THE ROLE OF OLFACTORY CUES IN THE HOST SELECTION BEHAVIOR OF A SPECIALIST WEEVIL USED FOR THE BIOLOGICAL CONTROL OF AN INVASIVE PLANT

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Authorization to Submit Thesis

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Abstract

Mogulones crucifer Pallas (Coleoptera: Curculionidae), root miner, was released in Canada in 1997 for the biological control of the rangeland weed *Cynoglossum officinale* L. (Boraginaceae). Release of this weevil in the United States was not permitted due to concerns on potential confamilial nontarget attack on federally listed threatened and endangered (T&E) plant species. For environmental safety assessment of this weevil to native Boraginaceae plant species: 1) Assessed the behavioral responses of *M. crucifer* to olfactory cues of *C. officinale* and selected four Eurasian and three Asian confamilial plants and electrophysiological experiments (GC-EAD) to assess antennal responses of *M. crucifer* and characterized the headspace volatile profiles of tested plant species (GC/MS) 2) Assessed the behavioral responses of *M. crucifer* to olfactory cues of *C. officinale* and selected five North American confamilial plants and electrophysiological experiments (GC-EAD) to assess antennal responses of weevil and characterized the headspace volatile profiles of tested plant species (GC/MS). *M. crucifer* strongly preferred its field host *C. officinale* over all tested nontarget plants. Among nontarget plants, *Hackelia micrantha* (Eastw.) J.L. Gentry shared the greatest number of compounds (10) with *C. officinale* whereas *Oreocarya rugulosa* Payson, *Oreocarya celosioides* (Eastw.) Payson and *Rindera umbellata* (Waldst. & Kit.) Bunge shared least (3). In addition, I identified six electrophysiologically active compounds in *C. officinale*, five electrophysiologically active compounds in *H. micrantha* and four electrophysiologically active compounds in *O. crassipes* (I.M. Johnst.) Hasenstab & M.G. Simpson. Results from these experiments suggest *M. crucifer* can locate its host plant and avoid nontarget plant species.

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Dedication

To my family

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Abstract

The root mining weevil *Mogulones crucifer* Pallas was released as a biocontrol agent for the control of the rangeland weed *Cynoglossum officinale* L. in Canada in 1997. Since then, the weevil has successfully suppressed the weed across southwestern Canada. Although not been permitted for release in the United States over concerns about potential nontarget attack on federally listed threatened and endangered (T&E) plant species in the Boraginaceae family, the weevil has immigrated into the United States and is now present in Idaho, Montana and Washington State. To investigate whether these concerns about the host-specificity of *M. crucifer* are merited, I examined the behavioral responses of female weevils to olfactory cues of *C. officinale* and select very closely related Eurasian and Asian confamilial plant species. I conducted dual-choice and multiple-choice behavioral bioassays with foliar volatiles of *C. officinale*, confamilial species and purified air (as control) in a four-armed olfactometer. In bioassays weevils always preferred *C. officinale* when available and it was the only plant species to which *M. crucifer* was attracted. Weevils discriminated against all closely related confamilial species and responded with indifference and/or repellence to their foliar volatiles. I then analyzed foliar headspace volatile organic compounds collected from *C. officinale* and seven confamilial plant species using gas-chromatography-mass spectrometry (GC-MS). I found that the European congener of *C. officnale*, *C. germanicum* (Jacq) shared the most volatiles with *C. officinale* (7) whereas the European *Rindera umbellata* (Waldst. & Kit.) shared least (3). Next, I performed gas chromatography coupled with electroantennographic

detection (GC-EAD) experiments with antennae of *M. crucifer* females using foliar volatiles of *C. officinale* and *Cynoglossum creticum* (Mill.). The antennae repeatedly elicited electrophysiological responses to six bioactive volatile compounds out of 25 volatiles identified in *C. officinale*. These are methyl isovalerate, (z)-3-hexen-1-ol, benzaldehyde, 6-methyl-5-hepten-2-one, (z)-3-hexen-1-ol acetate and (z)-β-ocimene. The antennae did not respond to any volatiles when tested with *C. creticum*. A principal component analysis based on these six bioactive volatiles separated the foliar odor from that of the seven confamilial plant species tested. The data presented here suggest that *M. crucifer* is much more host-specific than previously assumed based on its fundamental host range that includes most of the confamilials tested here. In fact, the host plant discrimination demonstrated here may in part explain how the weevil maintains its near-monophagous status on *C. officinale* in its native range.

Introduction

Non-native species when introduced into new areas can cause threats to agricultural ecosystems and natural habitats (Keane and Crawley 2002; Pimentel et al. 2005) and potentially reduce biodiversity (Mack et al. 2000; Vilà et al. 2011). Conventional control methods for invasive plants including chemical control (herbicides) or mechanical and cultural control strategies (tilling, mowing, hand pulling, etc.) are widely used but they can be costly depending on the scale of the invasions or their accessibility in remote areas (Culliney 2005; DiTomaso 2000; Kelton and Price 2011; Sheley et al. 2011). Classical weed biological control is an alternative approach for the management of invasive plants and has been used successfully for more than a century (McFadyen 1998; Müller-Schärer and Schaffner 2008; Schwarzländer et al. 2018a, 2018b; Winston et al. 2014).

Classical biological control of weeds (hereafter weed biocontrol) is defined as the control of exotic invasive plant populations through the importation of host-specific natural enemies (typically insect herbivores, mites and pathogens) from the native range of the plant with the aim of reducing invasive plant populations in their introduced range (Harris 1991; Müller-Schärer and Schaffner 2008; Van Driesche et al. 2010). The goal of biocontrol is to reestablish the specialist herbivore-host plant relationship in the range where the plant is invasive, controlling or at least weakening the competiveness of the invasive plant to levels below ecological and/or economic thresholds (Bellows 2001; Gurr et al. 2000; Hoddle 2004; Keane and Crawley 2002; Müller-Schärer and Schaffner 2008).

Despite these successes, there are potential problems with weed biocontrol implementation. Incidences of attack by deliberately introduced biocontrol agents on nontarget plant species that are confamilial with the target, in some cases severe, have been reported during the last 22 years (Louda et al. 1997; Louda et al. 2005; Strong 1997; Taylor et al. 2007). Two examples are the intentionally introduced seed-feeding weevil *Rhinocyllus conicus* (Froel.) (Coleoptera: Curculionidae) attacking native thistle species in the genus *Cirsium* in North America (Louda 2000; Louda and Arnett 2000) and nontarget effects on native *Opuntia* species caused by *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae), which was accidentally introduced from the Carribean to Florida (Johnson and Stiling 1998; Solis et al. 2004). Because of these and other examples of nontarget attack, weed biocontrol has been scrutinized in the United States (Fowler et al. 2012; Hinz et al. 2014; Simberloff 2012). Even small levels of nontarget attack from released biocontrol agents on plant species with which the biocontrol agent has no shared evolutionary history may raise concern over the ultimate scale of that nontarget effect on the respective plant species (Van Klinken and Edwards

2002). These concerns include an agent's ability for host range expansion, where the biocontrol agent uses a nontarget species as host plant in the introduced range, which is suitable to support complete development of the agent, and host-shifts, where a biocontrol agent adapts to utilize a nontarget species as a host plant that previoulsy was not usable (van Klinken and Edwards 2002). There are to our knowldege no known examples for host-shifts in weed biological control (van Klinken and Edwards 2002). Other forms of nontarget effects include temporary use of nontarget in the field under conditions of high agent density (collateral which is nontarget feeding in close proximity to target plants which are not related to target plants following the outbreaks of released biocontrol agents and spillover which is the nontarget feeding, growing in close proximity to target plant affecting comfamilial nontargtes occur at high biocontrol agent densities) (Hinz et al. 2019). It is important to note that indirect nontarget effects for example in the form of enrichment (in which the introduction of the agent enriches the diet of a native predator which impacts nontarget prey) (Holt and Hochberg 2001) are currently not tested in weed biocontrol (Pearson and Callaway 2005). For example the gall flies, *Urophora affinis* (Frauenfeld) and *U. quadrifaciata* (Meigen), both Diptera: Tephritidae was introduce to control spotted knapweed in North which have increased in number and have been exploited by many native consumers (Harris 1980; Muller-Shärer and Schroeder, 1993; Story et al. 1995).

In recent worlwide reviews of nontarget attack in classical biological weed control authors concluded that less than one percent of deliberately released biocontrol agents had serious negative effects on nontarget species (Hinz et al. 2019; Suckling and Sforza 2014). Post-release nontarget attack is most often occurring on nontarget species closely related to the weed that were not included in host-specificity testing or the nontarget cases were predictable

but ignored during release decisions for socio-political reasons prior to the 1970s (Hinz et al. 2019). Before prospective biocontrol agents can be released against invasive plants, host-specificity tests are conducted using confamilials of the target weeds from the native and introduced ranges in order to make predictions on the risk of potential nontarget attack (Schaffner 2001; Smith and Beck 2013, Wapshere 1974). Rare and endangered plant species as well as economically important species and crops within the family of the target weed are also added to the test plant list. Based on the centrifugal phylogenetic approach (Wapshere 1974), closely to distantly related confamilial plant species of the target weed are tested until the host range breadth of the biological control candidate is adequately well-defined (Schaffner 2001, Wapshere 1974). Host-specificity tests include no-choice and choice tests under varying environmental conditions, ranging from laboratory to open-field experiments, to determine the candidate agent's fundamental and ecological host range (Clement and Cristofaro 1995; Cullen 1990; Heard 2002; Schaffner 2001; Sheppard et al. 2005). The fundamental host range is defined as the range of plants physiologically suitable for the development of the biological candidate whereas the ecological host range is the range of plant species accepted by the agent under more natural conditions in choice tests (Schaffner 2001; Schaffner et al. 2018; Van Klinken and Heard 2000). Currently the terms "ecological" and "realized" host range are generally used interchangeably, though some practioners have distinguished and defined the ecological host range as the range of plant species which are utilized under natural field or field cage conditions determined pre-release from the realized host range, the range of plant species utilized by herbivore after release (Hinz et al. 2019). While release decisions relying solely on fundamental host range data are very safe, they may overestimate potential nontarget attack risks and limit the potential number of biocontrol

agents available for release (Blossey et al. 2001; Hinz et al. 2014; Pratt et al. 2009; Schaffner 2001). Introduction decisions based solely on fundamental host range data bear the risk that candidate agents may be rejected although they are environmentally safe (Hinz et al. 2014; Marohasy 1998; Wapshere 1989). Assessments of the ecological host range include experimental designs that use more natural field cage or open-field conditions allowing insect herbivores to express, at least to a certain degree, their host choice behaviors (Heard 2002). The ecological host range of a candidate agent depends upon its pre-alightment responses to plant cues before feeding and oviposition can occur (Knolhoff and Heckel 2014; Schaffner 2001; Wheeler and Schaffner 2013). Combined, pre-release assessments of the fundamental and ecological host range are considered an adequate predictor of the post-release realized host range of the biocontrol agent, i.e., the range of plant species actually used after its release in the area of introduction (Hinz et al. 2014; Hinz et al. 2019; Paynter et al. 2004; Schaffner 2001; Schwarzlander et al. 2018).

