., 2014; 2015).

The case of *M. crucifer* illustrates the importance of data that allow to better estimate the ecological host range of this insect specifically and of biological weed control organisms in general. While it may be true that most non-target attack caused by *M. crucifer* is limited and transitory (Catton et al., 2014), concerns about the environmental safety in the United States with regard to T&E species have to be addressed satisfactorily. The study of chemical factors mediating insect-plant interactions could improve the predictability of the host range of biological control organisms and its ecological impact (Wheeler & Schaffner, 2013). For *C. officinale* and its confamilial plant species, it is known that they emit volatile organic compounds (VOCs) and there are preliminary data that indicate that these VOCs could mediate the host selection in *M. crucifer* (Andreas et al., 2008a). In this study, we aim to clarify contradictory host-specificity data for *M. crucifer* in order to contribute to the debate about the environmental safety of this insect, which has dispersed into the United States. Specifically, we studied the behavioral response of *M. crucifer* during early host finding i.e. prior to contact with the host and its underlying phytochemical basis with regards to T&E listed and other rare confamilial Boraginaceae species.

Materials and Methods

Study system

Cynoglossum officinale is a Eurasian monocarpic, biennial to short lived perennial herb in the Boraginaceae family (De Jong et al., 1990; Forcella & Harvey, 1988; Upadhyaya & Cranston, 1991; Upadhyaya et al., 1988). Following vernalization, seeds germinate in spring

producing rosettes with long pubescent leaves in the first year (De Jong et al., 1990; Upadhyaya et al., 1988). With the first frost in fall, leaves die off and plants overwinter (Boorman & Fuller, 1984). In the subsequent spring, *C. officinale* plants will bolt and flower or stay in the rosette stage for another year depending on the size of the rosette at the end of the previous field season (De Jong et al., 1990). Bolting plants develop red to purple and sometimes brownish flowers and each flower can produce up to four flattened, tear drop-shaped, single seeded nutlets arranged in pyramidal pattern (Klinkhamer & de Jong, 1993). Small barbed nutlets facilitate dispersal via epizoochory by attaching to fur of grazing animals or mammalian wildlife (DeClerck-Floate, 1997). In its native range *C. officinale* grows as isolated plants or in smaller clusters along roadsides, in open woodland, sand dunes and fallow lands (Boorman & Fuller, 1984; De Jong et al., 1990). In North America, the plant invades rangelands, pastures, forests, and many disturbed habitats such as railroad and highway right of ways, gravel pits and fallow lands (Macoun, 1884; Upadhyaya & Cranston, 1991).

Mogulones crucifer Pallas (synonym *M. cruciger* Herbst) is a root-mining specialist weevil native to Eurasia that is associated with, feeds and develops on *C. officinale* (Dieckmann, 1972; Koch, 1992; Lohse, 1983; Schwarzländer, 1997). In early spring overwintering weevils become active and start feeding on foliage and with increasing temperatures, weevils begin to mate and lay eggs in the base of the petioles of newly developing leaves near to the crown root (Schwarzländer, 1997). Early instar larvae are found in root crowns, but later instars will also mine in taproots and secondary roots. Mature larvae leave the roots and form a cocoon out of soil particles for pupation. Adults emerge in early summer and start feeding on foliage before they aestivate throughout the summer and

fall and then hibernate in the leaf litter (Schwarzländer, 1997). A proportion of weevils will, however, become active again in September and continue to feed on foliage, mate and lay eggs before the first frost forces them into hibernation. Because of this partial second generation, larvae can be found throughout the year in roots of *C. officinale* (Schwarzländer, 1997).

During early spring of 2013, 2014 and 2015, adult overwintered *Mogulones crucifer* were collected at a *C. officinale* infestation from plants that just began to form new foliage near Bonners Ferry, Idaho (N 48.6913239^{o}, W 116.3308525^{o}) and then transported to the University of Idaho in Moscow, Idaho. At the laboratory the gender of weevils was determined by the presence of ventral abdominal depressions in males (Jordan et al., 1993). Weevils were maintained, separated by gender, in cylindrical plastic containers (11 cm diameter × 15 cm height), lined with paper towels, and covered with a muslin cloth in an environmental chamber (I-35VL, Percival Mfg. Co., Boone, Iowa) under 14:10 (L:D) and 17^{o}C day and 10^{o}C night. Weevils were fed fresh *C. officinale* foliage every second day. *Mogulones crucifer* was also reared in the laboratory by mating field-collected weevils. During the third week of April, five weevil pairs were released on a single-bolting *C. officinale* plant growing in a plastic tree pot (20 cm width × 32 cm height, Stuewe and Sons, Inc., Tangent, Oregon) and covered with a gauze bag (50 cm × 35 cm, Trimaco, LLC, Morrisville, North Carolina) and allowed to mate and lay eggs. Adult emergence of the new weevil generation commenced during the 4th week of June, and continued to the first week of August. A proportion of neonate weevils reared on potted plants does not emerge in summer, but instead aestivate and hibernate, and emerge during the subsequent spring. These weevils

were considered naïve and were used to conduct behavioral bioassays to test for effects of feeding experience.

Native confamilial plant species used for this study were selected based on their relatedness to *Cynoglossum officinale,* availability of propagules, and for federally protected species the availability of agency permits to obtain and propagate species for research. Selected species included the following native Boraginaceae species: *Adelinia grande* (Douglas ex Lehm.) J. I. Cohen (=*Cynoglossum grande* Douglas ex Lehm.), *Andersonglossum occidentale* (A. Gray) J. I. Cohen (=*C. occidentale* A. Gray), the single population, species and genus taxa *Dasynotus daubenmirei* I.M. Johnston, and the federally listed T&E species *Amsinckia grandiflora* (Kleeb. ex A. Gray) Kleeb. ex Greene, *Hackelia venusta* (Piper) H. St. John, *Plagiobothrys hirtus* (Greene) I.M. Johnston, *P. strictus* (Greene) I.M. Johnston. In addition, we included two congeners of *H. venusta*: *Hackelia californica* (A. Gray) I.M. Johnston, and *Hackelia micrantha* (Eastw.) J.L. Gentry. Finally, the European *Borago officinalis* L. was included in tests because it occurs abundantly throughout the distribution range of *M. crucifer* in Europe, and is within the fundamental host range of the weevil but not known to be a field host of the weevil (Dieckmann, 1972; Jordan et al., 1993; Lohse, 1983). The results from previous host-specificity testing are summarized in Table 2.1.

Plant materials for experiments was either propagated from seeds or collected at field sites. If not differently stated, plants were grown in tree pots in a 1:1:1 mix of topsoil, sand and Sunshine Mix #2 (Sun Gro Horticulture Canada Ltd., Vancouver, Canada) along with 2.5g trace elements (FRIT Industries, Inc., Ozark, Alabama), 1.25g chelated iron (Grow More Inc., Gardena, California), 47.5g limestone (Grow More Inc., Gardena, California), 47.5g triple super phosphate (Bonide Products Inc., Oriskany, New York), and 187.5g

Osmocote® (The Scotts Company LLC., Marysville, Ohio). The transplanted plants were watered every second day and were maintained in the University of Idaho's greenhouse at the Manis Entomological Laboratory and Parker Farm in Moscow, Idaho under 16:8 (L:D) and 25°C day and 18°C night.

Cynoglossum officinale were propagated both from seed and from roots collected at Idler's Rest Nature Preserve, Moscow, Idaho (N 46.804160°, W 116.948554°) and near Chief Timothy State Park, Clarkston, Washington (N 46.4144304°, W 117.1991536°). *A. occidentale* plants were collected from the Deschutes National Forest, near Camp Sherman, Oregon, USA (N 44.47011°, W 121.6282°) and transplanted at the site in tree pots with soil from their collection site to minimize the transplant shock. Plants were maintained in an environmentally controlled greenhouse at the Manis Entomological Laboratory and watered every second day. Similarly, *A. grande* (White Salmon, Washington, N 45.756892°, W 121.490535°), *D. daubenmirei* (Walde Lookout, Idaho, N 46.23528°, W 115.63528°), *H. californica* (Deschutes National Forest near Camp Sherman, Oregon, N 44.48194°, W 121.63917°), and *H. micrantha* (McCall, Idaho, N 44.9021882°, W 116.087851°) plants were collected in the field and maintained as described above. Seeds of the Eurasian *B. officinalis* were acquired from Swallowtail Garden Seeds (Santa Rosa, California), seeds of *A. grandiflora* were kindly provided by the California Department of Fish and Game's Native Plant Program, *H. venusta* seeds were provided by the University of Washington's Rare Plant Care and Conservation Program, *P. hirtus* seeds were provided by the Native Plant Conservation Program at Oregon State University, and *P. strictus* seeds were provided by the University of California at Berkley Botanical Garden.

Behavioral olfactometer bioassays

A four-armed olfactometer (Syntech Ltd., Hilversum, The Netherlands) as described by (Vet et al., 1983) was used to conduct behavioral bioassays testing the responses of female *M. crucifer* to volatiles from the host and non-host plants. In brief, the olfactometer is comprised of a four inlet-armed central rhomboid-shaped experimental choice arena (22 cm diameter) with a basal outlet and covered with a heavy clear glass plate (10 mm thickness) resting on an air-tight rubber seal (Fig. 2.1). Two perpendicular lines passing through the center of the olfactometer were drawn on the glass plate to delineate four quadrants of the arena, each with an area of 3,750 mm^2. Each of the four inlet arms was connected via a Tygon® tube (8 mm internal diameter, Fischer Scientific Co., Pittsburgh, Pennsylvania) to volatile sources (foliage of potted plants) that were placed inside airtight sealed sterilized polyvinyl acetate bags (20 cm×15 cm, Reynolds Consumer Products LLC., Richmond, Virginia). Four push pumps (Rena® Air 400, Mars Fishcare North America, Inc., Chalfont, Pennsylvania) were used to deliver air into the olfactometer directly (purified air treatment), or into the polyvinyl acetate bags containing plant foliage. Prior to pushing air into the olfactometer, it was purified by passing through activated charcoal (Sigma-Aldrich Co. LLC, St. Louis, Missouri)- filled polyethylene tubes (17 cm length ×1.6 cm internal diameter, Scienceware™, Bel-Art Products, Wayne, New Jersey) and humidified by passing through distilled water in a 500 ml gas-washing bottle (Chemglass Life Sciences LLC, Vineland, New Jersey) to create uniform humidity. The airflow in each arm was maintained at 300 ml/minute using four flowmeters (King Instrument Company, Inc., Garden Grove, California). Volatiles from plant species were offered in two opposite quadrants and the remaining two quadrants receiving purified air were considered control treatments. In

addition, air was drawn from the basal outlet at the rate of 1200 ml/min using a Rena® Air 400 pump that was modified to provide a pull by switching the direction of the pump diaphragm. The entire olfactometer setup was surrounded with white polyethylene vinyl acetate (PEVA) sheets to eliminate potential visual cue distractions to *M. crucifer*. A single full spectrum light source (Jansjö® LED lamp, Inter Ikea System B.V., Delft, The Netherlands), was used to illuminate the olfactometer arena uniformly from above.

Weevils were starved for 24 hrs prior to testing to enhance their responsiveness to treatments. At the beginning of each bioassay, the chamber outlet air hose was temporarily removed and an individual female *M. crucifer* was introduced into the olfactometer arena using a fine paintbrush. The hose was reconnected and the behavior of the weevil was observed and recorded for 30 min using a video camera (Contour Roam 2, Contour Inc., Seattle, Washington) fitted on top of the olfactometer arena. After every five bioassays, the odor sources were replaced and the olfactometer was rotated 90° to reduce positional effects. The central arena of the olfactometer and all connecting tubing were washed with 70% ethyl alcohol and distilled water after testing10 *M. crucifer* females. Each weevil was used only once. Weevils were recorded as "unresponsive" if they did not make any choice after five min of exposure and discarded from the experiment. The proportion of discarded weevils varied among bioassays but was always less than 20%. A weevil was considered to have made a choice for an odor when it entered into the respective quadrant and remained there for a minimum of 30 sec. The quadrant in which a weevil was located at the end of the 30-min observation period was considered the final choice of that weevil. Bioassays were only carried out between 0900 h and 1600 h. The video recordings with movement and

positioning of weevils were analyzed with the behavioral software program Noldus Observer XT 11 (Noldus Information Technology BV, Wageningen, The Netherlands).

To test the instrument, we placed *M. crucifer* females in the center of the olfactometer arena and exposed them to purified air in all four quadrants. Weevils typically were inactive following their placement into the arena, but once the airstream commenced, they would stretch their antennae prior to selecting a specific quadrant. In these blank trials, *M. crucifer* females were randomly distributed among the four quadrants with regard to initial choice (χ^2=4, p=0.2615, n=20), final choice (χ^2=4.8, p=0.1870, n=20) and proportional time spent in quadrants (χ^2=4.2, p=0.2598, n=20).

The following parameters were measured during behavioral bioassays: The Initial Choice of a weevil, defined as the quadrant that a weevil chose first after it was introduced into the olfactometer and remained there for a minimum of 30 sec., was recorded to evaluate *M. crucifer*'s ability to discriminate different odors. The Final Choice was defined as the location of each weevil at the end of the experiment and was assumed to be its ultimate preferred odor source. The proportional time spent in each quadrant of the olfactometer arena was recorded and considered an indicator for the Strength of Preference for each odor.

Naivety tests

Due to the phenology of *M. crucifer* naïve weevils were not available for all bioassays. Thus, tests were performed to determine if naïve and weevils with limited feeding experience responded differently to host plant cues. The phenology of *M. crucifer* and time constraints prevented the use of only naïve insects for bioassays because most weevils need to feed on foliage in summer or fall before they can overwinter and seek out host plants for oviposition

in the following spring (Schwarzländer, 1997). Most of the plant species tested in this study do not, however, produce foliage in late summer and in fall. To evaluate the potential effect of experience and a potential bias towards the odor of *C. officinale*, we conducted behavioral bioassays with as many naïve *M. crucifer* as we were able to rear and experienced weevils. Naïve weevils had no experience on any plants prior to experiment, whereas weevils fed with *C. officinale* leaves for several days were considered experienced. Because of the limited number of naïve *M. crucifer* females available, we could only conduct tests with three test plant species, i.e. *B. officinalis, H. californica,* and *H. micrantha.* We offered the odor of bagged foliage of potted test plant species and *C. officinale* to naïve and experienced *M. crucifer* females and compared results for the initial choice, final choice and proportion time spent in each quadrant. For each test plant species we conducted 20 replicates.

Dual-choice bioassays

To determine whether *M. crucifer* females were attracted to test plant species or able to identify them as potential hosts in the absence of other hosts, we conducted no-choice tests. For these tests, *M. crucifer* females were offered the choice between the volatile headspace of one test plant species in two opposing arms/quadrants and purified air in the remaining two arms/quadrants. There were 20 replicates for each plant species tested and as before the initial choice, final choice and proportion time spent in each quadrant were measured.

Multiple-choice bioassays comparing test species with *C. officinale*

To determine the relative attraction of female *M. crucifer* to confamilial plant species in the presence of its preferred host *C. officinale,* choice tests were conducted. For these tests, *M.*

crucifer females were offered volatile headspace from *C. officinale* and one of the confamilial plant species, and purified air (as control) in the remaining two quadrants. Tests were replicated 20 times for each plant species and the initial choice, final choice and proportion time spent in each quadrant were recorded for each weevil.

Statistical analysis

The choice data were discrete categorical responses. Hence, the proportion of initial choices and final choices of female *M. crucifer* in bioassays were initially assessed using χ-square tests of homogeneity. Logistic regression was subsequently used to model the odds of choice versus quadrants and assess pair-wise comparisons among quadrants.

The strength of preference for each choice was measured with the time (minutes) spent in each quadrant of the four-armed olfactometer. Differences among the four quadrants were assesses using log-linear categorical model assuming the time to be discrete counts. Within this model, single degree of freedom contrast allowed pair-wise comparison of the quadrants counts (times). For all analyses p-values <0.05 were regarded as significant. All analyses were conducted using the statistical software SAS Version 9.4 (SAS Institute Inc., 2013).

Results

Naivety Test

Naïve and experienced female *M. crucifer* exhibited behaviorally similar response patterns when exposed to volatiles of *C. officinale* and *B. officinalis, H. californica, and H. micrantha,* respectively. Both naïve and experienced females preferred *C. officinale* volatile

quadrants for their initial and final choice and spent more time in *C. officinale* quadrants than in quadrants with volatiles from confamilial plants or purified air (Table 2.2, Figs. 2.2, 2.3 and 2.4). However, there were no differences in all three parameters i.e. initial choice, final choice and time spent by female *M. crucifer* in test plant volatiles and purified air quadrants (Table 2.2, Figures 2.2, 2.3).

Dual-choice bioassays

In these bioassays, *M. crucifer* greatly preferred *C. officinale* volatile quadrants over those with purified air for the initial and final choice (Fig. 2.5). At the end of the bioassays, all females without exception were located in *C. officinale* quadrants (Fig. 2.5). Similarly, *M. crucifer* spent four times more time in *C. officinale* quadrants (81.12%) compared to quadrants with purified air (18.88%) (Table 2.3, Figure. 2.6). For all three plant species tested in the genus *Hackelia*, (i.e., *H. californica*, *H. micrantha*, and *H. venusta*), female weevils did not differentiate between *Hackelia* volatile quadrants and purified air quadrants in their initial choice, and they preferred purified air over *Hackelia* quadrants for their final choice (Table 2.3, Figure. 2.5). Similarly, *M. crucifer* females spent more time in quadrants with purified air than in those with volatiles from any of the three *Hackelia* species (Table 2.3, Figure. 2.6). In tests with *Andersonglossum occidentale*, *Adelinia grande*, and *Amsinckia grandiflora*, there were no differences in the initial and final choice of *M. crucifer* (Table 2.3, Figure. 2.5), but female weevils tended to spend more time in quadrants with purified air compared to those with volatiles from the respective plant species (Table 2.3, Figure. 2.6). For all other plant species, *M. crucifer* did not distinguish between plant volatiles and

purified air quadrants in their initial and final choices, or in the time spent in respective quadrants (Table 2.3, Figures. 2.5 and 2.6).

Multiple-choice bioassays with *C. officinale*

In choice bioassays, *M. crucifer* females universally preferred *C. officinale* volatile quadrants for their initial and final choice and spent more time in those quadrants than those with volatiles from confamilial plants or those with purified air. In addition, female weevils did not distinguish between confamilial plant volatiles and purified air for all measured behavioral responses, the initial and final choice and time spent in quadrants (Table 2.4, Figures. 2.6 and 2.7).

Discussion

Behavioral response of M. crucifer to volatiles emitted by host and non-host plants

The results from our experiments strongly suggest that volatile cues play an important role in the host finding of *M. crucifer* females. Not only were female weevils attracted to volatiles emitted by its field host *C. officinale*, the weevils also consistently preferred host plant from confamilial non-targets in our study. Our bioassays detected all three possible behavioral response outcomes: attraction (one plant's volatiles preferred over another's or to purified air), indifference (plant volatiles not preferred over purified air control) and repellence (purified air preferred over plant volatiles) (Martini et al., 2015; Vet et al., 1983). Female weevils were repelled, based on their Final Choice and proportion of time spent in quadrants, by VOC from all three *Hackelia* species tested, including the T&E species *H. venusta*. Weaker repellence was found for volatiles of the T&E species *Amsinckia*

grandiflora as well as the former North American congeners of *C. officinale Adelinia grande* and *Andersonglossum occidentale*. The effects of volatiles of the remaining confamilial plants (*B. officinalis*, *D. daubenmirei,* and the T&E species *P. hirtus* and *P. strictus*) were indifferent, as the female weevils were not able to differentiate those volatiles from purified air. Phytochemical similarity between potential and ancestral hosts is considered conducive to host range switching or expansion (Ehrlich & Raven, 1964; Futuyama, 1999). We characterized the volatile headspace from *C. officinale* and the other species tested in this study and found that one bioactive compound, methyl isovalerate, that was unique to *C. officinale* among these plants (Kafle unpublished data). In behavioral bioassays, weevils were able to perceive and were behaviorally attracted to methyl isovalerate when offered along with purified air in a four-armed olfactometer (Kafle unpublished data). In addition, non-target plants emitted several VOCs that were absent in *C. officinale*, which could act as repellents to *M. crucifer* (Kafle unpublished data). However, physiological responses of *M. crucifer* to those compounds remain unknown at this time.

Linking behavioral responses to plant volatiles with host use under field conditions

Plant-emitted volatiles have long been known to play an important role in the host selection of herbivorous insects (Bruce et al., 2005). Our approach to improve the predictability of the host range of biological control agents is based on the assumption that behavioral responses to olfactory cues by herbivorous insects reliably reflect their host use under field conditions (Wheeler & Schaffner, 2013). Several behavioral bioassays have reported the attraction of phytophagous insects towards the volatiles from their host plants (Bartlet et al., 1993; Bruce & Pickett, 2011; Fraser et al., 2003; Knolhoff & Heckel, 2014;

Nottingham et al., 1991; Quiroz et al., 2005; Yan et al., 1999) and indifference behavior or repellence of phytophagous insects to the volatiles from their non-host plants (Brielmann et al., 1999; Cao et al., 2015; Cha et al., 2008; Thomas et al., 1987). For example, when goldenrod beetles *Trirhabda Canadensis* (Coleoptera: Chrysomelidae) were tested in Y-tube olfactometers, it was found that beetles preferred the host odor to confamilial non-host odors and were either indifferent or repelled to non-host odors when offered along with purified air (Puttick et al., 1988). Those results were in accordance with colonization experiments, in which beetles preferred monoculture plots of its host plant to plots with confamilial non-hosts intermixed with host plants indicating the impact of non-host volatiles on host finding (Morrow et al., 1989). The indifference and repellence documented in our study suggest that the respective non-target species would not be sought out, or even would be avoided by *M. crucifer* during host selection in natural settings.

In CBC, the importance to elucidate the role of volatile compounds during the host selection has only very recently been recognized (Beck et al., 2014; Beck et al., 2008; Knolhoff & Heckel, 2014; Müller & Nentwig, 2011; Park et al., 2011; Piesik et al., 2015; Rendon et al., 2014; Smith & Beck, 2013; Wheeler, 2014; Wheeler et al., 2014; Wheeler & Schaffner, 2013). For example, it was found that the weevil *Ceratapion onopordi* Kirby (Coleoptera: Apionidae) preferred volatiles of its host plant *Cirsium arvense* (L) Scop. (Asteraceae) to purified air in a four-armed olfactometer (Müller & Nentwig, 2011). Behavioral responses of candidate biological control organisms towards host cues can help predict their host use post-release.

Towards a better understanding of host use by Mogulones crucifer under field conditions

There are major concerns about the environmental safety of *M. crucifer* in the United States, which was approved for release in Canada in 1997 (De Clerck-Floate & Schwarzländer, 2002a). While the weevil is considered a successful biocontrol organism in Canada (De Clerck-Floate et al., 2005), the petition for release in the United States was first denied in 2002 and the United States Department of Agriculture issued a pest alert for *M. crucifer* in 2010 (USDA, 2010). These concerns were partly based on the overall very broad fundamental host range with 55% of native confamilials tested supporting development of the weevil, with T&E species *A. grandiflora*, *H. venusta*, and *P. hirtus* causing the greatest concern (Andreas, 2004). For this study, we included four of the five T&E species in the Boraginaceae (*A. grandiflora*, *H. venusta*, *P. hirtus,* and *P. strictus*). Our data indicate that despite the ability of *M. crucifer* to develop on *A. grandiflora, H. venusta,* and *P. hirtus* (Andreas, 2004), none of these plant species may be at risk of being attacked by the weevil because their respective volatiles were repellant in the former two and the weevil's response was indifferent in the latter case. Although weevils were not repelled immediately (Initial Choice) by *A. grandiflora* and *H. venusta* during early host finding, they spent slightly more time in purified air quadrants of the olfactometer compared to those with *A. grandiflora* volatiles. For *H. venusta,* which is limited to a single population of about 500 plants in Washington State (Vance, 2013) we found repellence with regard to the Final Choice and the amount of time spent in quadrants. Our findings of consistent repellence with regard to *Hackelia* spp. are consistent with previous findings that suggested repellence of *M. crucifer* by *H. micrantha* volatiles when offered in four-armed olfactometer along with host volatiles (Andreas et al., 2008a). For *P. hirtus,* we found no indication that during early host finding

(prior to contact with the host) *M. crucifer* is capable of responding to the species as potential alternative host plant. The last T&E species included in our study, *P. strictus,* has never been tested with *M. crucifer,* but as for *P. hirtus,* we found no indication that during early host finding weevils are capable identify or distinguish this species as a potential alternative host.

The Eurasian *B. officinalis*, which is found abundantly throughout Europe in the same areas as *C. officinale* where it is often grown as a garden herb, supports development of *M. crucifer* as well as *C. officinale* (Jordan et al., 1993; Schwarzländer, 1996). Despite this, *B. officinale* has never been reported as a host of *M. crucifer* in its native range (Dieckmann, 1972; Schwarzländer, 1999). This species was included in the present study because of this ecological relevance and the presumed evolutionary history with *M. crucifer* and *C. officinale.* Consistent with its non-host status in its native range *M. crucifer* did not respond to volatiles from *B. officinalis*. Why the plant had indifferent effects on *M. crucifer* despite its evolutionary relationship and the fact that it readily can support weevil development is beyond the scope of this study. We speculate that differences in volatile emission from *B. officinalis* render the plant unrecognizable to *M. crucifer.*

While we report repellence of *M. crucifer* towards plant species in the genus *Hackelia* including *H. micrantha* here, the species is known to be utilized as host in the field by *M. crucifer* (Andreas et al., 2008b; Catton et al., 2015). However, patterns of that non-target attack show that *H. micrantha* was consistently the lesser preferred host in comparison to *C. officinale* (Catton et al., 2015). Non-target use of *H. micrantha* was explained as spillover because *M crucifer* females laid only eggs in sympatrically occurring *H. micrantha* for a few years in the presence of *C. officinale* but did not establish weevil populations on *H. micrantha* in the absence of *C. officinale* (Catton et al., 2015). Spillover or apparent

competition occurs when biological control organisms use a lower ranked host as a consequence of high herbivore densities (White & Whitham, 2000). Mechanisms such as central excitation and sensitization have been proposed to explain spillover. Central excitation occurs when an insect contacts a favorable host, which excites the insect's central nervous system, and results in the acceptance of lower preference hosts during the state of excitation that would otherwise be rejected (Marohasy, 1998; Menzel et al., 1993). Since central excitation is short-lasting, this indiscriminate host use may be better explained by sensitization. Sensitization is defined as the increase in responsiveness of an organism due to repeated presentation of an eliciting stimulus (Domjan & Burkhard, 1982). The impact of sensitization is not stimulus specific i.e., if an insect is aroused in response to one stimulus, its reactivity is stimulated by a variety of stimulus cues (Domjan & Burkhard, 1982).

As shown here and elsewhere, host finding in *M. crucifer* is mediated by olfactory stimuli in the form of plant volatile emissions (Andreas et al., 2008a). These volatile compounds form an odor plume as they are dispersed by air movement (Marques et al., 2003; Murlis et al., 1992). In an environment where there are multiple odor sources, insects will encounter odor plume admixtures (OPA) during host finding. Based on our data and that of Catton et al. (2014, 2015), we propose that *M. crucifer* is sensitized by *C. officinale* volatile OPA, which increases its responsiveness towards *H. micrantha* volatiles in the admixture and results in its non-target attack in the field. The repellence documented here would explain the reduced use of *H. micrantha* by weevil over time and the lack of non-target attack in the absence of *C. officinale* (Catton et al., 2014; 2015). Further, any protected confamilial non-target plant species would only be at risk of OPA mediated non-target attack if the plant

species occur sympatrically with *C. officinale.* To our knowledge that is not the case for any of the T&E species in the Boraginaceae family.

Overall, our results suggest that during the initial stage of host finding, *M. crucifer* uses odor cues to locate and discriminate its host plant from non-hosts and that that all confamilial T&E species tested here are not at risk of non-target attack or colonization by *M. crucifer* as long as they are not in immediate proximity of *C. officinale,* which can be ruled out given the distribution ranges of each of the species (Kartesz, 1999). We only evaluated the walking behavior of *M. crucifer* although it is known that the weevil is capable of flying and dispersing up to 0.5 km annually (De Clerck-Floate et al., 2005), still not nearly sufficient to bridge >100 miles to the closest populations of most of the plant species tested in this study (Kartesz, 1999).We tried repeatedly, but were unable to acquire propagules of *Oreocarya crassipes* for this study. It would have been interesting to test this T&E species because concern for this species prevented the release of *M. crucifer* in the United States. It would also have been important because another plant species within the genus *Oreocarya, O. spiculifera,* was attacked by *M. crucifer* in the field (Andreas et al., 2008b) and recent phylogenetic revisions of Boraginaceae based on morphological and molecular traits moved the genus *Oreocarya* more closely towards the Eurasian genus *Cynoglossum* (Cohen, 2014; Weigend et al., 2013). While *O. crassipes* and *C. officinale* distributions are not overlapping, *Oreocarya* (synonym *Cryptantha*) represent the most species-rich genus in Boraginaceae with more than 150 species in North America (Kartesz, 1999) and should be included in studies of behavioral responses of *M. crucifer* and their chemical ecological bases before final risk assessments about the environmental safety of the weevil are derived. This study

illustrates the utility of the chemical ecology of host finding to ensure sound predictions concerning the host fidelity of potential weed biological control organisms.

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Table 2.1: List of selected plant species for behavioral analysis of female *M. crucifer*.

Plant species	Synonyms	Rationale for testing	Previous host range data
Adelinia grande (Douglas ex Lehm.) J.I. Cohen[a]	*Cynoglossum grande* Douglas ex Lehm.	Among closest North American relative of *C. officinale*	Within fundamental host range[2]; Adult feeding & oviposition in laboratory testing[4]
Amsinckia grandiflora (Kleeb. ex A. Gray) Kleeb. ex Greene		T&E confamilial species	Within fundamental host range[4]; Adult feeding & oviposition in laboratory testing[4]
Andersonglossum occidentale (A. Gray) J.I. Cohen[a]	*Cynoglossum occidentale* A. Gray	Among closest North American relative of *C. officinale*	Adult feeding in laboratory testing[4]
Borago officinalis L.		Abundant Eurasian sympatric confamilial of *C. officinale*	Within fundamental host range[1,2]; Adult feeding & oviposition in laboratory testing [1,2]
Dasynotus daubenmirei I.M. Johnston		Single genus/species taxa closely related to *C. officinale.* Rare and limited to single population in Idaho	Within fundamental host range[4]; Adult feeding in laboratory testing[4]
Hackelia venusta (Piper) H. St. John		T&E confamilial	Within fundamental host range[4]; Adult feeding & oviposition in laboratory testing[4]
Hackelia micrantha (Eastw.) J.L. Gentry[b]	*Hackelia jessicae* Brand	North American confamilial recently studied with regard to *M. crucifer* attack	Within fundamental host range[4]; Adult feeding & oviposition in laboratory

			testing[4] Non-target attack in field[4,5,6]
Hackelia californica (A. Gray) I.M. Johnston		Congener of T&E *H. venusta*, sympatrically occurring with *A. occidentale*	Not tested
Plagiobothrys hirtus (Greene) I.M. Johnston		T&E confamilial species	Within fundamental host range[3,4]; Adult feeding & oviposition in laboratory testing[4]
P. strictus (Greene) I.M. Johnston		T&E confamilial species	Not tested

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Table 2.2: Summary statistics for behavioral response of naïve and experienced *M. crucifer* females in multiple-choice bioassays with three confamilial plant species compared with *C. officinale* and clean air. In all cases the weevils preferred *C. officinale* to the other species (see Figure 2.3 and text for details).

		Borago officinalis		***Hackelia californica***		***H. micrantha***	
		χ^2	**p-value**	**χ^2**	**p-value**	**χ^2**	**p-value**
Naïve	Initial Choice	32.4	<0.0001	10.0	0.0186	17.6	0.0005
	Final choice	32.4	<0.0001	24.1	<0.0001	16.2	<0.0001
	Time spent	245.44	<0.0001	226.38	<0.0001	229.69	<0.0001
Experienced	Initial Choice	17.2	0.0006	17.2	0.0006	26.8	<0.0001
	Final choice	32.4	<0.0001	19.6	<0.0001	60.0	<0.0001
	Time spent	279.27	<0.0001	380.42	<0.0001	224.23	<0.0001

Table 2.3: Summary statistics for behavioral response of *M. crucifer* females in dual-choice bioassays with volatile headspace of ten sensitive or threatened and endangered listed confamilial plant species in the Boraginaceae family vs. purified air. Weevils preferred *C. officinale* to purified air, and weevils preferred purified air to plant volatiles with final choice and time spent in all *Hackelia* species tested and time spent with *Amsinckia grandiflora* and *Andersonglossum occidentale*, (n=20 for all bioassays) (see Figures 2.4 and 2.5 and text for details).