During host selection, herbivorous insects pass through a sequence of stages during which they respond to visual, olfactory and other sensory plant cues alone or in combination before they accept a suitable host (Bernays and Chapman 1994; Schoonhoven et al. 2005). In the pre-alightment phase, visual and olfcatory cues are used to identify and locate potential hosts. Olfactory receptor neurons (ORNs) that are specialized in adult insects perceive airborne plant volatiles as olfactory cues and use these cues to distinguish host plants from non-hosts (Anholt 1992; Farkas and Shorey 1972; Mustaparta 2002; Visser 1986). Pre-alightment host selection can either depend on specific ORNs that detect species-specific volatiles, or combinations of ORNs that detect species-specific ratios of volatile blends (Bruce et al. 2005; Smart and Blight 1997; Visser 1986).

Recently, assessments of behavioral responses of biocontrol candidate species to plant cues have been added to weed biocontrol systems in addition to traditional choice and no-choice feeding and development tests as it has been long suggested that such data could potentially explain discrepancies between fundamental and ecological host ranges (Heard 2000; Hinz et al. 2014; Kafle 2016; Park et al. 2018; Park et al. 2019). This is important because behavioral and underlying physiological data explaining the relative attractiveness of target and nontarget plant species could further improve pre-release host range predictions of a biological control candidate and, thus improve environmental safety decisions (Hinz et al. 2019; Schaffner 2001).

At least theoretically, true host-shifts could occur post-release over evolutionary time and biocontrol agents may use nontarget species in the area of introduction that were not formerly part of their fundamental host range (Williamson 1992). The occurrence of rapid evolution has recently been demonstrated in a few biological control systems (McEvoy et al. 2012; Szűcs et al. 2011)**.** For example, the cinnabar moth *Tyria jacobaeae* L. (Lepidoptera: Arctiidae), a biocontrol agent for *Jacobaea vulgaris* Gaertn. (syn. *Senecio jacobaeae* L.) had shorter development times in mountainous regions that correspond to shorter growing seasons than to warmer valley climates (McEvoy et al. 2012). In another study with the flea beetle *Longitarsus jacobaeae* Waterhouse (Coleoptera: Chrysomelidae), also a biocontrol agent of *J. vulgaris*, it was demonstrated that the beetle phenology adapted over a short amount of time to different climatic conditions (Szűcs et al. 2011). Post-release host ranges could theoretically narrow or expand depending on genetic and phenotypic changes in biocontrol agent populations over time (Bernays and Graham 1988; Dennill et al. 1993; Futuyma 1991; Wasserman and Futuyma 1981). One potential driver of changes in host-

specificity could be the avilability of the invasive host plant or changes in the availability of related suboptimal alternative nontarget host plants (van Klinken and Edwards 2002). In addition to similar phylogenies of target and nontarget plant may lead to insect feeding on closely related plants species (Futuyma 1991; Jaenike 1990; Janz and Nylin 1998; Thompson 2005), changes in behavioral responses or their underlying physiological traits toward plant cues may also lead to evolution of specialization (Futuyma 1983). In the past, it has been argued that host-specificity is largely a function of the fundamental host range of an insect herbivore and therefore changes in the insects feeding and ovipositional behavior could drive the evolution of host-specificity (van Klinken and Edwards 2002).

Earlier behavioral and sensory studies with the root feeding weevil *M. crucifer* Pallas and its near-monophagous sibling species, the seed-feeding weevil *M. borraginis* (Fabricius) (both Coleoptera: Curculionidae), potential and/or released biocontrol agents for *Cynoglossum officinale* L., a Eurasian herbaceous plant that is an invasive rangeland weed in western North America, was done. It was found that both weevil species strongly preferred volatiles and visual cues from *C. officinale* over those from several Eurasian and North American confamilial nontarget plant species (Kafle 2016; Park et al. 2018; Park et al. 2019). Neither weevil species was attracted to any nontarget species and reacted with indifference (plant volatiles were not preferred over purified air control) or even repelled (purified air was preferred over plant volatiles) (Kafle 2016; Park et al. 2018; Park et al. 2019) to plant cues of North American and Eurasian nontarget species. Previous study assessed the nontarget attack risks of *M. crucifer* to native nine North American and one Eurasian confamilials using olfactory cues involved in host finding behavior (Kafle 2016). They found that *M. crucifer* did not differentiate between a Eurasian plant volatile and purified air, raising the question of

how *M. crucifer* distinguishes between its host and non-host in its native range. Here, I aim to study the responses of *M. crucifer* to olfactory plant cues of closely related Eurasian and Asian congeners and confamilials and representatives of genera in the target's subtribe, Cynoglossinae (*Cynoglossum* s.l.) (Chacon et al. 2016). I was specifically interested in whether degree of relatedness of Eurasian confamilials was associated with indifference and repellence by the weevil, and whether the responses could be explained by the volatiles emitted by these plant species. Specifically, this research aims to address host specificity maintenance of *M. crucifer* in its native range.

Materials and methods

Study system

Cynoglossum officinale L. or houndstongue is a monocarpic, biennial to short-lived perennial herbaceous plant in the Boraginaceae family (De Jong et al. 1990; Upadhyaya and Cranston 1991; Upadhyaya et al. 1988). Plants germinate from overwintered vernalized seeds in early spring and produce rosettes during their first year. They overwinter as hemicryptophytes, i.e., their aboveground plant parts die back following the first frosts and carbohydrates are stored in the tap roots (Boorman and Fuller 1984; De Jong et al. 1990; Upadhyaya et al. 1988). In the subsequent spring, plants typically bolt and flower (De Jong et al. 1990). Reddish-purplish flowers develop and each flower produces up to four tear-drop-shaped, single-seeded, burred nutlets (Klinkhamer and de Jong 1993). In mid-summer, plants usually die after seed set (De Jong et al. 1990). Seed production is correlated with rosette size at the end of the growing season of the previous year (De Jong and Klinkhamer 1988). Dispersal of seeds is exclusively via epizoochory of the barbed and hooked dried nutlets that easily attach to fur, wool, or hair of passing animals (De Clerck-Floate and Schwarzländer 2002).

Cynoglossum officinale is native to Eurasia and widely distributed throughout Europe, although it is becoming increasingly rare to the point that the species may soon be threatened in many central European countries (De Jong et al. 1990; Enßlin et al. 2011). The plant was first accidentally introduced into eastern North America in the late 1800s (Macoun 1884) and is now particularly problematic in the northwestern United States and southwestern Canada (Upadhyaya et al. 1988; USDA NRCS 2019). In the United States, the plant is declared noxious in seven western and northwestern states (i.e. Idaho, Montana, Colorado, Nevada, Oregon, Wyoming and Washington) (USDA NRCS 2019). *Cynoglossum officinale* invades open woodlands, rangelands, roadsides, sand dunes and other disturbed habitats (De Clerck-Floate 1997; Klinkhamer and de Jong 1988; Upadhyaya and Cranston 1991), and can impair forage production in these agroecosystems (De Clerck-Floate and Schwarzländer 2002). In addition, all plant parts but especially young leaves of *C. officinale* contain large quantities (1.41-4.30 mg per gram of leaves) of pyrrolizidine alkaloids (PAs) (van Dam et al. 1995), which are toxic to mammals including livestock and cause kidney and liver disease that can lead to death (van Dam et al. 1995; Baker et al. 1991; Knight et al. 1984; Stegelmeier et al. 1996). Finally, seeds attached to livestock fur can cause irritation and lead to losses in the market value of livestock (Upadhyaya and Cranston 1991).

Conventional control methods of *C. officinale* in North America include chemical control using different herbicides (such as 2, 4-D, chlorosulfuron, picloram, and metasulfuron), and cultural control methods (cutting shoots close to the ground at flowering stage or hand-pulling bolting plants in spring) (De Clerck-Floate and Schwarzländer 2002; Dickerson and Fay 1982; DiTomaso & Kyser 2013; Peachey et al. 2016; Upadhyaya and Cranston 1991). However, long-term use of herbicides, mechanical control or both for the management of *C.*

officinale populations can be costly and given the widespread distribution and mode of dispersal especially in remote areas, control success may not be easily achievable or even feasible (De Clerck-Floate and Schwarzländer 2002). A biological control project for the invasive plant was initiated in 1987 (Freese 1987). Since then, seven biocontrol candidate species have been studied for their host range (Hinz et al. 2007; Hinz et al., 2005; Hinz et al., 2003; Hinz et al., 2006; Schwarzländer 1999). These are *Mogulones crucifer* Pallas, *M. trisignatus* Gyllenhal, *M. borraginis* Fabricius, *Rabdorrhynchus varius* Herbst (all Coleoptera: Curculionidae), *Longitarsus quadriguttatus* Pontoppidan, *L. exoletus* Linnaeus (Coleoptera: Chrysomelidae), and *Cheilosia pasquorum* Becker (Diptera: Syrphidae). Of these seven candidate species, five, i.e. *M. trisignatus, R. varius, L. quadriguttatus, L. exoletus,* and *C. pasquorum* were not sufficiently host-specific to *C. officinale* to be petitioned for introduction in the United States (Hinz et al., 2005; Hinz et al., 2003; Hinz et al., 2006).

Mogulones crucifer (syn: *M. cruciger* Herbst) is a univoltine root mining weevil native to central Europe (Koch 1992; Schwarzländer 1997). In the literature, it is described as monophagous on *C. officinale* (Dieckmann 1972; Lohse 1983; Scherf 1964), although one account (Peyerimhoff 1911) also mentions the closely related Eurasian confamilials *Pardoglossum cheirifolium* (L.) Barbier & Mathez (syn: *Cynoglossum cheirifolium* L.) and *Cynoglossum creticum* Mill. (syn: *Cynoglossum pictum* Aiton.) in Algeria as host plants of the weevil. In its native range, overwintered weevils emerge from soil in early spring when temperatures increase and begin feeding on foliage of *C. officinale.* After approximately two weeks, they begin laying eggs at the base of *C. officinale* petioles near the root crown. Eggs hatch seven to 10 days following oviposition and early instar larvae tunnel down the leaf

petiole to develop inside the upper root and root crown, where they feed on the vascular cylinder (Schwarzlaender 1997). Older larval stages move from the root crown to the taproot and eventually to the secondary rootlets. Mature larvae leave the roots and spin a cocoon with soil particles for pupation in the soil (Schwarzlaender 1997). Because of partially overlapping generations, larvae can be found within roots throughout the year (Schwarzlaender 1997).

The experimental host range of *M. crucifer* was assessed between 1988 and 1996, using 32 Eurasian and 5 native North American Boraginaceae species (Jordan et al. 1993; Schwarzländer 1996). These studies resulted in the recommendation of the weevil for release by the Canadian Biocontrol Review Committee and the United States Technical Advisory Group (TAG), which advises the United States Department of Agriculture - Animal Plant Health Inspection Service (USDA APHIS), the agency responsible for biocontrol release decisions in the United States. The weevil was approved for release in Canada in 1997. Since its release in British Columbia and Alberta, weevil populations have reached outbreak densities and succesfully controlled the weed with 100% establishment at all release sites in southern British Columbia, Canada (De Clerck-Floate et al. 2005). However, the petition for release in the United States was denied by USDA APHIS in the early 2000s because of concerns by the United States Fish and Wildife Service (USFWS) over potential nontarget attack of the weevil with regard to federally listed threatened and endangered (hereafter, T&E) confamilials of *C. officinale* (De Clerck-Floate and Schwarzländer 2002). There are five T&E species in the Boragincaeae family and 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 (USFWS, 1997).

Complementary host range testing was conducted between 1997-2000 using six European and 22 North American confamilial species (De Clerck-Floate and Schwarzländer 2002). Although , *M. crucifer* seems to have a broad fundamental host range with mature larvae able to develop on 11 out of 23 tested North American species and on 18 out of 34 tested European species, rates of development on test plants generally very low when compared to C. officinale (De Clerck-Floate et al. 1996; De Clerck-Floate and Schwarzländer 2002; Jordan et al. 1993). Further host-specificity testing between 2001-2004 using eight North American confamilials, including three T&E species, showed that the T&E species *Amsinckia grandiflora* and *Plagiobothrys hirtus* supported complete development of *M. crucifer* albeit at very low rates (Andreas 2004). Development of a single larvae was also observed for the T&E species *Hackelia venusta* (Andreas 2004). *Mogulones crucifer* development rates on nontarget plant species were always lower when compared to *C. officinale* and the weevil typically preferred *C. officinale* over nontarget species in choice tests (Andreas 2004; De Clerck-Floate et al. 1996; De Clerck-Floate and Schwarzländer 2002; Jordan et al. 1993). In 2007, *M. crucifer* naturally dispersed into the United States from Canada and quickly built up large populations in northern Washington, Idaho and Montana. In 2010, USDA APHIS issued a pest alert for *M. crucifer* based on the broad fundamental host range of the weevil, which comprised 55% of native North American confamilials tested including the three T&E species *H. venusta, P. hirtus* and *A. grandiflora* (Andreas 2004; USDA, 2010).

Materials and Methods

Adult *M. crucifer* were collected at a *C. officinale* field site near Bonners Ferry, ID (N48.42373˚, W116.10759˚) during early spring in late April between 2017 and 2019, respectively. Weevils were transported to the University of Idaho in Moscow, ID (N46.7288˚, W117.0126˚). Weevils were used to maintain a laboratory colony at the University of Idaho. The gender of weevils was visually determined using the ventral abdominal depressions observable in males (Jordan et al., 1993). *M. crucifer* adults were separated by gender and 10 to 15 pairs were kept in cylindrical plastic containers (diameter: 11 cm, height: 15 cm) lined with a moistened paper towel and covered with a gauze cloth lid. To simulate natural conditions, cylinders were kept in an environmental chamber (I-35 VL, Percival Mfg. Co., Boone, Iowa) at 12:12 (L:D) at 7˚C day: 2˚C night during winter and 16:8 (L:D) at 17˚C day: 8˚C night during spring. Weevils were fed with fresh *C. officinale* foliage during active periods in spring and fall. During summer they were fed every second day and during winter they were fed once a week. Weevils were used throughout the year for bioassays as described below.

Emphasis for selecting Eurasian confamilial plant species used for this study were closely related to *Cynoglossum officinale* and the availability of propagules (Table 2.1). The following Eurasian and/or Asian confamilial species were included in this study: The European *Cynoglossum germanicum* Jacq., which overlaps in distribution with *C. officinale* (Joshi 2016; Marinov 2009; *C. creticum* Mill., which replaces *C. officinale* in the Mediterranean (Gams 1927); *C. amabile* Stapf & J. R. Drumm, a worldwide naturalized Asian ornamental congener (El-Shazly A et al. 1996); *C. lanceolatum* Forssk., a previously untested Asian congener (Chacón et al. 2016); *Rindera umbellata* (Waldst. & Kit.) Bunge, which is a representative of a different genus within the subtribe Cynoglossinae that occurs

sympatrically with *C. officinale* at field sites in Serbia (Ivo Toševski pers. communication) and was fed upon by *M. crucifer* in previous host-specificity tests (Jordan et al. 1993); and *Solenanthus circinatus* Ledeb., which is in the same subtribe as *C. officinale* and a congener of *S. apenninus* (L.) Fisch. & C.A. Mey., a plant mentioned as host plant of *M. crucifer* (Enzo Colonelli pers. Communication). Finally, we also included the western Himalayan *Lindelofia longiflora* (Benth.) Baill., a representative of an Asian genus that is within the same subtribe as *C. officinale* (Chacón et al. 2016) (Table 2.1).

Plants for experiments were propagated from seed (*Cynoglossum germanicum, C. creticum, C. lanceolatum, R. umbellata, S. circinatus,* and *L. longiflora*). *Cynoglossum officinale* plants were propagated as described below. In addition, *C. officinale* rosettes and bolting plants were collected from a population at Idler's Rest Nature Preserve, Moscow, ID (N46.804160, W116.948554˚). Seeds of *C. germanicum, C. lanceolatum, R. umbellata* and *S. circinatus* were kindly provided by CABI Switzerland, Delémont, Switzerland. Seeds of *C. amabile* were acquired from American Meadows, Shelburne, Vermont. *C. creticum* seeds were kindly received from the Royal Botanic Garden, Kew, Richmond, United Kingdom. Seeds of Himalayan *L. longiflora* were purchased from Jelitto Perennial Seed, Louisville, Kentucky. Seeds of all plant species were first soaked on wet filter paper in Petri dishes (90 mm diameter) for 24 hours. Then the seed coats were carefully peeled off seeds for those species that had thicker seed coats, i.e., *S. circinatus* and *L. longiflora*. Peeled seeds were sown into Sunshine Mix (Sun Gro Horticulture Canada Ltd., Vancouver, Canada) soil in seedling starter trays (15 cm x 12 cm x 5 cm) at the University of Idaho. After three to four weeks, seedlings were transplanted into tree pots (12.7 cm x 30.5 cm x 5 L) (Stuewe & Sons, Inc. Tangent, Oregon) in a 1:3 mixture of sand and Sunshine Mix No. 2 (Sun Gro Horticulture

Canada Ltd., Vancouver, Canada) along with 2.5 g of trace elements (FRIT Industries, Inc., Ozark, Alabama), 1.25 g chelated iron (Grow More Inc., Gardena, California), and 47.5 g limestone (Grow More Inc., 47.5 g triple super phosphate (Bonide Products Inc., Oriskany, New York), and 187.5 g Osmocote® (The Scotts Company LLC., Marysville, Ohio), per 12 kg of sand and Sunshine Mix No. 2 used. Transplants were watered heavily every second day and maintained in greenhouses at the University of Idaho's Manis Entomological Laboratory and Parker Research Farm in Moscow, Idaho at 16:8 (L:D) and 25˚C day and 18˚C night.