	Initial choice		**Final choice**		**Percent time spent in quadrants of olfactometer**	
	χ^2	**p-value**	**χ^2**	**p-value**	**χ^2**	**p-value**
Cynoglossum officinale	7.3	0.0260	20.4	0.0001	196.89	<0.0001
Adelinia grande	2.058	0.5603	1.6	0.6594	10.80	0.0129
Amsinckia grandiflora	2.8	0.4235	7.2	0.0658	12.71	0.0053
Andersonglossum occidentale	2.8	0.4235	2.8	0.2466	8.09	0.0443
Borago officinalis	2.8	0.4235	6.0	0.1116	2.26	0.5212
Dasynotus daubenmirei	3.6	0.3080	4.8	0.1870	7.47	0.0584
Hackelia californica	7.6	0.0550	8.0	0.0460	123.29	<0.0001
H. micrantha	6.0	0.1116	9.2	0.0267	122.61	<0.0001
H. venusta	0.4	0.9402	8.0	0.0460	44.75	<0.0001
Plagiobothrys hirtus	3.6	0.4235	2.8	0.4235	6.58	0.0866
P. strictus	3.6	0.3080	2.8	0.4235	1.02	0.7970

Table 2.4: Summary statistics for behavioral response of *M. crucifer* females in multiple-choice olfactometer bioassays with volatile headspace of ten sensitive or threatened and endangered listed confamilial plant species in the Boraginaceae family, *Cynoglossum officinale*, and purified air (n=20 for all bioassays). In all cases the weevil preferred *C. officinale* to the other species tested (see Figures 2.6 and 2.7 and text for details).

	Initial choice		**Final choice**		**Percent time spent in quadrants of olfactometer**	
	χ^2	**p-value**	χ^2	**p-value**	χ^2	**p-value**
Adelinia grande	17.2	0.0006	17.2	0.0006	319.82	<0.0001
Amsinckia grandiflora	13.2	0.0042	22.0	<0.0001	285.18	<0.0001
Andersonglossum occidentale	17.6	0.0005	26.8	<0.0001	290.49	<0.0001
Borago officinalis	17.2	0.0006	32.4	<0.0001	419.92	<0.0001
Dasynotus daubenmirei	22.0	<0.0001	17.2	0.0006	231.15	<0.0001
Hackelia californica	17.2	0.0006	19.6	<0.0001	286.03	<0.0001
H. micrantha	26.8	<0.0001	60.0	<0.0001	421.65	<0.0001
H. venusta	13.2	0.0042	27.2	<0.0001	301.31	<0.0001
Plagiobothrys hirtus	17.2	0.0006	38.4	<0.0001	302.55	<0.0001
P. strictus	29.63	<0.0001	28.9	<0.0001	346.42	<0.0001

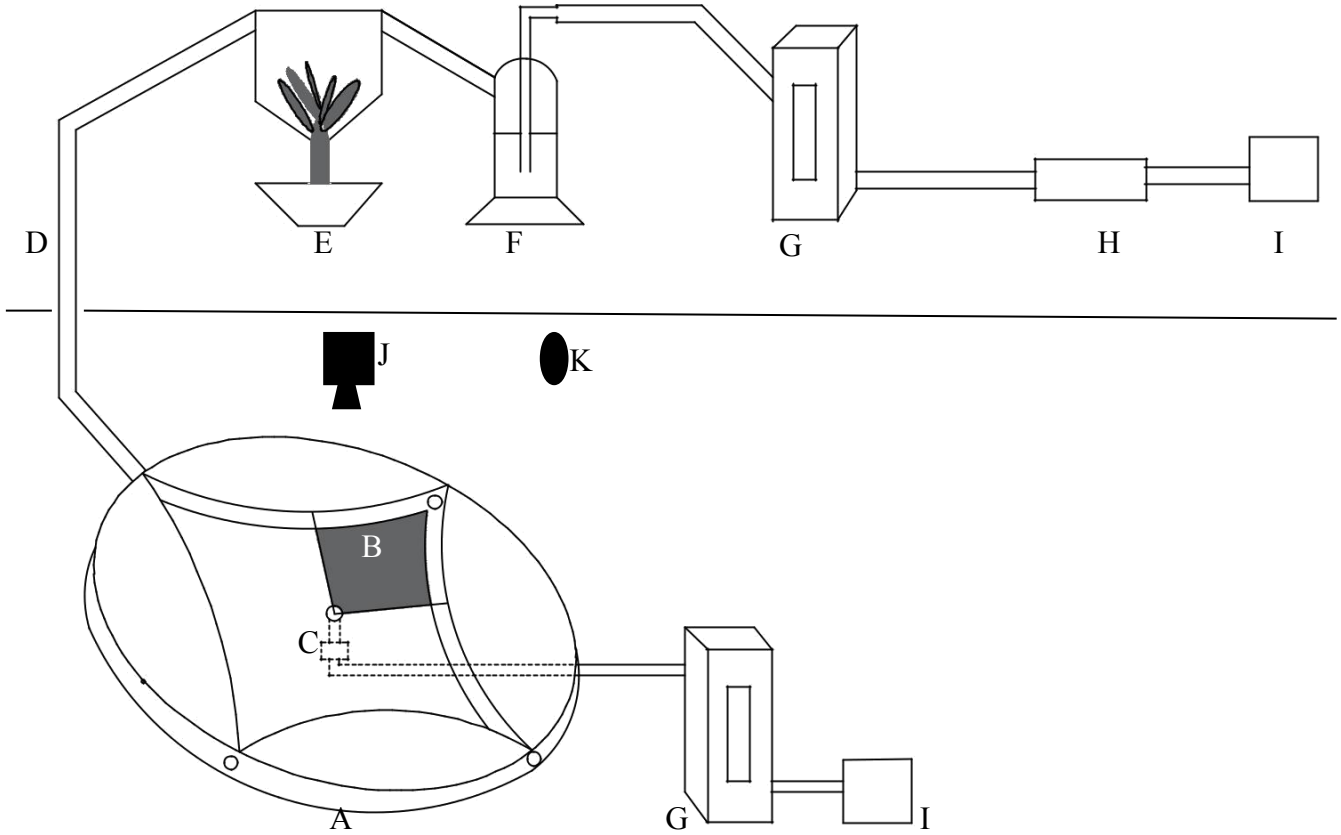


Figure 2.1: Schematic diagram of four-armed olfactometer (not drawn to scale).A: Central arena (22 cm diameter), B: individual quadrant (55mm×55mm, 10mm height), D: insect inlet port, D: Tygon® tube (8 mm internal diameter), E: odor source (foliage enclosed in bag), F: humidifier, G: flowmeter, H: activated charcoal filter, I: air pump, J: video camera, K: light source. Arrows indicate the direction of air flow.

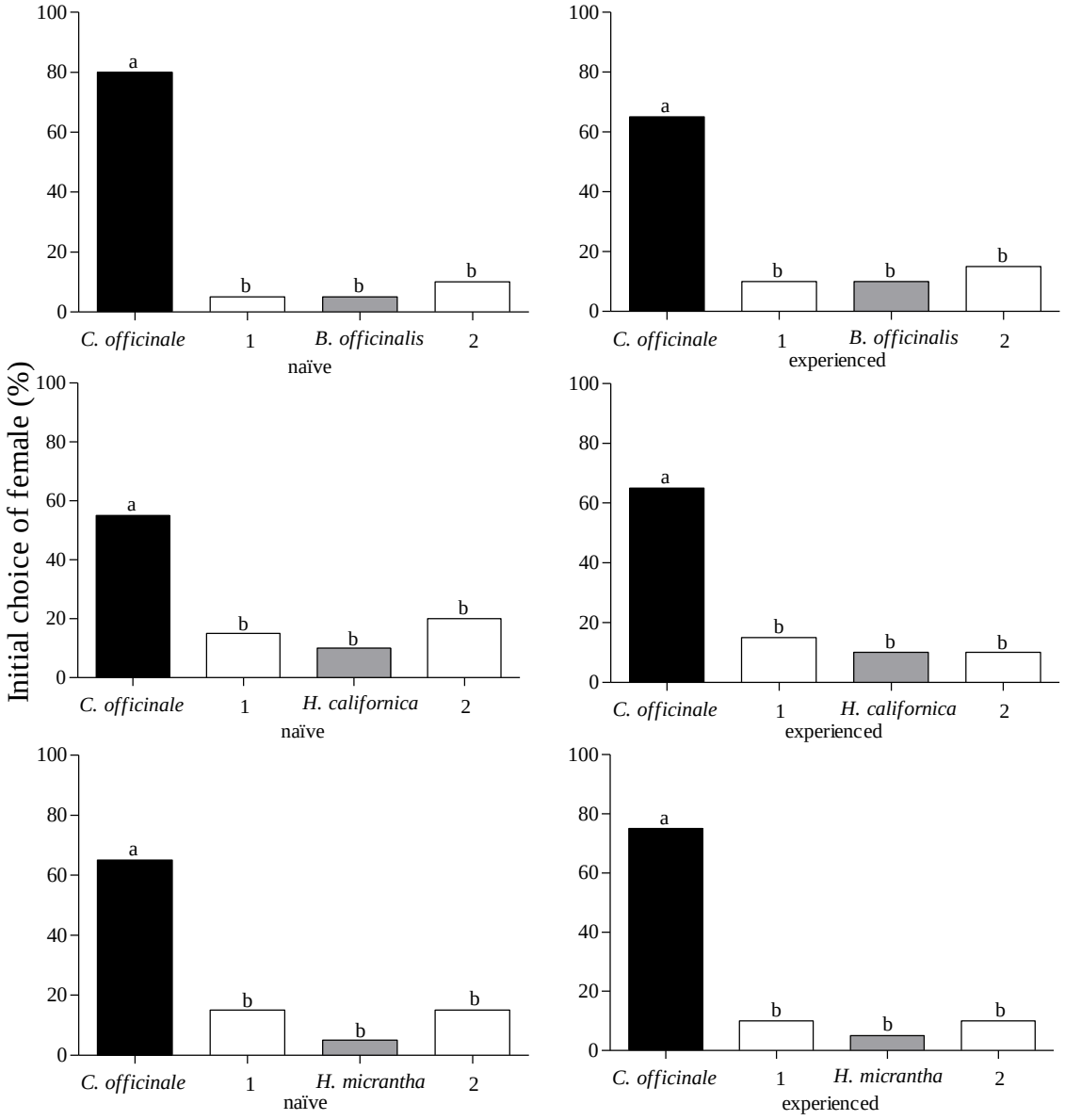


Figure 2.2: Proportion naïve (left graphs) and experienced (right graphs) female *Mogulones crucifer* initially choosing one of four quadrants of a four-armed olfactometer arena using volatile headspace of *Cynoglossum officinale*, one test plant species, and purified air in two quadrants (1 and 2). Differing letters on top of bars denote significant differences. (n=20) (χ^2-test followed by logistic regression analysis, $p<0.05$, ns=not significant) (see text for details).

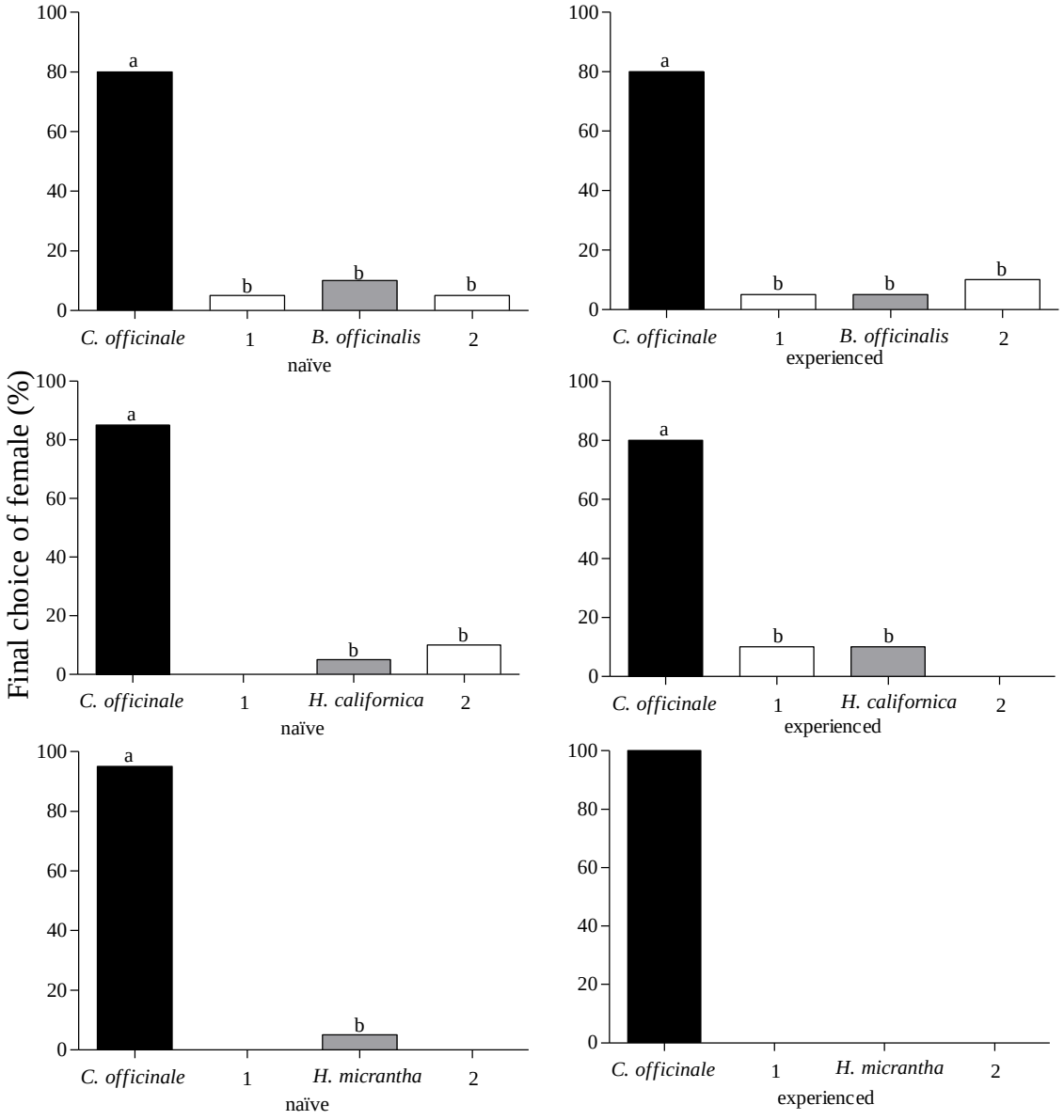


Figure 2.3: Proportion naïve (left graphs) and experienced (right graphs) female *Mogulones crucifer* finally choosing one of four quadrants of a four-armed olfactometer arena using volatile headspace of *Cynoglossum officinale*, one test plant species and purified air in two quadrants (1 and 2). Differing letters on top of bars denote significant differences. (n=20) (χ^2-test followed by logistic regression analysis, $p<0.05$, ns=not significant) (see text for details).