Behavioral olfactometer bioassays

A four-armed olfactometer (Syntech Ltd., Hilversum, The Netherlands) as described by Vet et al. (1983) was used to assess behavioral responses of female *M. crucifer* to volatiles from the host plant *C. officinale* and nontarget plants (Fig. 2.1). We used methods previously developed for four-armed olfactometer tests with *M. crucifer* (Kafle 2016). In brief, the olfactometer allows four different odors to be pushed through four-inlet arms into a central rhomboid-shaped experimental arena (diameter: 22 cm) with a basal outlet, and covered with a heavy clear glass plate (thickness: 10mm), within the glass plate an individual insect can freely move (Vet et al. 1983). To separate the arena into four quadrants, perpendicular lines were drawn on the glass plate meeting in the center and dividing the experimental area in four 37.50 cm^2 quadrants. Tygon ® tube (8 mm internal diameter, Fisher Scientific Co., Pittsburgh, Pennsylvania) was used to connect each of the four inlet arms to volatile sources: foliage of potted plants, which were placed inside sealed, sterilized polyvinyl acetate bags (20 cm x 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 into the olfactometer air that was purified using activated charcoal (Sigma-Aldrich

Co. LLC, St. Louis, Missouri) and humidified by passing through distilled water in a 500 ml gas-washing bottle (Chemglass Life Sciences LLC, Vineland, New Jersey). Air flow rate was set to 300 ml/minute, measured and maintained with flow meters (King Instrument Company, Ins., Garden Grove, California). From the basal outlet of the olfactometer, air was drawn at the rate of 1200 ml/minute using a Rena ® Air 400 pump. Plants used as volatile sources were kept diagonally in two opposite directions and purified air (control treatments) in the two remaining quadrants. For dual-choice experiments, volatiles from a single plant species were delivered to the opposing quadrants. For multiple-choice experiments, volatiles from *C. officinale* were delivered opposite to volatiles of a different plant species, while the remaining quadrants received purified air. Details of these two bioassay designs are provided below. White polyethylene vinyl acetate (PEVA) sheets were used to cover all four sides of the olfactometer arena to eliminate visual cues distraction to *M. crucifer*. For uniform light in the olfactometer, a single full spectrum LED light source (Jansjö ® LED lamp, Inter Ikea Syatem B. V., Delft, The Netherlands) was used.

We used female *M. crucifer* with previous contact with *C. officinale* in experiments assuming that females are more responsive than males to suitable hosts because they must find hosts for oviposition. We determined in previous tests that female *M. crucifer* were reactive in the olfactometer bioassays and responses did not differ from those of males or naïve females (Kafle 2016). Weevils used during experiments 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 arena from the outlet using a fine paintbrush. The hose was reconnected, and the behavior of the weevil was recorded for 30 min. with a video camera (Contour Roam

2, Contour Inc., Seattle, Washington) fitted above the olfactometer arena. Weevils were recorded as 'unresponsive' if they did not make any choices after 5 min. of exposure and were discarded from the experiments. Each weevil was used only once. After every five replicates, the odor sources were replaced and the olfactometer was rotated 90 degrees to reduce positional effects. After 10 replicates all the tubes were washed with distilled water and 70 % ethyl alcohol. The initial choice of a weevil was determined when it entered a 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 conducted between 0900 hrs. and 1600 hrs. The video recordings containing movement and positions of weevils were analyzed using the behavioral software program Noldus Observer XT 11 (Noldus Information Technology BV, Wageningen, The Netherlands).

The following response variables were measured in bioassays: initial choice, final choice and the proportion time spent by *M. crucifer* females in each quadrant. The initial choice is defined as the quadrant first entered by weevil for a minimum of 30 sec. and was used as a measure to evaluate *M. crucifer*'s ability to discriminate between different odors quickly. The final choice, i.e., the quadrant in which a weevil was found at the end of the 30 min. reporting period was because it was assumed to be *M. crucifer's* ultimate preferred odor source. The time spent in each quadrant of the olfactometer arena was considered a measure for the strength of preference of an odor.

Dual-choice bioassays

We conducted dual-choice bioassays in which the weevil was given a choice between headspace volatiles of a confamilial plant species and purified air to determine whether *M.*

crucifer females were able to identify these plant species as potential hosts in the absence of *C. officinale*. These bioassays allowed for three possible behavioral outcomes: attraction, when the plant volatiles were preferred over purified air; indifference, when plant volatiles were not preferred over purified air; and repellence, when purified air was preferred over plant volatiles (Martini et al. 2015; Kafle 2016). For this, two opposing arms of the olfactometer were provided with headspace volatiles from one plant species and the two perpendicular arms of the olfactometer were provided with only purified air as control. The responses of weevils in relation to the control (purified air) were measured as described above. For each plant species tested, there were 20 replicates.

Multiple-choice bioassays

We conducted bioassays in which female *M. crucifer* could choose between odors of *C. officinale* and of one nontarget plant species to determine the relative attraction of nontarget confamilial plant species to the weevil in the presence of its preferred field host. For these tests, volatile headspace from *C. officinale* was provided in one arm and volatile headspace from one non-target confamilial was provided in the opposing arm of the olfactometer. Purified air was provided in the other two perpendicular arms. The responses of weevils to confamilial plant volatiles in relation to *C. officinale* volatiles and purified air were assessed. As for dual-choice tests, for each *C. officinale* vs. nontarget species test, 20 replicates were conducted, and the response variables were recorded for each weevil as described above.

Collection and analysis of plant volatiles

Plant volatile organic compounds (VOC) were collected in 2017 and 2018 at the University of Idaho's Manis Entomological Laboratory and the Parker Research Farm in greenhouses from propagated plant species using a portable volatile collection system (PVCS) (Park et al.

2019). Volatiles were collected from *C. officinale* and the following Eurasian confamilials of *C. officinale* that were used in behavioral bioassays: *Cynoglossum germanicum, C. creticum, C. amabile, C. laneolatum, Rindera umbellata,* and *Solenanthus circinatus*.

Pre-sterilized (140˚C for 1 hr.) polyvinyl acetate bags (20 cm x 15 cm, Reynolds Consumer Products LLC., Richmond, Virginia) were used for the collection of foliar volatiles from individual plants. While collecting foliar volatiles, the end of the bags was gently sealed using pre-sterilized cotton balls and cable ties. Rena ® Air 400 pumps (Mars Fishcare North America, Inc., Chalfont, Pennsylvania) were modified to create push-pull pumps by switching the direction of the diaphragm within the pump assemblage to create a uniform airflow. Air was purified using activated charcoal filters (Orbo ^{TM}, Sulpelco, Sigma-Aldrich Co. LLC, St. Louis, Missouri), and pushed into the bag at a rate of 300 ml/min. through a perforation (5 mm) made at the upper corner of the bag. A modified Rena ® Air 400 pump was used to draw foliar volatile headspace air out of the bag at a rate of 300ml/min. and VOC were collected in volatile collection traps (VCTs hereafter) containing 40 mg of 80-100 mesh Porapak-Q adsorbent (Southern Scientific Inc. Micanopy, Florida). Prior to collection, VCTs were rinsed with 1000 µL of dichloromethane (EMD Chemicals Inc., Gibbstown, New Jersey) to remove contamination in the adsorbent. Four pairs of flowmeters (King Instrument Company Inc., Garden Grove, California) were used to maintain airflow through the bags. Based on the number of volatile peaks obtained from gas chromatography-mass spectrometry analysis of collected volatile samples and previous research (Kafle 2016), collection time was set to 6 hrs. (0900 h to 1500 h). Volatiles were collected from 3 plant individuals along with 1 control (surrounding air) simultaneously. After each collection, the VOC in the VCTs were extracted by eluting with 200 µL of dichloromethane into a glass vial (National C5000-

180, Thermo Fisher Scientific Inc., Rockwood, Tennessee) and stored in a freezer (-80˚C) for later use.

A Hewlett-Packard 7890 Gas Chromatograph (Agilent Technologies Inc., Palo Alto, California) equipped with a fused silica HP-5MS capillary column (30 cm x 0.25 mm x 0.25 μm, Agilent Technologies Inc.), which was coupled with a Hewlett-Packard 5973 Mass Selective Detector (Agilent Technologies Inc., Palo Alto, California) was used for identification and analysis of headspace volatile organic compounds. Temperature of the injection port was set to 250˚C. The initial oven temperature was set to 40˚C and the temperature was held 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., then held isothermally for 2 min. Helium was used as a carrier gas at 3.0 mL/min. Volatile extract (1 μL) was injected into the gas chromatograph using splitless mode. Mass spectra were obtained using electron impact (EI, 70 eV). The relative amount of each identified component was determined based on peak area normalization of the total ion concentration (Puttick et al. 1988). Those compounds with a relative peak area of 1% or more of the total chromatogram in any one of three samples were included. Peaks detected in both plant volatile samples and purified air control samples were regarded as contaminants and subtracted from the total peak area. 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)

Gas chromatography coupled with electroantennographic detection (GC-EAD) is considered an appropriate technique for identifying specific compounds triggering insect behaviors from complex mixtures of volatile compounds (Arn et al. 1975; Gouinguené et al. 2005; Kafle 2016; Weissbecker et al. 2004; Zhang et al. 2015). Headspace volatiles of *C. officinale* and *C. creticum* were subjected to coupled gas chromatography-flame ionization detection/electroantennographic detection (GC-FID/EAD) analysis with female *M. crucifer* to detect electrophysiologically active compounds in the volatile blend. We used a HP Agilent 6890 GC equipped with FID and coupled to an electroantennogram detector. For antennal responses of *M. crucifer*, females were first decapitated, and the antennal tips were cut off using a sharp scalpel. The excised head was then placed over a reference electrode, while the antennae were connected to the recording electrode by submerging them in Spectra® 360 electrode gel (Parker Laboratories Inc., Fairfield, New Jersey) that coated the electrode. The electrodes conduct signals generated by the antenna to a high-impedance input ampliflier (IDAC-2, Syntech Ltd., Hilversum, The Netherlands) that feeds the signal to a graphical readout on a PC equipped with GC-EAD 2014 Software (Syntech Ltd., Hilversum, The Netherlands). Simultaneous changes in voltage in both antennal and FID signals indicate olfactory sensitivity to the compound eluting at that retention time.

For GC/EAG tests, volatile samples (1 μL) injected splitless into an Agilent 6890N GC equipped with a capillary column (30 cm x 0.25 mm x 0.25 μm, Agilent Technologies Inc., Palo Alto, California). The initial oven temperature was set to 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,

then held at that temperature for 2 min. Helium was used as a carrier gas at 3.0 ml/min. The effluent from the column was split into two parts, where 50 % was transferred to the FID and the other 50 % to the EAD via an 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). Electrodes mounted with excised antennae 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 i.e. the voltage changes in the antenna was distinguishable from background noise which arise from antennal preparations and the GC-EAD associated hardware in three or more of the antennal preparations. The antennal responses to the plant compounds were selected while responses to potential impurities (as identified by GC-MS analysis of respective volatile samples) were discarded (Kafle 2016).

Statistical analysis

The choice data (IC, FC) in bioassays were treated as discrete categorical responses. The proportion of initial choices and final choices of female *M. crucifer* in bioassays were initially assessed using χ-square tests of homogeneity. A log-linear categorical model was subsequently used to assess pair-wise comparisons among quadrants. The strength of preference (TS) for each choice was measured as the time (sec.) spent in each quadrant of the four-armed olfactometer. Differences between the four quadrants were assessed using a log-linear categorical model assuming time in sec to be a discrete count. Within this model, a

single degree of freedom contrast allowed pair-wise comparison of the quadrants counts (times). For all analyses, tests with p-values <0.05 were regarded as significant. All analyses were conducted using the statistical software SAS Version 9.4 (SAS Institute Inc., 2013). The relative concentration of each compound identified through GC-MS was based on peak area normalization of the total ion concentration. The electrophysiologically active compounds, as identified by GC-FID/EAD, in percentage of total ion concentration 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).

Results

Multiple-choice bioassays with C. officinale*, confamilials and purified air*

In multiple-choice bioassays, based on their initial choice *M. crucifer* females did not prefer *C. officinale* VOCs over any of the four Eurasian and three Asian confamilials (Fig. 1.2, Table 1.2). Based on their final choice, the weevils preferred *C. officinale* VOC over those from *C. lanceolatum* ($p = 0.0046$) and *S. circinatus* ($p = 0.0125$) (Fig 1.2, Table 1.2). *M. crucifer* spent more time in quadrants with *C. officinale* VOC compared to all confamilial plant species tested or purified air ($p < 0.0001$ for all species) (Fig. 1.3, Table 1.2).

Dual-choice bioassays

Previous study (Kafle 2016), found that *M. crucifer* consistently attracted to *C. officinale* volatiles in dual-choice bioassays, spending more time in those quadrants than in purified quadrants. In my dual-choice bioassays, *M. crucifer* responded with indifference or were repelled to volatiles of the seven confamilial plant species when compared to purified air. There was no preference between VOC of any of plant species tested and purified air in the initial choice of *M. crucifer* females (Fig. 1.4, Table 1.3) or in the final choice for any of the

Cynoglossum species tested and *R. umbellata. M. crucifer* females responded with repellence in their final choice to *L. longiflora* ($p = 0.0008$) and *S. circinatus* ($p = 0.0298$) (Fig. 1.4, Table 1.3). Females spent more time in quadrants with purified air for all confamilial plant species ($p < 0.0001$ for all species) (Fig 1.5 Table 1.3).

Plant species VOC

The volatile headspace of *C. officinale* comprised 25 volatile chemical compounds and contained green leaf volatiles, terpenes and esters (Table 1.5). The total number of identified compounds obtained from each of the seven confamilial species were: *C. amabile* (10), *C. germanicum* (10), *C. creticum* (18), *C. laneolatum* (14), *R. umbellata* (13), and *S. circinatus* (14) (Table 1.5). *Rindera umbellata* shared the fewest individual VOC with *C. officinale* (three), while *C. germanicum* shared the most (seven) (Table 1.5).

GC-EAD

In our experiment using the same instrumentation (Kafle 2016) we found identical results for *C. officinale* VOC. Weevil antennae responded to all six volatiles mentioned above and none of the remaining 19 found in *C. officinale* (Table 1.5). When tested with *C. creticum* volatiles, *M. crucifer* antennae did not respond to any VOC.

Based on the six electrophysiologically active compounds from *C. officinale,* PCA separated *C. officinale* from all seven Eurasian and Asian confamilial species tested. The first principal component (PC1) explained 52.88% of variability and separates *C. officinale* from *C. creticum, C. germanicum, C. amabile, C. lanceolatum,* and *R. umbellata* (Fig. 2.5). The second principle component (PC2) explained 27.55 % of variation and separates *C. officinale* from *R. umbellata, C. germanicum,* and *S. circinatus* (Fig. 1.6).

Discussion

Behavioral responses of M. crucifer *to volatiles emitted by* C. officinale *and closeley related confamilials*

Our results show that volatile cues play an important role during the pre-alightment host finding stage of *M. crucifer*'s host selection. The data are consistent with previous research conducted with other confamilial species that showed that *M. crucifer* females were attracted to volatiles from *C. officinale* but not to VOC of nine related North American and a few Eurasian confamilial species (Kafle 2016). In the bioassays conducted here, we observed for all possible behavioral outcomes of *M. crucifer*: attraction (where one plant's volatiles are preferred over another's or to purified air), indifference (plant volatiles were not preferred over purified air control), and repellence (purified air was preferred over plant volatiles) (Kafle 2016; Park et al. 2018; Park et al. 2019; Martini et al. 2015; Vet et al. 1983). While attraction was solely observed with regard to *C. officinale,* behavioral responses of *M. crucifer* to volatiles of the nontarget species ranged from indifference to repellence.

M. crucifer were repelled by VOC of the closest relatives of *C. officinale* tested in this study, *C. creticum* and *C. germanicum*, in the proportion time spent. Both species are Eurasian congeners of *C. officinale* and *C. germanicum* is morphologically very similar to *C. officinale.* (Chacón et al 2016, Tutin et al. 1972). *Cynoglossum creticum* replaces *C. officinale* in similar habitats in southern Europe and the Mediterranean (Gams 1927; De Jong et al. 1990). According to one record, *M. crucifer* has been observed on *C. creticum* (syn: *C. pictum* Aiton) (Enzo Colonelli, pers. communication; Schwarzländer 1996) although the plant is not mentioned as a host in the literature (Dieckman 1972, Lohse 1983, Scherf 1964). *Cynoglossum germanicum* overlaps in distribution and habitat in central Europe with *C. officinale* (Joshi 2016; Marinov 2009) but has never been reported as a field host of *M.*

crucifer (Dieckman 1972, Lohse 1983, Scherf 1964). The repellence in time spent would support literature records that *C. germanicum* is indeed not used as a host plant by *M. crucifer.* However, our observed repellence in time spent with regard to *C. creticum* while supported by literature records would call into question the observation that the plant is used as host by *M. crucifer* in Italy (Enzo Colonelli, pers. communication). Additionally, *M. crucifer* were indifferent to *Rindera umbellata* VOC with regard to time spent. *Rindera umbellata* grows sympatrically with *C. officinale* at field sites in Serbia (Ivo Toševski pers. communication) and the genus *Rindera* is within the subtribe Cynoglossinae (*Cynoglossum sensu lato*) (Chacón et al 2016). While adult feeding was observed during conventional host-specificity tests, *R. umbellate* is not known as a field host and not attacked by the weevil in populations sympatric with *C. officinale* populations (Dieckman 1972, Lohse 1983, Scherf 1964) (Ivo Toševski pers. communication). Based on these data, *M. crucifer*'s host fidelity to *C. officinale* seems sufficiently strong to discriminate against plant species that are i) geographically (at large and small scales) overlapping with *C. officinale,* and ii) phylogenetically very closely related to *C. officinale.* All of the three plant species, *C. creticum, C. germanicum* and *R. umbellate,* are well within the fundamental host range and able to support development of *M. crucifer* at rates comparable to *C. officinale* (Jordan et al. 1993, Schwarzländer 1996). We cannot explain what factors drove this specialization of *M. crucifer* over evolutionary time. But the behavioral responses during pre-alightment host selection and the VOC driving these responses seem to be examples for the mechanisms involved in maintaining this host plant specialization.

M. crucifer were repelled by VOC of *S. circinatus* in proportion time spent (TS) and in final choice (FC). *S. circinatus* is native to Eurasia (Otero et al. 2014), is a representative of a

genus within the same subtribe as *C. officinale* (Chacón et al. 2016) and *M. crucifer* has been reported to use one of its congeners, *S. apenninus* (L.) Fisch. & C.A. Mey (syn: *Cynoglossum apenninum* L.) as field host in Italy (Enzo Colonelli pers. communication). We do not know whether the report of *M. crucifer* on *S. apenninus* is restricted to the presence of the weevils on those plants or whether they really use the plant for development (Enzo Colonelli pers. communication). It would be interesting to verify that host record as *M. crucifer* were repelled with one of the congeners, *S. circinatus* in time spent. *Mogulones crucifer* has been reported to use nontarget plants as hosts in the area of introduction (Catton et al. 2015, 2016), despite the fact that the weevil responded with repellence to the plant species in question (Kafle 2016). That attack on the native North American *Hackelia micrantha* (Eastw.) J.L. Gentr. is, however, considered spillover attack, i.e. only occurs because of the presence of *C. officinale* at those field sites (Catton et al. 2015, 2016). Again, surveys of populations of *S. apenninus* in Italy for the presence of the weevil and the presence of *C. officinale* could verify the host record and potentially provide an explanation between observed repellence and the host record. Alternatively, the potential existence of additional field hosts in *M. crucifer's* native range may suggest that the weevil does not discriminate as clearly between *C. officinale* and some closely related congeneric or confamilial plant species at some stage during host selection as observed here under laboratory conditions. Although *C. officinale* is the only host plant recorded for *M. crucifer* in its native range (Dieckman 1972, Lohse 1983, Scherf 1964), if there are additional host plants this may indicate adaptation of host preference in *M. crucifer* to alternative confamilial species. Selection favoring such a range expansion could occur when *C. officinale* becomes rare (Enßlin et al. 2011). It would also contradict conclusions of our data above on *C. germanicum* and *R. umbellate* if *M. crucifer*s

utilizes nontarget confamilial plant species based on pre-alightment cues in the field and contradict ecological theory that specialist herbivores tend to adapt towards increased specialization (Loxdale et al. 2011).