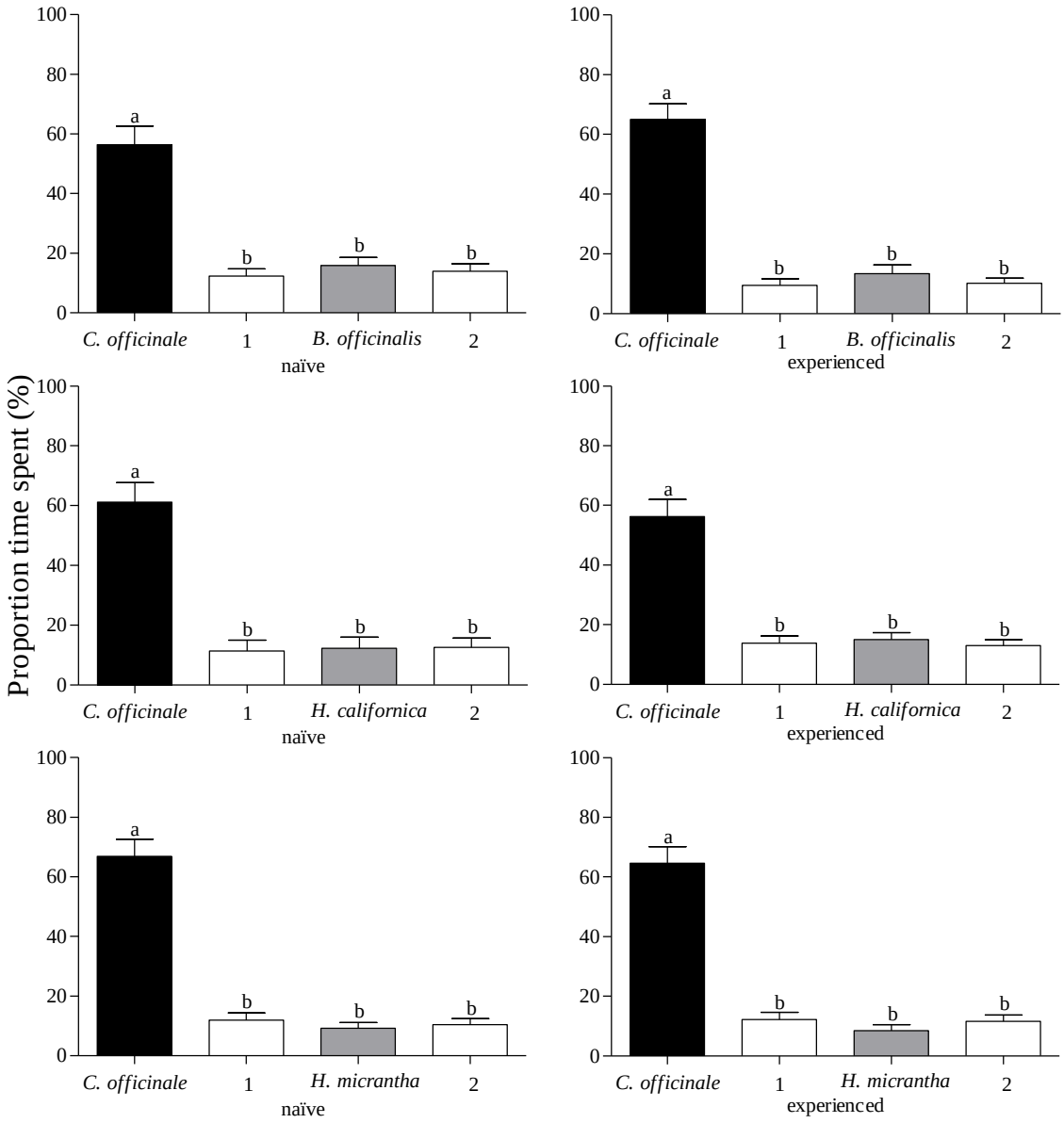
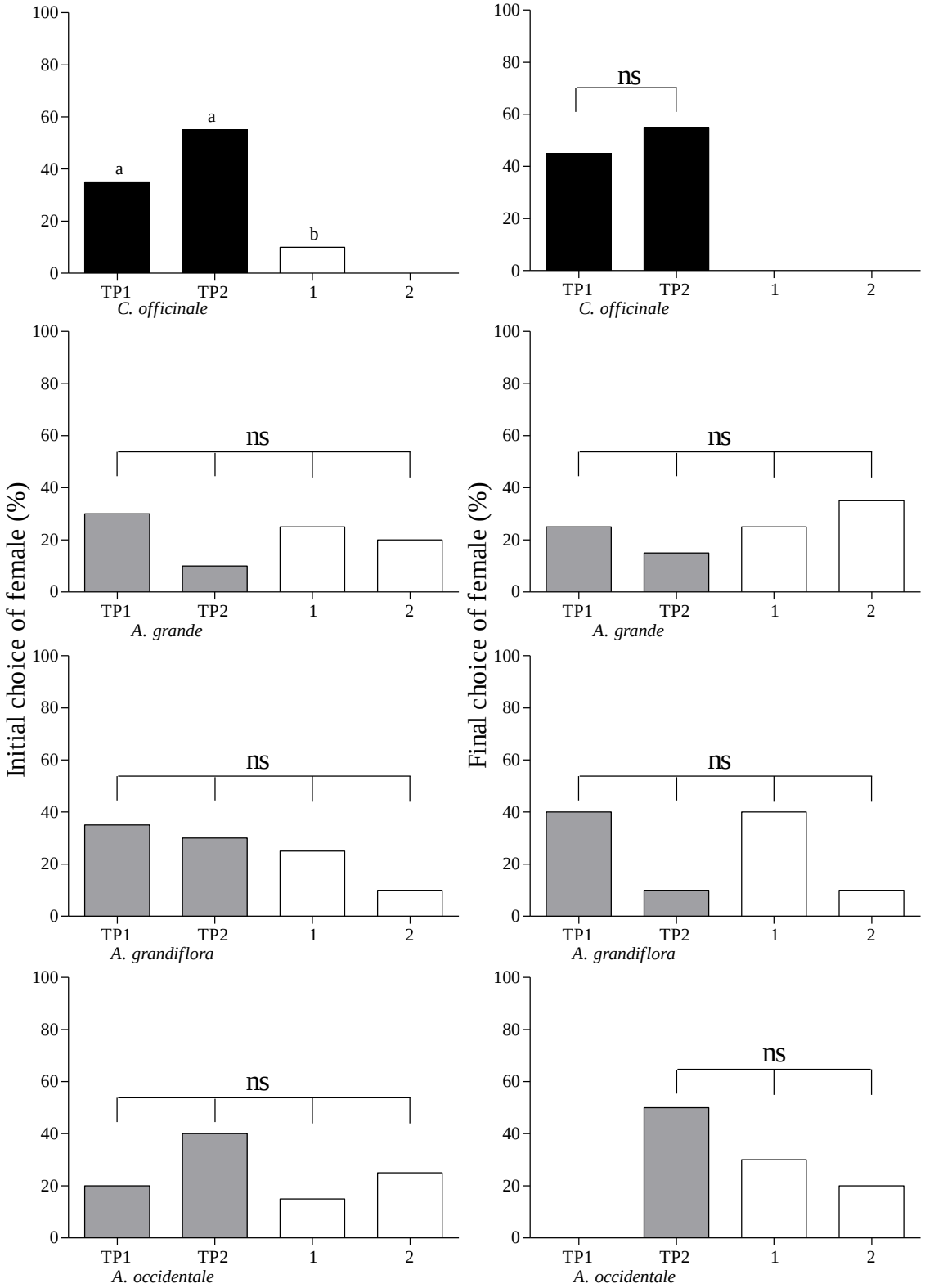
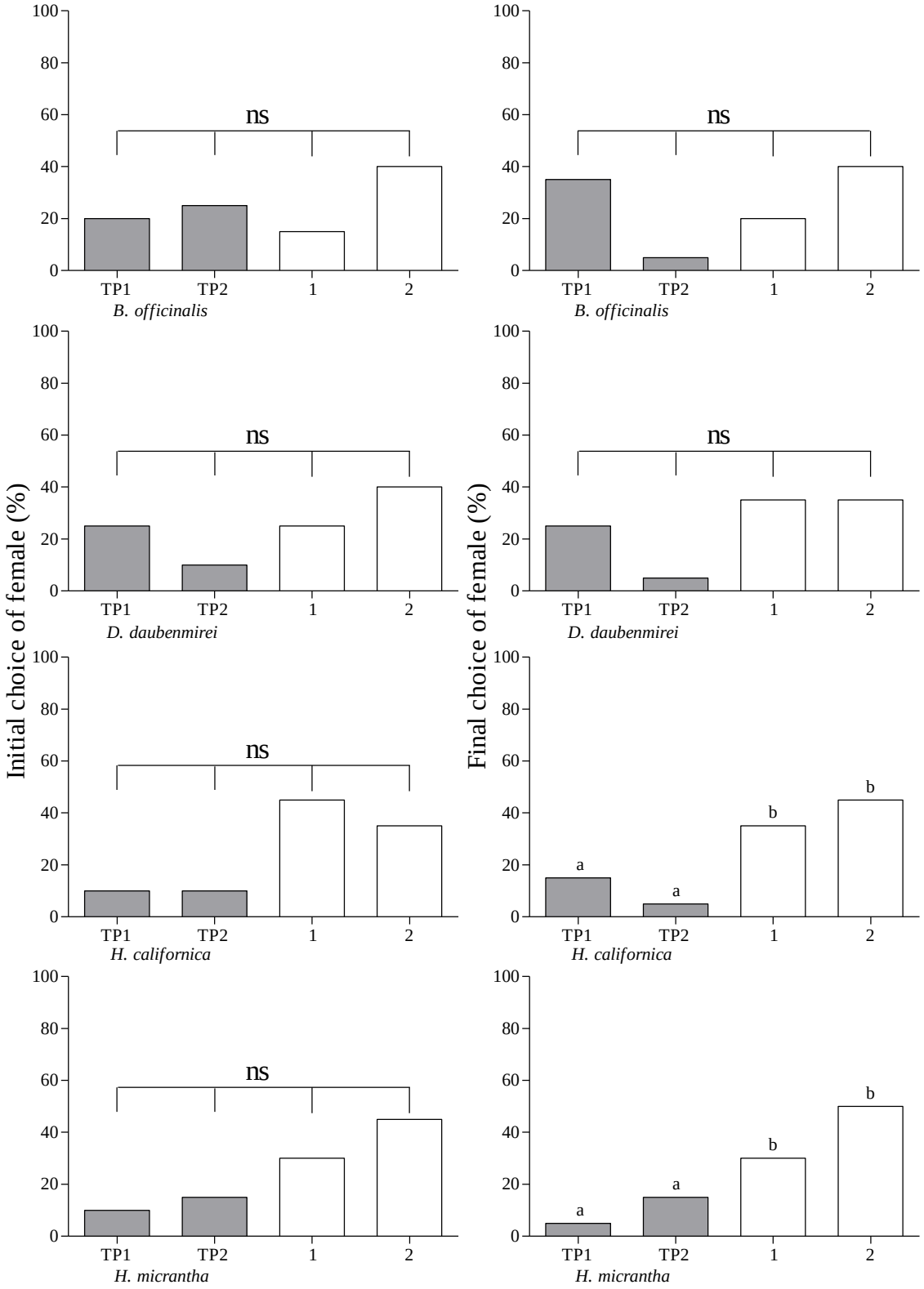


Figure 2.4: Proportion of time spent by naïve (left graphs) and experienced (right graphs) female *Mogulones crucifer* in each of four quadrants of a round four-armed olfactometer arena using volatile headspace of *Cynoglossum officinale*, one test plant species, and purified air in two quadrants (1 and 2). Differing letters on top of bars denote significant differences

(n=20) (Categorical log linear model followed by single degree of freedom contrast analysis, $p<0.05$) (see text for details).


Initial choice of female (%)
Final choice of female (%)
100
80
60
40
20
0
a
a
b
ns
TP1
TP2
1
2
C. officinale
A. grande
A. grandiflora
A. occidentale


Initial choice of female (%)
Final choice of female (%)
100
80
60
40
20
0
TP1
TP2
1
2
ns
a
b
B. officinalis
D. daubenmirei
H. californica
H. micrantha

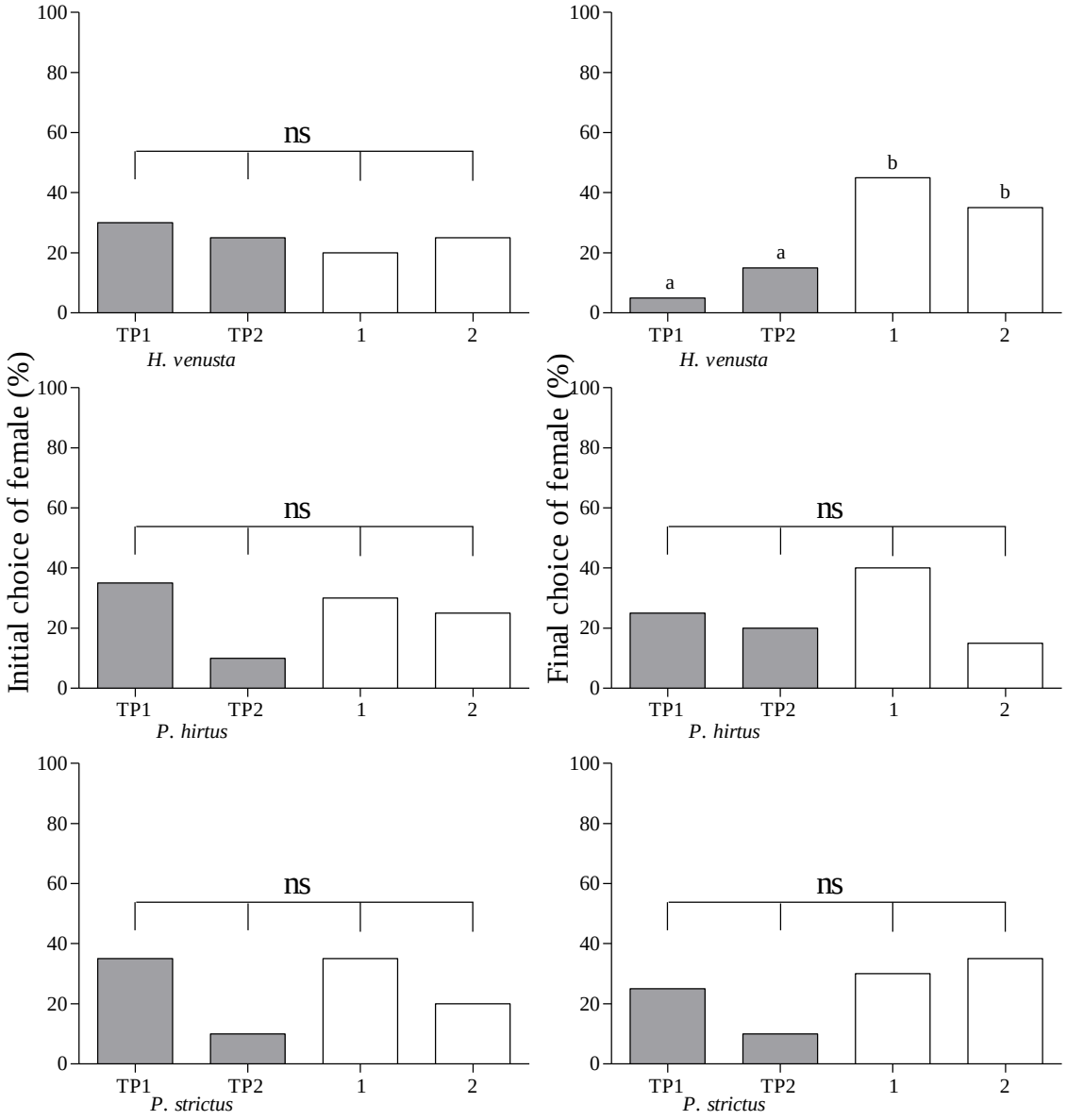
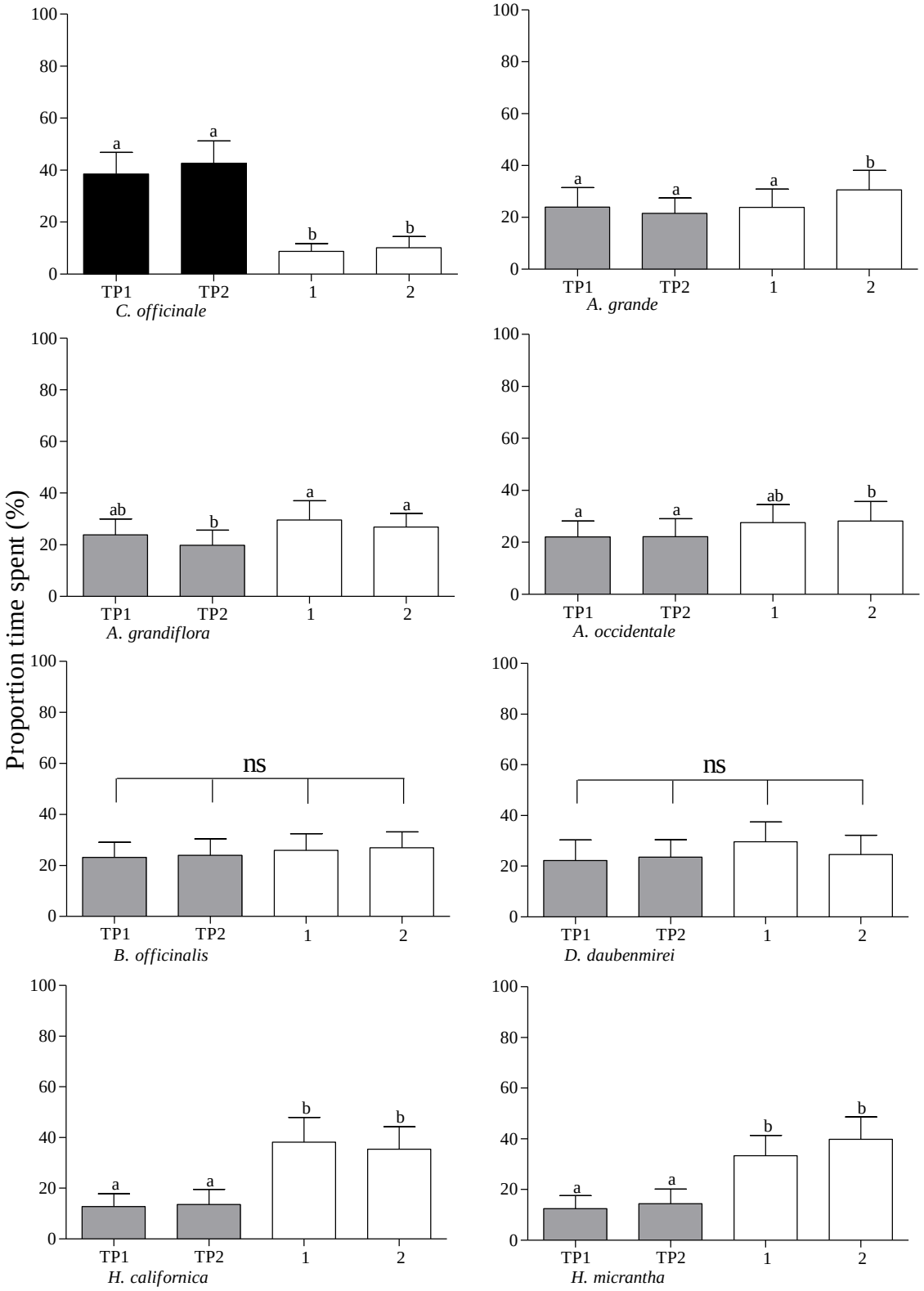


Figure 2.5: Proportion of female *Mogulones crucifer* initially (left graphs) and finally (right graphs) choosing one of four quadrants of a four-armed olfactometer arena using volatile headspace of one test plant species in two quadrants (TP1 and TP2) and purified air in two quadrants (1 and 2). Differing letters on top of bars denote significant differences (n=20) (χ^2-test followed by logistic regression analysis, $p<0.05$, ns=not significant) (see text for details).