M. crucifer were repelled by *L. longiflora* VOC with regard to TS and FC of females. Similarly, weevils were repelled by *C. amabile* and *C. lanceolatum* VOC with regard to TS. *L. longiflora* is native to the western Himalayas (Singh and Lal 2007), which is geographically distant from the native range of *C. officinale*, but a recent phylogenetic revision has aligned the genus within the same subtribe as *Cynoglossum*, i.e., Cynoglossinae (Chacón et al 2016). *Cynoglossum amabile* is native to Asia (Joshi 2016) and *C. lanceolatum* is native to sub-Saharan Africa and Asia (Joshi 2016), and within the genus *Cynoglossum* (Chacón et al 2016). *Cynoglossum amabile* is naturalized in South America, Europe and in the United States and widely used as an ornamental. It is commonly found in central Europe and similarly to *C. creticum*¸ it is well within the fundamental host range of *M. crucifer* (Jordan et al 1993), but it has never been reported as a host plant of the weevil (Dieckman 1972, Lohse 1983). A systematic survey of *C. amabile* in Europe for *M. crucifer* would be of particular interest because this confamilial species and the weevil have no co-evolutionary history, analogous to confamilials in North America where *M. crucifer* has been intentionally released (Canada) or accidentally been introduced (United States). Our data would predict that under field conditions, *C. amabile* is not used as host plant by the weevil.

Evolution of host plant specialization by insects has been long discussed (Ehrlich and Raven 1964; Farrell 1998; Futuyma and Moreno 1988; Loxdale et al. 2011; Rasmann and Agrawal 2011). It is believed that insects specialize and adapt to toxic metabolites in their host plants by tolerating those toxic metabolite (Wheat et al. 2007), sequestering plant toxins to protect

against predation (Nishida 2002), or possibly avoiding plant toxins using either visual or olfactory cues (Chapman 2003). Especially among Curculionidae, many species are highly specialized and host-specific (Farrell 1998), and coincidently species within the Curculionidae are among the most frequently used and effective biological weed control agents (Clewley et al. 2012; Heimpel and Mills 2017; Schwarzländer 2018; Winston et al. 2014). Behavioral responses of *M. crucifer* to VOC have been tested with *C. officinale* and a total of 26 confamilial plant species and the only species the weevil was attracted to was its field host *C. officinale* (data preseneted in this thesis, Andreas et al. 2008; Kafle 2016). This coincides with the fact that nontarget attack by *M. crucifer* thus far has not been reported despite the fact that the weevil has been released for 22 years (De Clerck-Floate and Schwarzländer 2002), with the exception of instances in which nontarget sympatrically occurred with *C. officinale* during the time of attack (Andreas et al. 2008; Catton et al. 2014; Catton et al. 2015).

VOC from the field host and nontarget plants

During host finding, herbivorous insects need specialized olfactory systems to process cues so that they can discriminate host plants from very complex olfactory environments (Baker 1988; Beck et al. 2008; Bruce and Pickett 2011; Schröder and Hilker 2008). The headspace volatile blend emitted by *C. officinale* and its Eurasian confamilials includes alcohols, ketones, aldehydes, esters, and mono- and sesquiterpenes. Some chemical compounds that are present in Eurasian and Asian confamilial nontarget species are species-specific and are not present in *C. officinale*. The possible presence of species-specific volatile compounds in the tested confamilials could explain the repellence of *M. crucifer* to some of the tested plant species in the genus *Cynoglossum,* i.e., *C. amabile*, *C. creticum, C. germanicum*, and *C.*

lanceolatum, as well as *S. circinatus* and *L. longiflora* in behavioral bioassays. In a previous study (Kafle 2016), *M. crucifer* antennae responded to six chemical compounds in the volatile headspace of *C. officinale*: methyl isovalerate, (z)-3-hexen-1-ol, benzaldehyde, 6-methyl-5-hepten-2-one, (z)-3-hexen-1-ol acetate, and (z)-β-ocimene. In our GC-FID/EAD experiments and those conducted previously (Kafle 2016), female *M. crucifer* responded to six volatile compounds emitted by *C. officinale*, some of which are used by other insects during host selection (El-Sayed 2016; Knudsen et al. 2006). For example, female wheat stem sawflies *Cephus cinctus* Norton, (Hymenoptera: Cephidae) were attracted in a Y-tube olfactometer to 60, 120 and 180 ng/h of (z)-3-hexenyl acetate, 6 ng/h of (z)-3-hexen-1ol and 0.96 ng/h β-ocimene relative to the control (Piesik et al. 2008). Benzaldehyde and β-ocimene within a volatile blend from Mexican marigold, *Tagetes erecta* L. (Asteraceae) attracted female cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) when tested in a wind tunnel (Bruce and Cork 2001). Methyl isovalerate, an ester compound, was found to be unique to the volatile headspace of *C. officinale* and the closest related confamilial *C. germanicum* but absent in the headspaces of the other Eurasian and Asian confamilials tested here, and absent in Eurasian and North American confamilials tested previously (Kafle 2016). In olfactometer trials, *M. crucifer* was attracted to purified methyl isovalerate relative to purified air (Kafle 2016), suggesting that the *C. officinale* compound methyl isovalerate is an important olfactory cue during host-selection and host discrimination of *M. crucifer*. The use of species-specific compounds during host finding has been found elsewhere. The cabbage seed pod weevil, *Ceutorhynchuc obstrictus* (Marsham) (Coleoptera: Curculionidae), for example, uses host plant volatile compound isothiocyanates (3-butenyl, 4-pentenyl, 2-phenylethyl) during host recognition (Blight et al. 1995; Smart and Blight

1997). While methyl isovalerate is found in at least seven plant families (Baser et al. 1993; Brielmann et al. 1999; El-Sayed 2016), there are very few studies examining its role in insect-plant interactions. One study compared a number of *Drosophila* species to evaluate the ecological shift in host preference at different stages of fruit development (Keesey et al. 2015). In that study, methyl isovalerate was identified as a compound associated with the fruit ripening process in strawberries and in electrophysiological experiments using gas chromatography-coupled with single sensillum recordings (GC-SSR), *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) responded to methyl isovalerate (Keesey et al. 2015). Methyl isovalerate was also present in *C. germanicum*, the closest Eurasian congener of *C. officinale* we tested. It should have had the same attractive effect on *M. crucifer*, but instead *M. crucifer* responded in behavioral bioassays with repellence to *C. germanicum* with regard to time spent. We cannot explain this contradiction but assume that other compounds in the VOC blend of *C. germanicum* are repelling to *M. crucifer* and that repellence is offsetting the attractive effect of methyl isovalerate. Unfortunately, we were not able to conduct GC-FID/EAD experiments with this plant species because of time constraint.

We used GC-EAD/FID to assess whether compounds in the VOC blend of *C. creticum* caused the repellence observed in behavioral bioassays. Unfortunately, none of the *C. creticum* headspace volatiles elicited an electrophysiological response in female *M. crucifer* during GC-EAD/FID. It is possible that our methods for extracting VOC is not sufficiently comprehensive for collecting volatile compounds which may be present in very small concentrations.

Weigend et al. (2013) estimate that there are 100 *Cynoglossum* species in the Mediterranean and adjacent Asia Minor. Even if *M. crucifer* uses *C. creticum*, the weevil would still be

regarded as near-monophagous despite its broader fundamental host range (Dieckmann 1972; Lohse 1983; Scherf 1964; Weigend et al. 2013). If phylogenetic relationship is a good predictor of host-specificity (Wapshere 1974) and/or if VOC headspace similarity is a predictor for host-specificity, then it would be much more likely that *M. crucifer* would adapt to or use more *Cynoglossum* species in its native range before attacking any of the more distantly related North American confamilials (Chacón et al. 2016) in the introduced range regardless of the fundamental host range of the weevil.

In summary, though it is believed that the fundamental host range may be an appropriate predictor for host range expansion in the area of introduction (Van Klinken and Edwards 2002), our data shows that an insects complex host selection process allows strict maintenance of host specificity and we agree with Hinz et al. (2014) that too much emphasis is placed on the fundamental host range when release decision are made. Data on the pre-alightment stage of host-finding can be effective in accurately assessing the risks posed by an agent with a broader fundamental host range as is the case with *M. crucifer* (Catton et al. 2015). The repellence behavior reported here by *M. crucifer* to two of its closest European congeners suggests that the weevil has adapted to one or very few field hosts in its native range over evolutionary time in the presence of up to 100 potentially suitable congeneric field hosts (Weigend et al. 2013). Host finding behavior evidently contributes to that specialization.

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Table 1.1: List of selected European plant species for behavioral analysis of female *Mogulones. crucifer*.