Proportion time spent (%)
C. officinale
A. grande
A. grandiflora
A. occidentale
B. officinalis
D. daubenmirei
H. californica
H. micrantha
TP1
TP2
1
2
ns

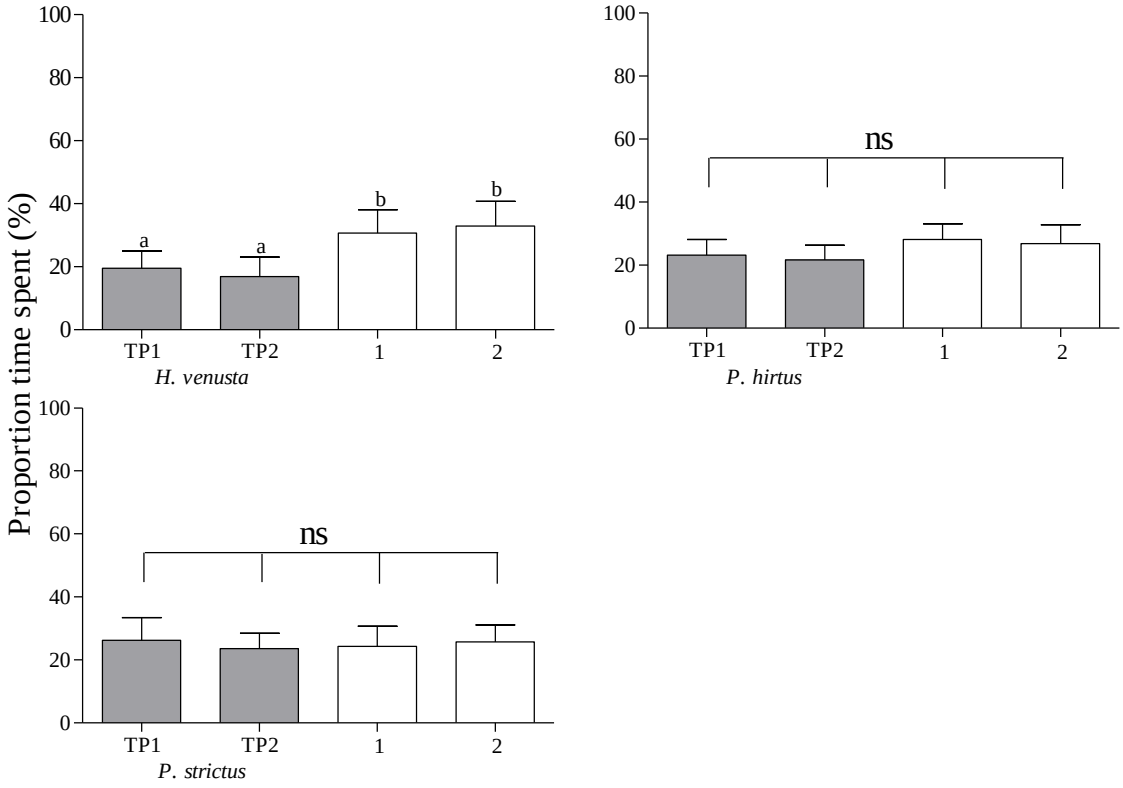
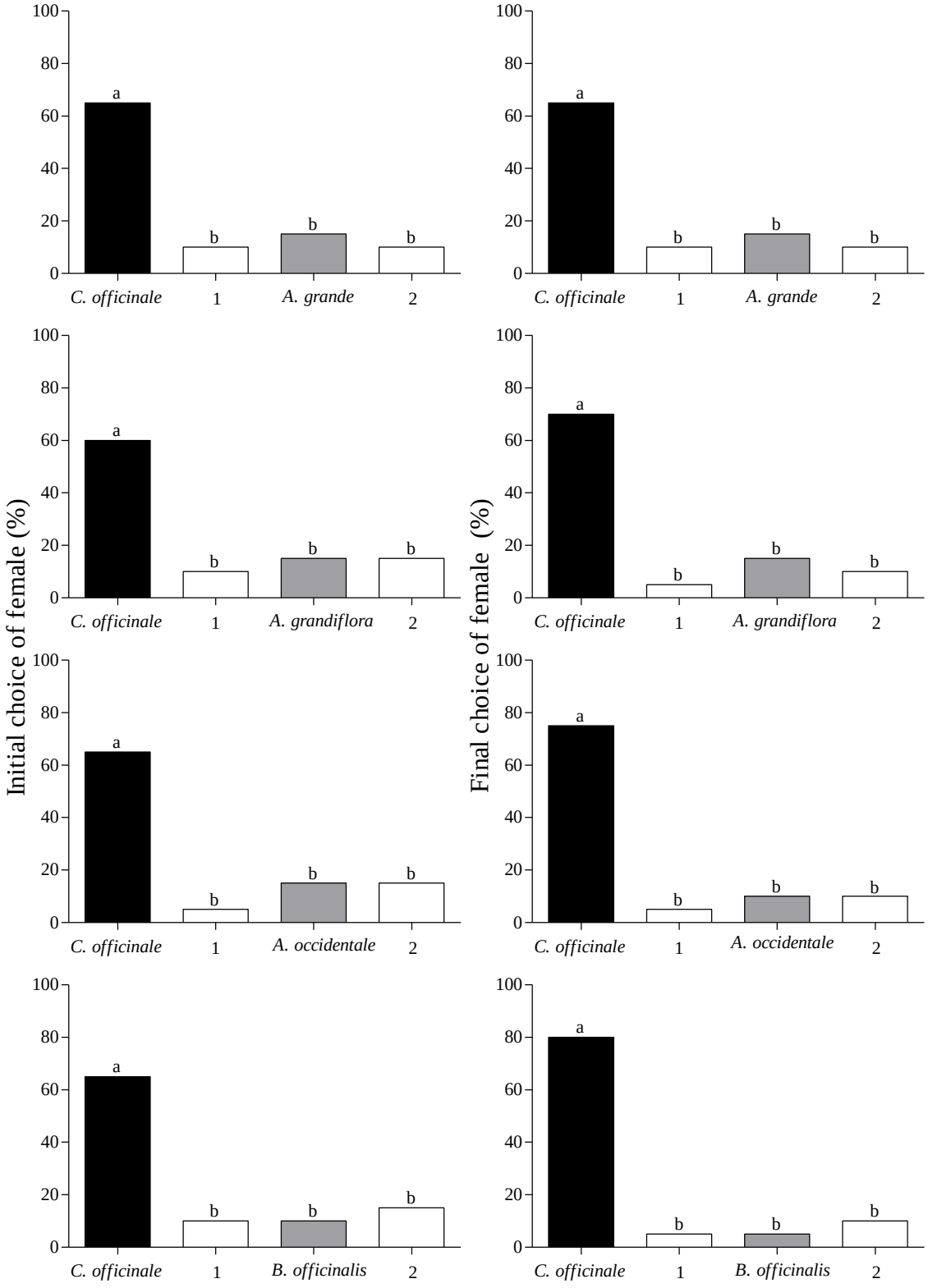
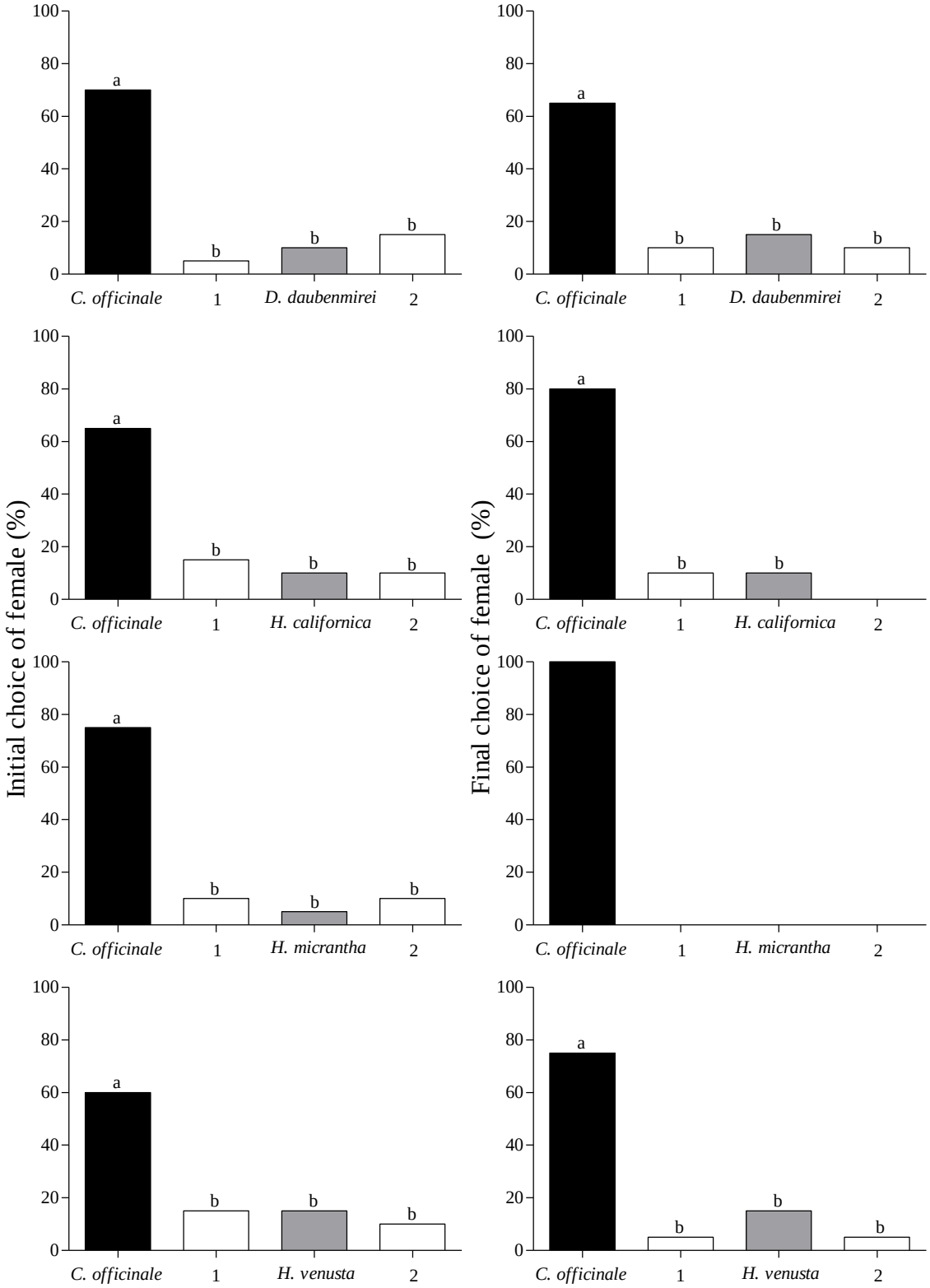


Figure 2.6: Proportion of time spent by female *Mogulones crucifer* in each of four quadrants of a four-armed olfactometer arena using volatile headspace of one test plant species in two quadrants (TP1 and TP2) and purified air in two quadrants (1 and 2). Differing letters on top of bars denote significant differences (n=20) (Categorical log linear model followed by single degree of freedom contrast analysis, $p<0.05$) (see text for details).


Initial choice of female (%)
Final choice of female (%)
100
80
60
40
20
0
a
b
C. officinale
1
A. grande
2
A. grandiflora
A. occidentale
B. officinalis


Initial choice of female (%)
Final choice of female (%)
100
80
60
40
20
0
a
b
C. officinale
1
D. daubenmirei
H. californica
H. micrantha
H. venusta
2

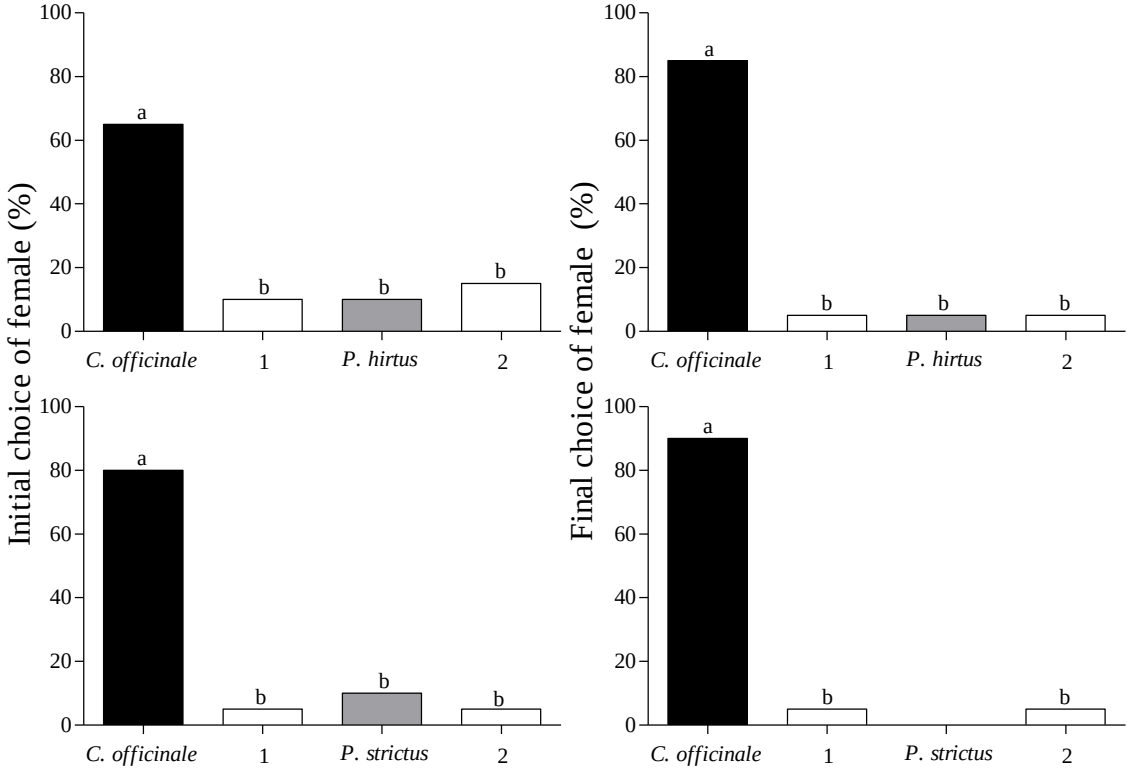
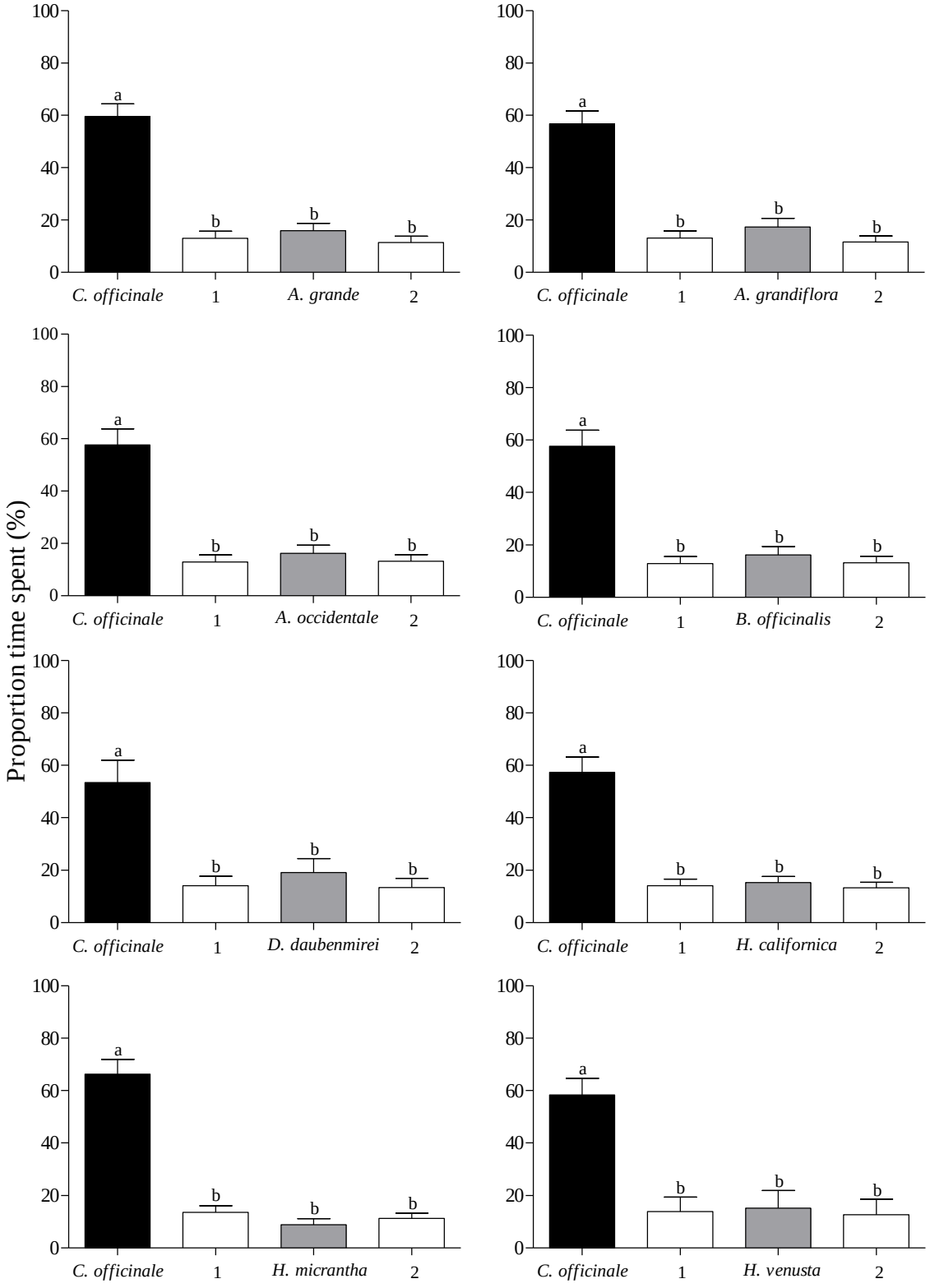


Figure 2.7: Proportion of female *Mogulones crucifer* initially (left graphs) and finally (right graphs) choosing one of four quadrants of a four-armed olfactometer arena using volatile headspace of *Cynoglossum officinale*, one test plant species and purified air in two quadrants (1 and 2). Differing letters on top of bars denote significant differences. Differing letters on top of bars denote significant differences (n=20) (χ^2-test followed by logistic regression analysis, $p<0.05$, ns=not significant) (see text for details).


Proportion time spent (%)
100
80
60
40
20
0
a
b
C. officinale
1
A. grande
2
A. grandiflora
A. occidentale
B. officinalis
D. daubenmirei
H. californica
H. micrantha
H. venusta

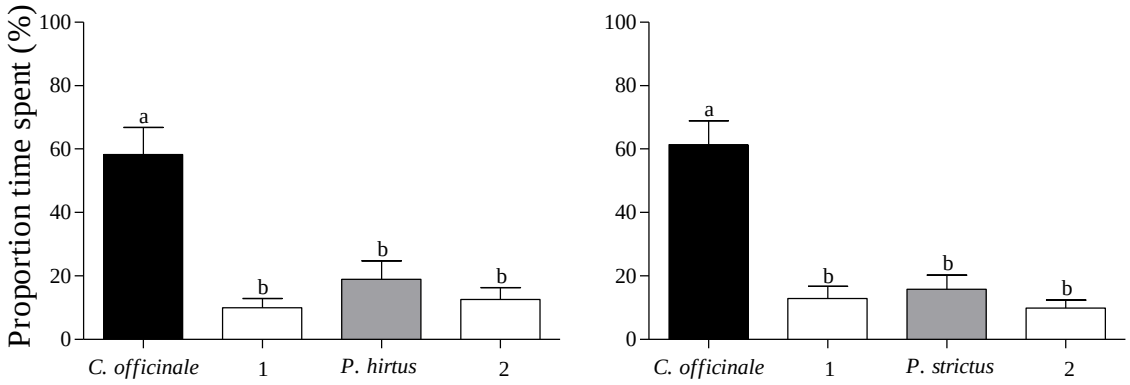


Figure 2.8: Proportion of time spent by female *Mogulones crucifer* in each of four quadrants of a four-armed olfactometer arena using volatile headspace of *Cynoglossum officinale*, one test plant species and purified air in two quadrants (1 and 2). Differing letters on top of bars denote significant differences (n=20) (Categorical log linear model followed by single degree of freedom contrast analysis, $p<0.05$) (see text for details).

Chapter 3

ELECTROPHYSIOLOGICAL RESPONSES OF THE ROOT-MINING WEEVIL *MOGULONES CRUCIFER* TO VOLATILES EMITTED BY ITS FIELD HOST *CYNOGLOSSUM OFFICINALE* EXPLAIN HOST PLANT DISCRIMATION

Abstract

Host-finding in herbivorous insects is partially mediated by plant primary and secondary metabolites that are emitted into the environment. In specialist insect herbivores including those used for classical weed biological control, adult insects perceive these olfactory plant cues to identify appropriate host plants for feeding and/or oviposition and discriminate against non-hosts. Behavioral bioassays with the weevil *Mogulones crucifer* Pallas (Coleoptera: Curculionidae), a biological control agent of the invasive plant *Cynoglossum officinale* L. (Boraginaceae), revelaed that females readily detected and preferred their field host, while responses towards volatiles from confamilial non-target plant species were either indifferent or weevils were repelled. To assess qualitative and quantitative variation in volatile blends emitted by target and non-target species, I collected and analyzed headspace volatile organic compounds (VOCs) from *M. crucifer*'s field host, *C. officinale,* and eight selected confamilial plant species using gas-chromatography-mass spectrometry (GC-MS). I found that among non-target plants *H. californica* shared greatest number of compounds (11) with *C. officinale* whereas *A. occidentale* shared least (7). Next, I conducted electrophysiological experiments using gas chromatography coupled with electroantennographic detection (GC-EAD), to determine volatile compounds in *C. officinale* that elicit a response from *M. crucifer* antennae. Of the 21 VOCs identified in *C. officinale*

headspace, six volatiles consistently and repeatedly triggered antennal responses in *M. crucifer*: methyl isovalerate, (z)-3-hexen-1-ol, benzaldehyde, 6-methyl-5-hepten-2-one, (z)-3-hexen-1-ol acetate, and (z)-β-ocimene. Of these, only methyl isovalerate, was specific to *C. officinale* and undetectable in any of the other tested plant species. Finally, I tested the behavioral response of *M. crucifer* females to methyl isovalerate in a four-armed olfactometer. I found that the compound was attractive to female *M. crucifer* when tested at ecologically relevant concentrations. The data suggest that the *C. officinale*-specific compound methyl isovalerate is an important olfactory cue during the host-selection and host discrimination of *M. crucifer*. The indifference of *M. crucifer* to confamilial plant species may in part be explained by the absence of this volatile in all tested non-target confamilials. To our knowledge, this is one of the first accounts of a unique host plant volatile contributing to host-fidelity of a biological weed control organism.

Introduction

Host-finding in herbivorous insects is partially mediated by plant volatiles synthesized as products of the plant metabolism and emitted into the environment (Becerra, 1997; Nishida, 2014; Pophof et al., 2005; Visser, 1986). Adult insects, through their specialized olfactory receptor neurons (ORNs), perceive these plant-emitted olfactory cues and utilize them to identify appropriate host plants for feeding and/or oviposition and discriminate against non-hosts (Anholt, 1992; Mustaparta, 2002; Visser, 1986). Specialist insect herbivores differentiate host plants from non-hosts based on the specific composition and ratio of a set of ubiquitous and unique chemical compounds emitted from host plants (Bruce et al., 2005; Smart & Blight, 1997; Visser, 1986). While the role of plant emitted volatile compounds in

host selection mediation of specialist insects has received considerable attention in theoretical ecology and arthropod pest management (Bernays & Chapman, 1994; Bruce & Pickett, 2011; Bruce et al., 2005; Hartlieb & Anderson, 1999; Visser, 1986), this is not the case in classical biological control (CBC) of invasive plants. In CBC, which deploys the release of specialist herbivores from the native range of an exotic invasive plant, few studies have identified or elucidated the role of plant volatiles in the host plant finding as part of host specificity assessments of prospective biological control candidates (Beck et al., 2014; Beck et al., 2008; Knolhoff & Heckel, 2014; Park et al., 2011; Piesik et al., 2015; Pophof et al., 2005; Rendon et al., 2014; Smith & Beck, 2013; Wheeler, 2014; Wheeler et al., 2014; Wheeler & Schaffner, 2013).

To better predict the ecological host range of prospective biological control organisms, which is defined as the range of plant species an insect chooses to attack under field conditions (Schaffner, 2001), traditional no-choice or choice feeding and developmental host specificity testing should be combined with studies assessing the behavioral responses of herbivorous insects to sensory cues during emitted by target and non-target species (Knolhoff & Heckel, 2014; Schaffner, 2001; Wheeler & Schaffner, 2013). Knowledge of key chemical compounds that mediate behavioral response during host selection may provide insight into the relative attractiveness, indifference or repellence of prospective biological control organism towards target and non-target plant species and, thus improve our understanding of the host fidelity of biological control candidates (Wheeler & Schaffner, 2013).

The root-mining weevil *Mogulones crucifer* Pallas (= *Ceutorhynchus cruciger* Herbst, *Mogulones cruciger* Herbst, Coleoptera: Curculionidae) was petitioned for release as a biological weed control agent in North America in 1996 and was subsequently released in

Canada in 1997 to control the Eurasian invasive rangeland weed *Cynoglossum officinale* L. (Boraginaceae). The weevil has since been successful in reducing or completely controlling *C. officinale* populations and dispersing to nearby populations in Canada (Clerck-Floate & Wikeem, 2009; De Clerck-Floate et al., 2005). In the United States, however, the release of the weevil was not recommended due to concerns by the United States Fish and Wildlife Service about potential non-target attack on a federally listed threatened and endangered (hereafter T&E) confamilial species, *Oreocarya crassipes* (I.M. Johnst.) Hasenstab & M.G. Simpson (USFWS, 1997). Pre-release and post-release host-specificity testing has shown that the weevil has a broad fundamental host range, defined as the range of plant species on which a herbivore can complete its life cycle (Schaffner, 2001) that spans across various genera and includes the native North American listed T&E species *Amsinckia grandiflora* (Kleeb. ex A. Gray) Kleeb. ex Greene, *Hackelia venusta* (Piper) H. St. John, and *Plagiobothrys hirtus* (Greene) I.M. Johnston (Andreas, 2004; De Clerck-Floate & Schwarzländer, 2002b; Jordan et al., 1993; Schwarzländer, 1996). In choice tests, *M. crucifer* typically and strongly preferred its co-evolved field host *C. officinale* (Andreas, 2004; De Clerck-Floate & Schwarzländer, 2002b; Jordan et al., 1993; Schwarzländer, 1996). After its release in Canada, the weevil sporadically attacked native confamilial non-target species including *Hackelia micrantha* (Eastw.) J.L. Gentry, *Lithospermum ruderale* Douglas ex Lehm., and *Oreocarya spiculifera* Piper (=*Cryptantha spiculifera* (Piper) Payson) at release sites in Canada (Andreas et al., 2008b; Catton et al., 2014; De Clerck-Floate & Schwarzländer, 2002b). However, *M. crucifer* attack rates on these plant species were variable among years and release sites, and were generally low (Andreas et al., 2008b; Catton et al., 2015). In a detailed post-release monitoring study using *H. micrantha*, which

sympatrically occurs with *C. officinale* in Canada, , it was shown that non-target herbivory was limited to spillover and that population level impacts on *H. micrantha* are consequently highly unlikely (Catton et al., 2014; 2015). In recent behavioral studies using plant volatiles as sensory cues in a multi-chambered olfactometer we found that female *M. crucifer* are able to distinguish *C. officinale* from ten tested non-target confamilial plant species (Andreas et al., 2008a, Kafle unpublished data). Females were either indifferent or repelled by volatiles emitted from non-target plant species (Kafle unpublished data). Volatile blends emitted by the different plant species in that study may quantitatively and/or qualitatively differ from each other and these differences are assumed to play a role in the behavioral response of *M. crucifer* (Bruce & Pickett, 2011).

Here, we investigate the mechanisms underlying the host plant identification and discrimination against confamilial non-targets demonstrated for *M. crucifer* based on plant volatile cues. For this, we characterized the volatile profiles of *C. officinale* and confamilial plant species used in our previous work. Also, we collected data on electrophysiological responses of *M. crucifer* to *C. officinale* volatiles in a series of laboratory trials. We identified electrophysiologically active volatiles and compared their presence among *C. officinale* and the confamilial non-targets. In addition, we studied the response of female *M. crucifer* to one electrophysiologically active volatile compound that is only found in *C. officinale* to test whether females can detect that compound and whether it triggers a behavioral response. The aim of this study is to provide physiological data that help explaining the strict host discrimination observed in *M. crucifer*.

Materials and Methods

Insects

In early spring 2015, adult *Mogulones crucifer* were collected at a *Cynoglossum officinale* infestation Bonners Ferry, Idaho (N 48.6913239°, W 116.3308525°) from plants that just began to form new foliage and then transported to the lab at the University of Idaho in Moscow, Idaho. In the laboratory the gender of weevils was determined by the presence of a ventral abdominal depression in males (Jordan et al., 1993). Weevils were maintained, separated by gender, in cylindrical plastic containers (11 cm diameter × 15 cm height), lined with paper towels and covered with muslin cloth in an environmental chamber (I-35VL, Percival Mfg. Co., Boone, Iowa) under 14: 10 (L:D) and 17°C day and 10°C night. Every second day, weevils were fed fresh *C. officinale* leaves.

Collection and analysis of plant volatiles

Plant volatiles organic compounds (VOCs hereafter) were collected in 2015 at the University of Idaho's Manis Entomological Laboratory's greenhouse in Moscow using a portable volatile collection system (Park 2016). Volatiles were collected from individual plants that were used in behavioral bioassays i.e., *Cynoglossum officinale* along with the following native Boraginaceae species; *Adelinia grande* (Douglas ex Lehm.) J. I. Cohen (=*C. grande* Douglas ex Lehm.), *Andersonglossum occidentale* (A. Gray) J. I. Cohen (=*C. occidentale* A. Gray), the single population species *Dasynotus daubenmirei* I.M. Johnston, and the federally listed threatened or endangered (hereafter, T&E) plant species, *Hackelia venusta* (Piper) H. St. John, *Plagiobothrys hirtus* (Greene) I.M. Johnston. In addition, we tested two congeners of *H.* venusta: *Hackelia californica* (A. Gray) I.M. Johnston, and

Hackelia micrantha (Eastw.) J.L. Gentry and a European sympatric confamilial of *C. officinale*: *Borago officinalis* L.

Polyvinyl acetate bags (20 cm×15 cm, Reynolds Consumer Products LLC., Richmond, Virginia), pre-sterilized through heating at 250°C for 1 hr., were placed over foliage of plants and gently sealed at the stem using cotton balls and plastic cable ties. Purified air using activated charcoal filter (Orbo™, Sulpelco, Sigma-Aldrich Co. LLC, St. Louis, Missouri) was introduced into the bag using a push pump (Rena® Air 400, Mars Fishcare North America, Inc., Chalfont, Pennsylvania) at the rate of 300 ml/min through a perforation (5 mm) made at the upper end of bag. A modified Rena® Air 400 pump, by switching the direction of diaphragm within pump assemblage, was used to draw air with foliar volatile out of the bag at the rate of 300ml/min and VOCs were collected into the volatile collection traps (VCT hereafter) containing 40 mg of 80-100 mesh Porapak-Q adsorbent (Southern Scientific Inc. Micanopy, Florida). Airflow inside the bags was maintained by using four pairs of flowmeters (King Instrument Company Inc., Garden Grove, California). Prior to use VCTs were rinsed with 1000 μL of dichloromethane (EMD Chemicals Inc., Gibbstown, New Jersey) to remove any contamination in the adsorbent. A collection time of 6 hrs (0900 h to 1500 h) was chosen based on the number of volatile peaks obtained from gas chromatography-mass spectrometry analysis of collected volatile samples of using various time spans (i.e., 3, 6, 9 and 12 hrs. respectively). Volatiles were collected from 3 plants along with 1 control (surrounding air) simultaneously. After each collection, the VOCs in the VCT were extracted by eluting with 200 μL of dichloromethane in a glass vial (National C5000-180, Thermo Fisher Scientific Inc., Rockwood, Tennessee) and stored in a freezer (-20°C) for further use.