Plant species	Synonyms	Life history trait	Rationale for testing	Native range	Previous host range data
Cynoglossum amabile Stapf. & J. R. Drumm.		Annual[3]	Asian congener of *C. officinale* [2]	Asia[3]	Within fundamental host range[1] Adult feeding and oviposition in laboratory testing[1]
Cynoglossum creticum Mill.	*Cynoglossum pictum* Aiton	Biennial[3]	Congener of *C. officinale*[3] which replaces *C. officinale* in south of Europe[7 10]. *M. crucifer* was found on *C. creticum* (Colonelli, pers. records)[4]	Mediterranean[3]	Within fundamental host range[1] Adult feeding and oviposition in laboratory testing[1]
Cynoglossum germanicum Jacq.		Biennial[3]	Sympatric congener with very similar morphological habitat, overlaps in distribution with *C. officinale*[3 8]	Central Europe[8]	Within fundamental host range[1] Adult feeding and oviposition in laboratory testing[1]
Cynoglossum lanceolatum Forssk.		Perennial[3]	Asian/African congener of *C. officinale*[3]	China, Africa, Asia, Pakistan, India[3]	Previously not included in host range testing
Rindera umbellata (Waldst & Kit.) Bunge	*Cynoglossum umbellatum* Waldst. & Kit. and *Mattia*	Biennial to perennial [6]	Eurasian confamilial in the same subtribe as *C. officinale*[5.]	Central Balkan Peninsula to SW. Ukraine	Within fundamental host range[1] Adult feeding in

	umbellata (Waldst. & kit.) Schult.[6]		Occurs sympatrically with *C. officinale* in Serbia (Ivo Toševski pers. communication)		laboratory testing[1]
Solenanthus circinatus Ledeb.		Perennial	Eurasian confamilial of *C. officinale* Eurasian representative of genus in same subtribe as *C. officinale*[5]	Europe and Asia	Previously not included in host range testing
Lindelofia longiflora (Benth.) Baill.		Perennial[9]	Confamilial of *C. officinale* Representative of genus in same subtribe as *C. officinale*[5]	Western Himalayas[9]	Previously not included in host range testing

[1] (Jordan et al. 1993); [2] (El-Shazly et al. 1996); [3](Joshi 2016); [4](Schwarzländer 1996); [5](Chacón et al. 2016); [6](Perić et al. 2012); [7](De Jong et al. 1990); [8](Marinov 2009); [9](Singh and Lal 2007); [10](Gams 1927).

Table 1.2: Summary statistics for the behavioral response of *Mogulones crucifer* females in multiple-choice bioassays with the volatile headspace of *Cynoglossum officinale*, seven closely related Eurasian and Asian congeners and confamilials, and purified air. Test statistics are log-linear categorical model. Within this model, single-degree-of-freedom contrasts were used for pair-wise comparison between four choices and total time spent in each odor quadrant. n = 20 for all bioassays (see text for details).

	Initial choice		**Final choice**		**Percent time spent in quadrants of olfactometer**	
Plant species	**χ^2a**	**p-value**	**χ^2a**	**p-value**	**χ^2a**	**p-value**
Cynoglossum amabile	2.00	0.5724	0.7	0.7047	304.69	<0.0001
Cynoglossum creticum	6.40	0.0937	3.6	0.3080	245.60	<0.0001
Cynoglossum germanicum	6.00	0.1116	4.8	0.1870	237.31	<0.0001
Cynoglossum lanceolatum	0.40	0.9409	13.02	0.0046	497.52	<0.0001
Lindelofia longiflora	6.26	0.0996	6.63	0.0847	427.22	<0.0001
Rindera umbellata	7.57	0.0557	7.18	0.0662	220.63	<0.0001
Solenanthus circinatus	7.41	0.0599	10.87	0.0125	371.08	<0.0001

a= Logistic regression analysis; df= 3 for all statistics, $p<0.05$

Table 1.3: Summary statistics for behavioral responses of *Mogulones crucifer* females in dual-choice bioassays with volatile headspace of seven Eurasian plant species confamilial to target weed *Cynoglossum officinale* vs. purified air. Test statistics are log-linear categorical model. Within this model, single-degree-of-freedom contrasts were used for pair-wise comparison between four choices and total time spent in each odor quadrant. n = 20 for all bioassays (see text for details).

	Initial choice		**Final choice**		**Percent time spent in quadrants of olfactometer**	
Plant species	**χ^2a**	**p-value**	**χ^2a**	**p-value**	**χ^2a**	**p-value**
Cynoglossum amabile	0.80	0.8495	4.40	0.2214	170.59	<0.0001
Cynoglossum creticum	2.00	0.5724	5.20	0.1577	220.63	<0.0001
Cynoglossum germanicum	3.57	0.3116	4.00	0.2615	192.21	<0.0001
Cynoglossum lanceolatum	1.90	0.3867	2.80	0.2466	199.28	<0.0001
Lindelofia longiflora	1.17	0.7591	16.76	0.0008	99.16	<0.0001
Rindera umbellata	3.95	0.2674	3.95	0.2674	287.24	<0.0001
Solenanthus circinatus	3.95	0.2674	8.96	0.0298	192.78	<0.0001

a= Logistic regression analysis; df= 3 for all statistics, $p<0.05$

Table 1.4: Relative total ion concentration (TIC) peak area percentage based on peak area normalization of the total ion concentration of electrophysiologically active compounds in *Cynoglossum officinale* and selected Eurasian confamilial species. The relative concentration of each compound identified through GC-MS was based on peak area normalization of the total ion concentration.

	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)	3.35	4.76	7.26	8.43	9.35	9.72
Cynoglossum officinale	5.79	2.52	1.66	2.55	16.69	3.14
Cynoglossum amabile	-	1.48	5.50	-	8.47	-
Cynoglossum creticum	-	-	6.35	-	4.20	-
Cynoglossum germanicum	1.10	2.50	3.24	-	19.00	-
Cynoglossum lanceolatum	-	-	3.60	-	-	-
Rindera umbellata	-	-	2.00	-	-	-
Solenanthus circinatus	-	4.11	1.50	-	-	6.04

Table 1.5: Relative total ion concentration (TIC) peak area percentage based on peak area normalization of the total ion concentration of volatile organic compounds collected in the headspace of plant species: *Cynoglossum officinale* (CO), *Cynoglossum amabile* (CA), *Cynoglossum creticum* (CC), *Cynoglossum germanicum* (CG), *Cynoglossum lanceolatum* (CL), *Rindera umbellate* (RU), *Solenanthus circinatus* (SC). RT: Retention time. The relative concentration of each compound identified through GC-MS was based on peak area normalization of the total ion concentration. Tentative identification of compound 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.

	RT (min)	CO	CYA	CC	CG	CL	RU	SC
Propyl aldoxime, 2-methyl	3.039	-	-	-	-	2.70	-	-
Heptanoic acid, 3-oxo-, methyl ester	3.361	-	-	4.05	-	-	-	-
Methyl isovalerate	3.358	5.8	-	-	1.10	-	-	-
Piperidine, 1,1'-dithiobis	3.609	-	-	-	-	1.90	-	-
Octane	3.617	1.44	-	-	-	4.30	-	-
Hexane, 3-ethyl	3.624	-	-	-	-	2.00	3.00	1.50
Hexanal	3.720	-	2.44	-	-	-	-	-
Butanoic acid, 2-methyl-, methyl ester	3.381	-	-	1.64	-	-	-	1.20
Pentanol, 5-amino	3.728	-	-	-	-	-	2.00	1.90
1,2-Benzisothiazol-3-amine	4.011	-	-	2.15	-	-	-	-
3-Hexen-1-ol, (Z)-	4.762	2.52	1.48	-	2.50	-	-	4.11
3(5H)-Furanone	4.881	-	-	-	-	-	-	7.40
Cyclopropane, 1,1,2-trimethyl-3(-2-methylpropyl)	5.242	-	-	1.36	-	-	-	-
Heptanal	5.803	2.81	-	-	-	-	-	-
2(5H)-Furanone	6.077	5.03	-	-	-	-	-	-
Cyclopentane, 1,2,3,4,5-pentamethyl	6.136	-	2.96	2.82	-	-	-	-
Ethanone, 1-(1-methylcyclohexyl)	6.196	2.97	-	-	-	-	2.00	-
2,4,4-Trimethyl-1-hexene	6.841	-	-	3.40	-	-	-	-
1-Hexene, 4,5-dimethyl	6.853	1.22	-	6.85	-	1.00	2.00	-
Cyclohexane, 1-methyl-2-propyl	6.903	-	-	2.52	-	2.80	-	1.40
3-Heptene, 4-propyl	6.905	-	-	-	2.50	-	-	-

m-Menthane	6.913	-	-	2.79	3.40	-	-	2.00
Benzaldehyde	7.261	1.70	5.50	6.35	3.24	3.60	2.00	1.50
1-Heptanol	7.575	1.29	-	-	-	-	-	-
6-methyl-5-heptene-2-one	8.432	2.60	-	-	-	-	-	-
Octanal	8.453	3.64	-	-	-	-	-	4.30
Cyclotetrasiloxane, octamethyl	8.457	-	-	-	-	-	5.00	-
3-Hexen-1-ol, acetate, (Z)-	8.585	17.00	8.47	6.87	19.00	-	-	-
4-Hexen-1-ol, acetate, (Z)-	8.602	-	-	-	-	5.20	1.00	6.10
Hexyl ester acetic acid	8.782	2.51	-	3.09	2.70	2.10	-	-
1-Hexanol, 2-ethyl	9.177	-	3.59	-	-	-	-	-
Cyclohexanone, 2,2,6-trimethyl	9.321	2.14	-	-	-	-	-	-
β-Ocimene	9.723	3.10	-	-	-	-	-	6.04
3-Carene	9.729	-	-	-	-	-	1.00	-
2H-Pyran-2-one, tetrahydro	9.854	-	-	-	-	1.20	-	-
2(5H)-Furanone, dihydro-4-methyl	9.863	-	-	-	-	-	1.00	-
Acetophenone	10.174	-	3.64	-	-	-	-	-
1-octanol	10.381	3.84	-	-	-	-	-	-
Cyclohexen-1-cabonitrile	11.187	-	-	5.29	-	-	-	-
Undecane	11.191	-	5.82	6.21	-	2.50	-	-
Nonanal	11.328	9.50	1.46	5.92	2.20	11.00	2.00	3.70
Linalool	11.453	5.83	-	-	-	-	-	-
Cyclohexane, 2-ethenyl-1, 1-dimethyl-3-methylene-	11.674	-	-	-	-	-	3.00	-
Methyl salicylate	12.89	3.34	-	-	-	-	-	-
Heptasiloxane	13.349	-	-	-	-	-	-	1.80
Decanal	14.19	2.16	3.45	-	-	-	-	2.00
Cyclohexanone, 2-(2-butynyl)	15.499	4.23	-	-	-	-	-	-
2-Decenal	14.186	1.96	-	-	-	-	-	-
Benzaldehyde, 4-(1methylethyl)	15.715	-	-	-	-	-	2.00	-
3-(Prop-2-enoyloxy) dodecane	15.718	-	-	-	-	1.40	-	-