Identification and analysis of headspace volatile organic compounds were conducted using a Hewlett-Packard 7890 Gas Chromatograph (Agilent Technologies Inc., Palo Alto, California) equipped with a fused silica HP-5MS capillary column (30cm×0.25mm×0.25μm, Agilent Technologies Inc., Palo Alto, California) and coupled with a Hewlett-Packard 5973 Mass Selective Detector (Agilent Technologies Inc., Palo Alto, California). Temperature of the injection port was set to 250°C. The initial oven temperature was set to 40°C and held at that temperature for 1 min and increased to 200°C at a rate of 5°C per min and then further increased to 300°C at a rate of 10°C per min and held isothermally for 2 min. Helium was used as the carrier gas at 3.0 mL/min. Volatile extract (1 μL) was injected into the Gas Chromatograph using a splitless mode. Mass spectra were obtained using electron impact (EI, 70 eV). Chromatograms were used to identify the chemical compounds present in volatile samples. The relative amount of identified component was determined based on peak area normalization of the total ion concentration (Puttick et al.). Only those compounds with a relative peak area of 1% or more of the total peak area in any one of the three samples were considered. Peaks detected in both, plant volatile samples and control samples were regarded as contamination and subtracted from the total peak area. Tentative identification of volatile compounds was done by comparing fragmentation patterns with the NIST library database (National Institute of Standards and Technology, Gaithersburg, Maryland). The retention indices (RIs) of identified compounds were calculated using a homologous series of n-alkanes on the HP-5MS column and compared with published retention indices. Furthermore, confirmation of compounds was made by comparing retention time, retention index and fragmentation pattern with authentic compounds whenever available.

Gas Chromatography – Flame Ionization Detector/ Electroantennographic Detection (GC-FID/EAD)

In insect chemical ecology, gas chromatography coupled with electroantennographic detection (GC-EAD) has proven to be a powerful technique for identifying compounds of biological significance from the complex mixture of various odors (Arn et al., 1975; Gouinguené et al., 2005; Weissbecker et al., 2004; Zhang et al., 2015). Headspace volatiles of *C. officinale* were subjected to coupled gas chromatography-flame ionization detector/electroantennogram detection (GC-FID/EAD) analysis with female *M. crucifer* to detect electrophysiologically active compounds in the volatile blend. Briefly, the system was based on a HP Agilent 6890 GC equipped with FID and coupled to an electroantennogram detector. For antennal preparation, female weevils were first decapitated and the antennal tips were cut off using sharp scalpel blades. The excised head was placed over a reference electrode, whereas the antennae were connected to the recording electrode. The antennal tips were partly submerged in Spectra® 360 electrode gel (Parker Laboratories Inc., Fairfield, New Jersey). The electrodes conduct signals generated by the antenna to a high-impedance input amplifier (IDAC-232, Syntech Ltd., Hilversum, The Netherlands) that feeds the signal to a graphical readout on a PC equipped with GC-EAD2000 software (Syntech Ltd., Hilversum, The Netherlands). Synchronous changes in voltage in both antennal and FID signals indicate olfactory sensitivity to the compound eluting at that particular retention time.

Volatile samples (1 μL) were injected splitless into an Agilent 6890N GC equipped with a capillary column (30cm×0.25mm×0.25um, Agilent Technologies Inc., Palo Alto, California). Initial oven temperature was set at 40°C for 1 min and increased to 200°C at the rate of 5°C per min and then further increased to 300°C at 10°C per min and held at that

temperature for 2 min. Helium gas was used as the carrier gas (3.0 mL/min). The effluent from the column was split into two parts, 50% were transferred to the FID and the other 50% to the EAD interface heated to 250°C using a temperature controller (Syntech Ltd., Hilversum, The Netherlands). The column of the EAD outlet was introduced into a 5 mm diameter glass tube with a constant stream of purified and humidified air (300 ml/min) generated with a stimulus controller (CS-05; Syntech Ltd., Hilversum, The Netherlands). Excised antennae mounted on electrodes were placed 5 mm away from the end of the glass tube.

GC-EAD recordings using *C. officinale* volatiles were performed with five different female antennal preparations. Compound peaks from the GC column were identified as electrophysiologically active if they elicited antennal responses in three or more of the antennae. The antennal responses to the compounds were selected and any contributions from potential impurities (as identified by GC-MS analysis of respective volatile samples) were discarded.

Behavioral bioassay with a *Cynoglossum officinale* specific volatile compound

Dual-choice behavioral bioassays were conducted in a four-armed olfactometer (Syntech Ltd., Hilversum, The Netherlands) to evaluate the behavioral response of female *M. crucifer* to the *C. officinale*-specific volatile compound methyl isovalerate. Each of the four inlet arms was connected via a Tygon® tube (8 mm internal diameter, Fischer Scientific Co., Pittsburgh, Pennsylvania) to volatile sources, i.e. 10 µL of 10ng/µL of methyl isovalerate dissolved in mineral oil (Paraffin oil, light, Thermo Fisher Scientific Inc., Fair Lawn, New Jersey) in two quadrants and remaining two quadrants were considered as control. Four Rena® Air 400 push

pumps were used to deliver air into the olfactometer. Prior to pushing air into the olfactometer, it was purified by passing through activated charcoal (Sigma-Aldrich Co. LLC, St. Louis, Missouri)-filled polyethylene tubes (17 cm length ×1.6 cm internal diameter, Scienceware™, Bel-Art Products, Wayne, New Jersey) and humidified by passing through distilled water in a 500 ml gas-washing bottle (Chemglass Life Sciences LLC, Vineland, New Jersey) to create uniform humidity. The airflow in each arm was maintained at 250 ml/min using four flowmeters (King Instrument Company, Inc., Garden Grove, California). In addition, air was drawn from the basal outlet at the rate of 1000 ml/min using a Rena® Air 400 pump that was modified to provide a pull by switching the direction of the pump diaphragm. A single light source (Jansjö® LED lamp, Inter Ikea System B.V., Delft, The Netherlands), was used to illuminate the olfactometer arena uniformly from above.

For each bioassay, an individual female *M. crucifer* was presented with four olfactory choices in the olfactometer chamber. Weevils were starved for 24 hrs prior to testing to enhance their responsiveness to treatments. At the beginning of each bioassay, the chamber outlet air hose was temporarily removed and an individual female *M. crucifer* was introduced into the olfactometer arena using a fine paintbrush. The hose was reconnected and the behavior of the weevil was observed and recorded for 30 min using a video camera (Contour Roam 2, Contour Inc., Seattle, Washington) fitted on top of the olfactometer arena. After every five bioassays, the odor sources were replaced and the olfactometer was rotated 90° to reduce positional effects. The central arena and all connecting tubing were washed with 70% ethyl alcohol and distilled water after testing each 10 *M. crucifer* females. Weevils were recorded as "unresponsive" if they did not make any choice after five min. of exposure and discarded from the experiment (<20%). A weevil was considered to have made a choice for

an odor when it entered into the respective quadrant and remained there for a minimum of 30 sec. The quadrant in which a weevil was located at the end of the 30min observation period was considered the final choice of that weevil. Bioassays were only carried out between 0900 h and 1600 h. The video recordings with movement and positioning of weevils were analyzed with the behavioral software program Noldus Observer XT 11 (Noldus Information Technology BV, Wageningen, The Netherlands).

The following parameters were measured during behavioral bioassays: The Initial Choice of a weevil, defined as the quadrant that a weevil chose first after it was introduced into the olfactometer and remained there for a minimum of 30 sec, was recorded to evaluate *M. crucifer*'s ability to discriminate different odors. The Final Choice was defined as the location of each weevil at the end of the experiment and was assumed to be its ultimate preferred odor source. The proportional time spent in each quadrant of the olfactometer arena was recorded and considered an indicator for the Strength of Preference for each odor.

Statistical Analysis

The relative concentration of each compound identified through GC-MS was based on peak area normalization of the total ion concentration. No quantitative analyses were performed with the volatile compounds.

The electrophysiologically active compounds, as identified by GC-FID/EAD, were subjected to principal component analysis (PCA) to differentiate volatile profiles of tested plant species based on the relative concentrations of the compounds (PROC PRINCOMP, SAS 9.4).

In behavioral bioassays, the choice data were discrete categorical responses. Hence, the proportion of Initial Choice and Final Choice of female *M. crucifer* in bioassays were initially assessed using χ-square tests of homogeneity. Logistic regression was used to model the odds of choice versus quadrants and assess pair-wise comparison among quadrants. The strength of preference for each choice was measured with the time (min) spent in each quadrant of the four-armed olfactometer. Differences among the four quadrants were assessed using a log-linear categorical model, while assuming the time to be discrete counts. Within this model, single degree of freedom contrasts allowed pair-wise comparison of the quadrants counts (times). For all analyses *p*-values of <0.05 were regarded as significant. All analyses were conducted using the statistical software SAS Version 9.4 (SAS Institute Inc., 2013).

Results

The volatile profile of *C. officinale* comprised 21 chemical compounds with terpenes and esters constituting the majority of emitted chemicals and seven compounds that were unique to the plant in our study, namely: methyl isovalerate, (z)-2-hexen-1-ol acetate, 2,4-hexadien-1-ol, 2,2,6-trimethyl-cyclohexanone, trans-β-ocimene, 1,5,5,6-tetramethyl-1,3-cyclohexadiene and β-sesquiphallendrene. The total number of identified peaks obtained from other plant species were, *A. grande* (18), *A. occidentale* (15), *B. officinalis* (16), *D. daubenmirei* (18), *H. californica* (22), *H. micrantha* (17), *H. venusta* (23), and *P. hirtus* (16), respectively. Only one compound, α-farnesene, was shared by all tested plant species. *Hackelia californica* shared the largest number of compounds (11) with *C. officinale*, whereas *A. grande* shared the least (7) (Table 3.1).

Six chemical compounds in the volatile headspace of *C. officinale*, i.e. methyl isovalerate, (z)-3-hexen-1-ol, benzaldehyde, 6-methyl-5-hepten-2-one, (z)-3-hexen-1-ol acetate, and (z)-β-ocimene elicited consistent antennal responses in female *M. crucifer* (Table 3.2).

The principle component analysis based on these electrophysiologically active compounds separated *C. officinale* from all other tested plant species indicating that the electrophysiologically active compounds blend in headspace volatiles of *C. officinale* is specific and different from other selected confamilial plant species tested (Fig 3.1). The first two principal components (PC1 and PC2) accounted for 67.60% of the variation (Fig. 3.1). The first principal component explains 36.34% of variability and separates *C. officinale* from *A. grande, A. occidentale, B. officinalis, D. daubenmirei, H. californica,* and *H. venusta.* The second principal component (PC2) explains 31.26% of variation and separates *C. officinale* from *A. grande, A. occidentale, H. californica, H. venusta,* and *P. hirtus*.

In behavioral bioassays with the *C. officinale*-specific compound methyl isovalerate, *M. crucifer* did not differentiate between methyl isovalerate quadrants and control quadrants for their initial choice (Table 3.3, Figure. 3.2), but they preferred methyl isovalerate over control quadrants for their final choice (Table 3.3, Figure. 3.2). Similarly, *M. crucifer* females spent more time in quadrants with methyl isovalerate than in control quadrants (Table 3.3, Figure. 3.3).

Discussion

Comparison of volatile profiles of C. officinale and confamilial non-target plants

The volatile blend emitted by *C. officinale* and confamilial plant species included alcohols, aldehydes, esters, ketones and mono- and sesquiterpenes. Within the tested non-target plant species, *Hackelia californica* shared the greatest number of compounds (11) with *C. officinale* whereas *A. occidentale* shared the least (7). Similarly, several of the tested confamilial plant species emitted compounds specific to respective plants that were absent in *C. officinale* volatiles. *Hackelia* species contained the greatest number of volatiles not found in *C. officinale*. Findings from behavioral analysis in which female *M. crucifer* were repelled by volatiles from three *Hackelia* species when offered with purified air as alternative choice (Kafle unpublished data), suggest that one or more of these *Hackelia* species-specific compounds may be responsible for the observed repellence but it was beyond the scope of this study to specifically test that assumption (Beck et al., 2008; Cao et al., 2015).

Identification of volatiles involved in olfactory host recognition.

In the GC-EAD experiment, *M. crucifer* females responded to six volatile compounds emitted by *C. officinale*, all of which are known to mediate olfactory host recognition in various specialist insects (El-Sayed, 2016; Knudsen et al., 2006). Among the electrophysiologically active compounds, the ester, methyl isovalerate was only found in *C. officinale* in our study. Methyl isovalerate has been extracted from plants of at least seven plant families (Baser et al., 1993; Brielmann et al., 1999; El-Sayed, 2016). To the best of our knowledge, this is a novel report of this ester compound for the Boraginaceae family. There are very few studies on the role of methyl isovalerate in insect plant interactions. In a study

comparing olfactory response of different members of genus *Drosophila* in evaluating the ecological shift in host preference of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) to different stages of fruit development in strawberry, methyl isovalerate was identified as a volatile associated with the fruit-ripening process in strawberry and *D. suzukii* has responded to the ester in electrophysiological experiments (Keesey et al., 2015). Similar to the attractiveness of methyl isovalerate to *M. crucifer* documented here, the related ester, ethyl isovalerate which is part of the plum volatile blend, was attractive to the plum curculio, *Conotrachelus nenuphar* (Herbst) (Coleoptera: Curculionidae) (Leskey et al., 2001). Generally, electrophysiological findings of insects towards chemical cues must be supported by behavioral bioassays to assess whether the compounds are truly used by herbivorous insects (Zhang et al., 2015) as the ability of herbivores to detect certain chemicals does not necessarily indicate its role or any role in behavioral ecology (Bruce et al., 2005; Hallem et al., 2006). The attraction of *M. crucifer* to methyl isovalerate in behavioral bioassays supports the inference that the weevil uses this specific compound during host-finding or recognition. Other insects have been shown to use specific compounds of their respective host plants to recognize them (Fraenkel, 1959; Szafranek et al., 2006). For example *Ceutorhynchus obstrictus* (Marsham) (syn. *C. assimilis* (Paykull)) (Coleoptera: Curculionidae) uses the host specific isothiocyanates (3-butenyl, 4-pentenyl, 2-phenylethyl) to detect their appropriate Brassicaceae host plants (Blight et al., 1995; Smart & Blight, 1997). An important role of methyl isovalerate in the host-finding of *M. crucifer* is also supported by the fact that female weevils were behaviorally indifferent to all confamilial plant species that lacked methyl isovaerate.

The antennal responses of female *M. crucifer* to compounds other than methyl isovalerate are also interesting in the context that these compounds are shared between the target and confamilial non-target plant species. The chemical similarity between plant species is considered an important factor mediating host range expansion of herbivores (Becerra, 1997; Becerra & Venable, 1999; Futuyma & McCafferty, 1990). The findings from our behavioral bioassays (Kafle unpublished data) and the presence of these compounds in more than one non-target plant species may indicate that any of these shared compounds alone does not act as a signature compound for host-finding.

The behavioral response of *M. crucifer* towards methyl isovalerate was lower than to the whole volatile profile of *C. officinale*. Although methyl isovlaerate seems to be a plausible determinant of host discrimination in *M. crucifer*, it is well known that the specific blend of ubiquitous chemical compounds in host plant volatiles is crucial in mediating host-plant recognition by specialist herbivores (Bruce & Pickett, 2011; Bruce et al., 2005; Cunningham, 2012; Visser, 1986). Several insect-plant interaction studies have proposed that specific mixtures of compounds in the volatile blend are more attractive than any individual compound (Birkett et al., 2004; Bruce et al., 2005; Natale et al., 2003). Even within a specific mixture of compounds in a blend, the physiological activity of insects can differ with the changes in concentrations of these compounds (Leskey et al., 2001; Najar-Rodriguez et al., 2010; Tamiru et al., 2015). For example, changing ratios of three behaviorally active compound (E)-β-caryophyllene, (E)-4,8-dimethyl- 1,3,7-nonatriene and (E)-β-farnesene from the host plant ratio to non-host plant ratios resulted in the disappearance of attraction in the grapevine moth *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae) (Tasin et al., 2006). In addition, the presence of all electrophysiologically active compounds might

be essential for optimal attraction in specialist insects. For example, removal of any of the individual compounds from the blend of seven electrophysiologically active compounds from *Vitis riparia* Michx. (Vitaceae) volatiles greatly reduced the attractiveness of the blend to *Paralobesia viteana* Clemens (Lepidoptera: Tortricidae)(Cha et al., 2008). As for other specialist herbivores, host discrimination in *M. crucifer* could be mediated by the specific ratio of electrophysiologically active compounds present in the volatile blend of *C. officinale* and the lack of behavioral attraction to the non-target plants could be further explained by differences in the ratios of the electrophysiologically active compounds in non-target plants (Najar-Rodriguez et al., 2010; Tasin et al., 2006). In future studies it will be important to test behavioral responses of the weevil to varying host and non-host concentrations of the remaining six shared active compounds. Methyl isovalerate is a known component of the floral VOC released from plants species of least seven families (El-Sayed, 2016) and may be present more widely in the environments in which *C. officinale* occurs. It is likely that the weevil relies on other cues from its host, in combination with methyl isovalerate to efficiently locate its host.

In conclusion, we demonstrated here that it is possible to identify electrophysiologically active compounds which are mediating the host-finding behavior of a specialist herbivore, in this case *M. crucifer,* using analytical techniques such as GC-MS and GC-EAD. We therefore propose that information on plant chemistry that determines the host utilization of prospective biological control organisms could valuably supplement the current approach for selecting test plants for pre-release host specificity testing (Schaffner, 2001; Wapshere, 1974; Wheeler & Schaffner, 2013). More importantly, incorporating behavioral bioassays and testing the electrophysiological and chemical basis of host plant selection in

pre-release host specificity testing would greatly improve the predictability of the ecological host range of biological control organisms (Wheeler & Schaffner, 2013). Since there is no host utilization without host-finding and/or recognition, we propose that such data should be prioritized in decision-making processes about the introduction of biological control organisms, particularly in those cases where the fundamental host-range of a candidate organism is broader than its ecological host-range in the native range (Hinz et al., 2014). While more data on the exact host discrimination of *M. crucifer* is needed, our data suggest that the absence of methyle isovalerate in the tested confamilial non-target plants diminishes the probability of non-target attack for any of those species, rendering them environmentally safe. To our knowledge, this is one of the first accounts attributing the host-fidelity of a biological weed control organism to an individual host plant-emitted volatile organic compound. This study is particularly relevant because *M. crucifer* is considered a risk to many native confamilial non-targets due to its broad fundamental host range (Andreas, 2004; De Clerck-Floate & Schwarzländer, 2002b; Jordan et al., 1993; Schwarzländer, 1996; USDA, 2010). In contrast, based on the data presented here and those by Catton et al. (2014), we propose that *M. crucifer* is more host-specific than previously assumed.

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Table 3.1: Relative Total Ion Concentration (TIC) peak area percentage of volatile organic compounds collected in the headspace of plant species. CO: *Cynoglossum officinale*, AG: *Adelinia grande*, AO: *Andersonglossum occidentale*, BO: *Borago officinalis*, DD: *Dasynotus daubenmirei*, HC: *Hackelia californica*, HM: *H. micrantha*, HV: *H. venusta*, PH: *Plagiobothrys hirtus*. Tentative identification of compounds is based on comparison of their mass-spectra with data in the NIST library (National Institute of Standards and Technology) and comparing calculated retention indices.

	Retention Time (min)	CO	AG	AO	BO	DD	HC	HM	HV	PH
methyl isovalerate	3.33	4.24	-	-	-	-	-	-	-	-
(z)-3-hexen-1-ol	4.75	1.92	4.77	-	3.39	1.16	3.83	2.52	1.85	-
heptanal	5.81	-	-	-	-	-	-	-	1.67	-
(z)-3-hexen-1-ol formate	6.26	-	-	-	-	-	-	-	1.22	-
benzaldehyde	7.25	2.77	1.73	2.38	2.13	0.82	-	5.41	3.3	17.5
β-phellandrene	7.6	-	1.94	-	-	6.61	-	-	6.88	1.34
1-octen-3-ol	7.82	-	-	3.66	-	-	0.47	-	-	-
6-methyl-5-heptene-2-one	8.05	1.66	-	-	-	-	0.84	-	1.05	1.10
β-myrcene	8.15	-	-	-	-	4.10	-	0.72	1.74	0.99
2-octanone	8.18	-	-	-	1.67	-	-	-	-	-
octanal	8.47	-	-	-		-	1.26	-	-	-
(z)-3-hexen-1-ol acetate	8.61	33.61	4.42	4.54	14.15	16.95	23.5	22.41	-	0.94
hexyl ester acetic acid	8.8	2.11	-	-	5.59	1.09	0.72	2.08	-	1.28
(z)-2-hexen-1-ol acetate	8.87	1.64	-	-	-	-	-	-	-	-
d-limonene	9.13	-	3.79	-	-	3.95	1.64	-	7.52	0.82
2,4-hexadien-1-ol	9.2	0.66		-	-	-	-	-	-	-
3-cyclohexen-1-ol acetate	9.21	-		-	1.42	-	-	-	-	-
eucalyptol	9.22	-	22.18	-	-	-	-	-	20.7	-
2,2,6-trimethyl-cyclohexanone	9.31	0.85		-	-	-	-	-	-	-

benzyl alcohol	9.32	-	1.29	1.65	-	-	0.97	1.07	3.18	32.34
trans-β-ocimene	9.44	1.08	-	-	-	-	-	-	-	-
indene	9.53	-	-	4.44	-	-	-	-	-	-
benzeneacetaldehyde	9.58	-	-	-	-	-	1.78	-	-	-
(z)-β-ocimene	9.74	20.03	-	16.75	5.85	9.14	22.51	20.98	10.09	27.92
1,2-cyclohexanediol	10.10	-	-	-	3.83	-	-			-
acetophenone	10.2	-	1.96	3.62	3.02	-	-	1.72		-
2,4-undecadien-1-ol	10.43	-	-	-	-	-	-	-	1.06	-
benzyl formate	10.57	-	-	-	-	-	-	-	1.26	0.89
linalool	11.22	3.6	4.69	3.94	-	5.22	7.37	8.8	3.27	-
nonanal	11.35	1.45	3.2	1.46	2.62	0.97	4.94	-	5.72	-
hexyl propanoate	11.44	-	-	-	-	-	-	-	-	0.51
phenylethyl alcohol	11.57	-	-	-	-	-	0.98	-	-	-
2-ethenyl-1,1-dimethyl-3-methylene cyclohexane	11.7	-	-	-	-	-	-	-	4.92	-
1,5,5,6-tetramethyl-1,3-cyclohexadiene	12.03	1.81	-	-	-	-	-	-	-	-
phenylmethyl ester acetic acid	13.07	-	-	-	-	-	-	-	-	0.89
cinamaldehyde	13.49	-	-	-	20.16	-	-	-	-	-
l-α-Terpineol	13.78	-	1.04	2.16	-	7.14	0.79	1.51	1.98	1.18
methyl salicylate	13.86	3.25	2.09	5.55	-	6.09	4.44	2.77	-	8.03
decanal	14.44	1.37	2.83	-	-	7.65	1.10	-	2.21	-
1,4-benzenedicarboxaldehyde	14.79	3.43	2.17	-	10.66	-	-	4.27	-	-
isopathaldehyde	14.98	-	2.67	-	-	-	-	5.48	-	-
p-cymen-7-ol	15.32	-	-	-	7.87	-	-	1.6	-	1.49
1-indanone	16.13	-	-	10.30	-	-	-	-	-	-
[1,1'-bicyclopentyl]-2-one	16.29	7.31	10.21	-	-	-	2.49	-	-	-
α-cubebene	18.06	-	-	-	-	1.07	-	-	-	-
4-methylphthalaldehyde	18.45	-	-	-	10.17	-	-	-	-	-
.alfa.-copaene	18.75	-	-	-	-	2.25	-	-	-	-
β-cubebene	19.12	-	-	-	-	6.58	-	-	-	-

dodecanal	19.63	-	-	-	-	-	-	-	2.36	-
caryophyllene	19.88	-	-	27.85	1.74	3.97	-	2.38	0.55	-
trans-β-bergamotene	20.27	-	-	-	-	-	2.18	-	4.24	-
β-sesquiphellandrene	20.45	1.83	-	-	-	-	-	-		-
cis-β-farnesene	21.5	1.82	-	-	-	-	0.77	-	2.94	-
α-farnesene	22.08	3.45	27.22	5.47	5.66	14.41	15.2	16.54	10.49	0.46
β-cedrene	22.43	-	-	-	-	-	1.33	-	-	-
(z)-3-bexen-1-ol benzoate	23.5	-	1.71	1.60	-	-	0.80	-	-	-
dendrolasin	23.75	-	-	-	-	-	-	1.04	-	-
Number of volatile compounds (Shared compounds with *C. officinale*)		21	18 (10)	15 (7)	16 (8)	18 (10)	22 (11)	17 (9)	23 (9)	16 (8)

Table 3.2: Relative Total Ion Concentration (TIC) peak area percentage of electrophysiologically active compounds in *C. officinale* and selected confamilial Boraginaceae species. Retention indices (RI) were calculated using a homologous series of n-alkanes on the HP-5MS column. Compound identity was confirmed by comparing mass spectra and retention time using authentic standard except for (z)-β-ocimene, which was tentatively identified by comparison with published calculated retention indices and mass spectral data in the NIST database.

	Compounds					
	methyl isovalerate	**(z)-3-hexen-1-ol**	**benzaldehyde**	**6-methyl-5-hepten-2 one**	**(z)-3-hexen-1-ol acetate**	**(z)-β-ocimene**
Retention time (min)	3.34	4.75	7.25	8.05	8.61	9.74
Calculated RI	849	886	956	979	996	1031
C. officinale	4.24	1.92	2.77	1.66	33.61	20.03
A. grande	-	4.77	1.73	-	4.42	-
A. occidentale	-	-	2.38	-	4.54	16.75
B. officinalis	-	3.39	2.13	-	14.15	5.85
D. daubenmirei	-	1.16	0.82	-	16.95	9.14
H. californica	-	3.83	-	0.84	23.50	22.51
H. micrantha	-	2.52	5.41	-	22.41	20.98
H. venusta	-	1.85	3.30	1.05	-	10.09
P. hirtus	-	-	17.5	1.10	0.94	27.92

Table 3.3: Summary statistics for behavioral responses of female *M. crucifer* females in dual-choice bioassays using the *C. officinale*-specific compound methyl isovalerate (See Figs. 3.1 - 3.3 and text for details, MI-1 and MI-2 = methyl isovalerate, C-1 and C-2 = control, n=20).

	MI-1	**MI-2**	**C-1**	**C-2**	χ^2	**p-value**
Initial choice	5	8	4	3	2.8	0.4235
Final choice	6	10	2	2	8.8	0.0321
Percent time spent on quadrants of olfactometer	26.42	36.38	19.88	17.32	50.46	<0.0001

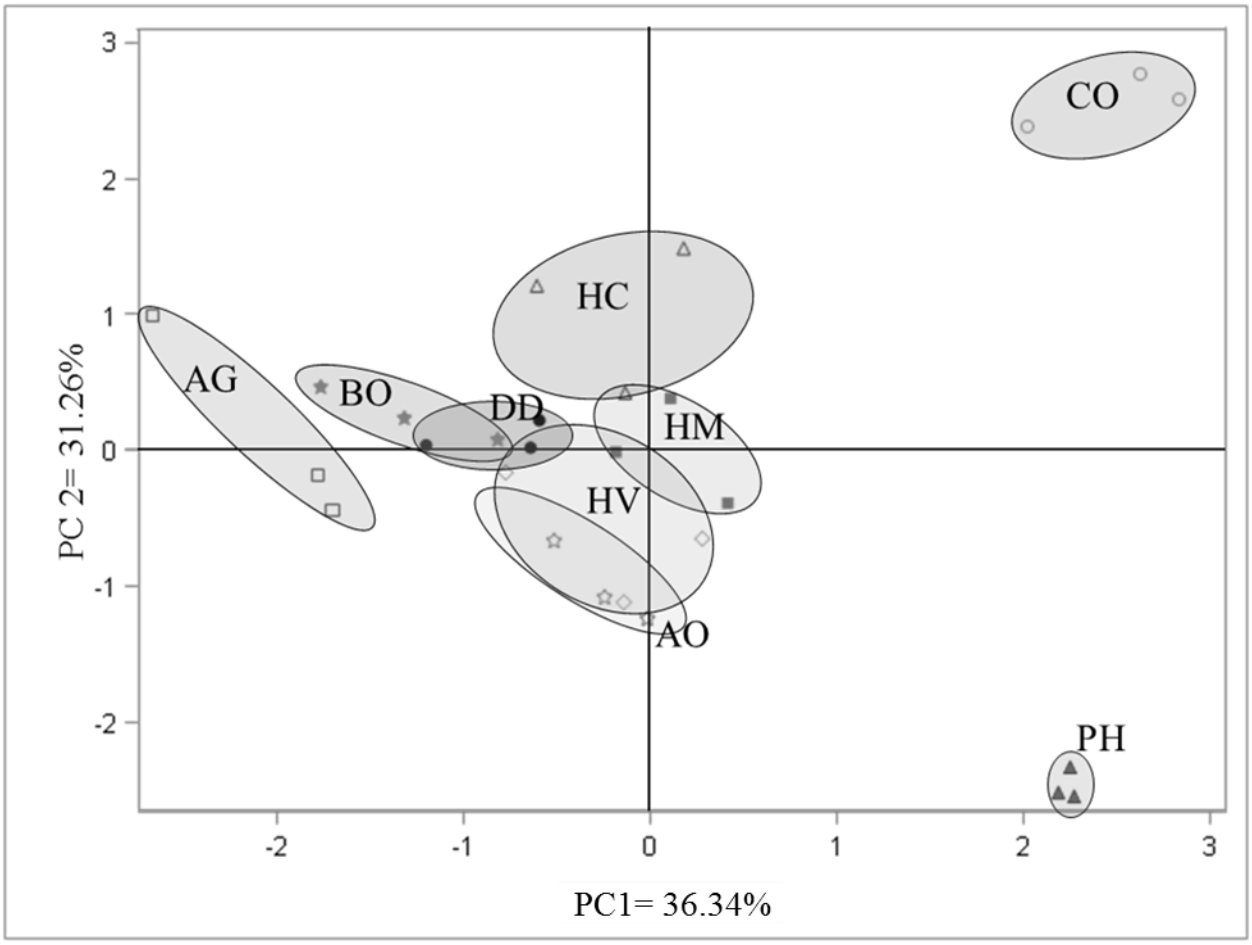

Figure 3.1: Principal component analysis score plot based on relative TIC peak area percentage electrophysiologically active volatile organic compounds identified in *C. officinale*. Plant species, CO: *Cynoglossum officinale*, AG: *Adelinia grande*, AO: *Andersonglossum occidentale*, BO: *Borago officinalis*, DD: *Dasynotus daubenmirei*, HC: *Hackelia californica*, HM: *H. micrantha*, HV: *H. venusta*, PH: *Plagiobothrys hirtus*.

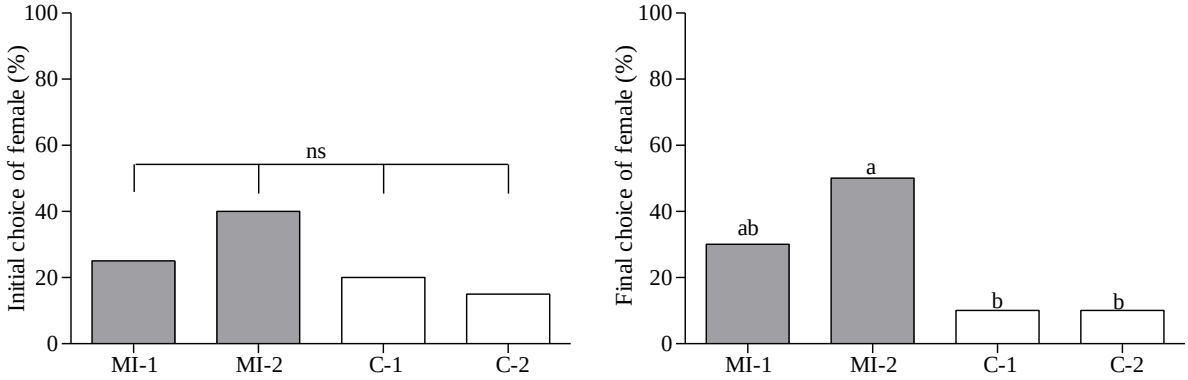

Figure 3.2: Proportion of female *Mogulones crucifer* initially (left graphs) and finally (right graphs) choosing one of four quadrants in a four-armed olfactometer arena using methyl isovalerate (10 ng/µL) in two quadrants (MI-1 and MI-2) and remaining two quadrants as control (C-1 and C-2). Differing letters on top of bars denote significant differences (χ^2-test followed by logistic regression analysis, $p<0.05$, ns=not significant) (see text for details).

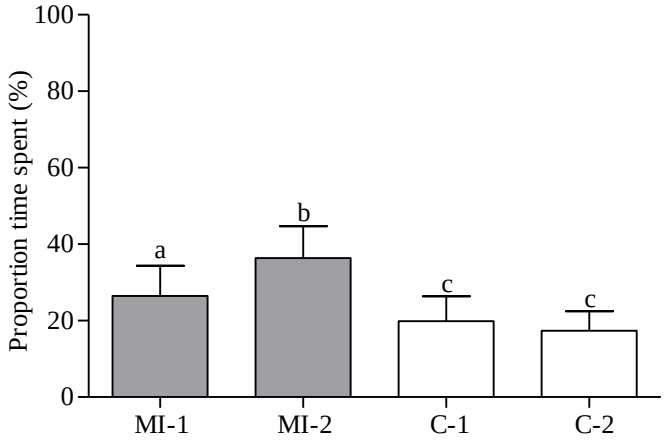

Figure 3.3: Proportion of time spent by female *Mogulones crucifer* in each of four quadrants of a round four-armed olfactometer arena using methyl isovalerate (10 ng/µL) in two quadrants (MI-1 and MI-2) and remaining two quadrants as control (C-1 and C-2). Differing letters on top of bars denote significant differences (Categorical log linear model followed by single degree of freedom contrast analysis, $p<0.05$) (see text for details)