Ethanone, 1-(4-ethyphenyl)	15.72	-	-	-	-	-	2.00	-
2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester	16.243	-	-	-	1.80	-	-	-
2-Trifluoroacetoxytridecane	16.759	-	-	1.65	-	-	-	-
β-sesquiphellandrene	19.881	2.59	-	-	-	-	-	-
α-Farnesene	21.851	2.63	-	-	-	-	-	-
Heptasiloxane, hexadecamethyl	32.197	-	-	1.31	1.80	3.40	-	-
Pentanoic acid	42.642	-	-	2.77	-	-	-	-
Number of volatile compounds (Shared compounds with *C. officinale*)		25	10 (5)	18 (5)	10 (7)	14(5)	13 (3)	14 (6)

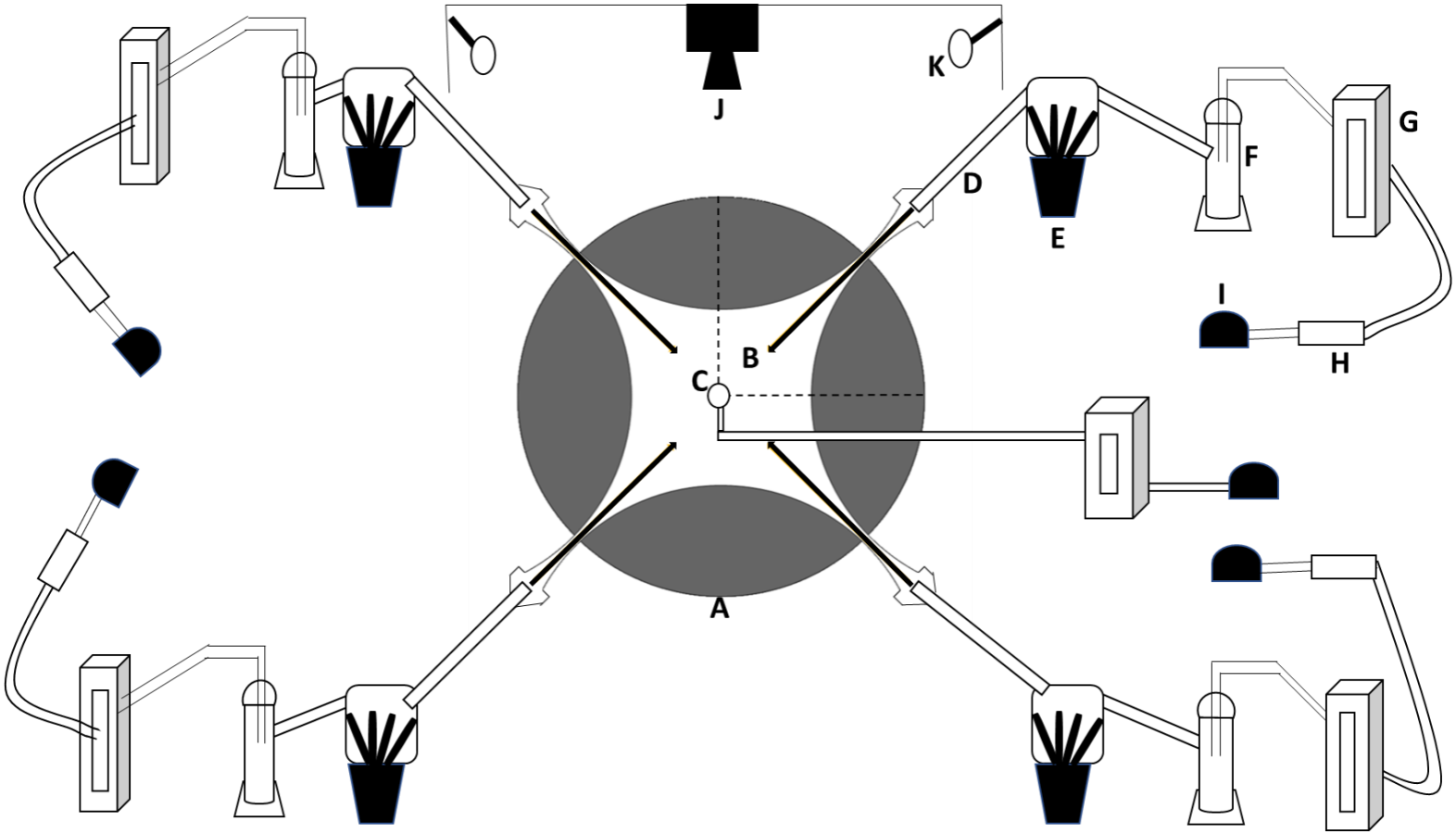
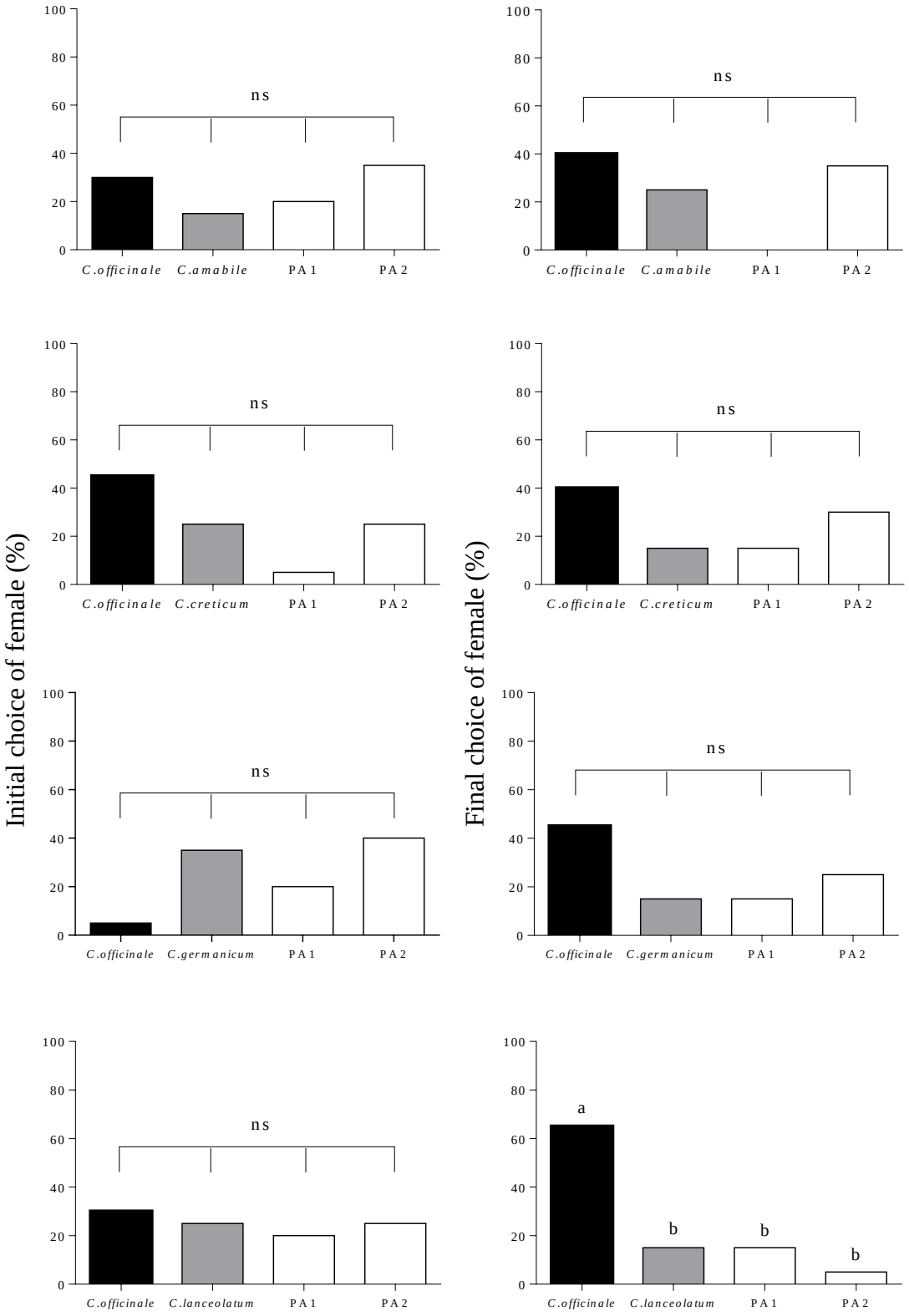


Figure 1.1: Schematic diagram of the four-armed olfactometer used for experiments (not drawn to scale). A: Central arena (22 cm diameter), B: Individual quadrant (55 mm x 55 mm, 10 mm height), C: Insect inlet port, D: Tygon® tube (8 mm internal diameter), E: Odor source (plant foliage enclosed in bag), F: Humidifier, G: Flowmeter, H: Activated charcoal filter, I: Air pump, J: Video camera, K: Light source.


Initial choice of female (%)
Final choice of female (%)
100
80
60
40
20
0
ns
a
b
C.officinale
C.amabile
C.creticum
C.germanicum
C.lanceolatum
PA 1
PA 2

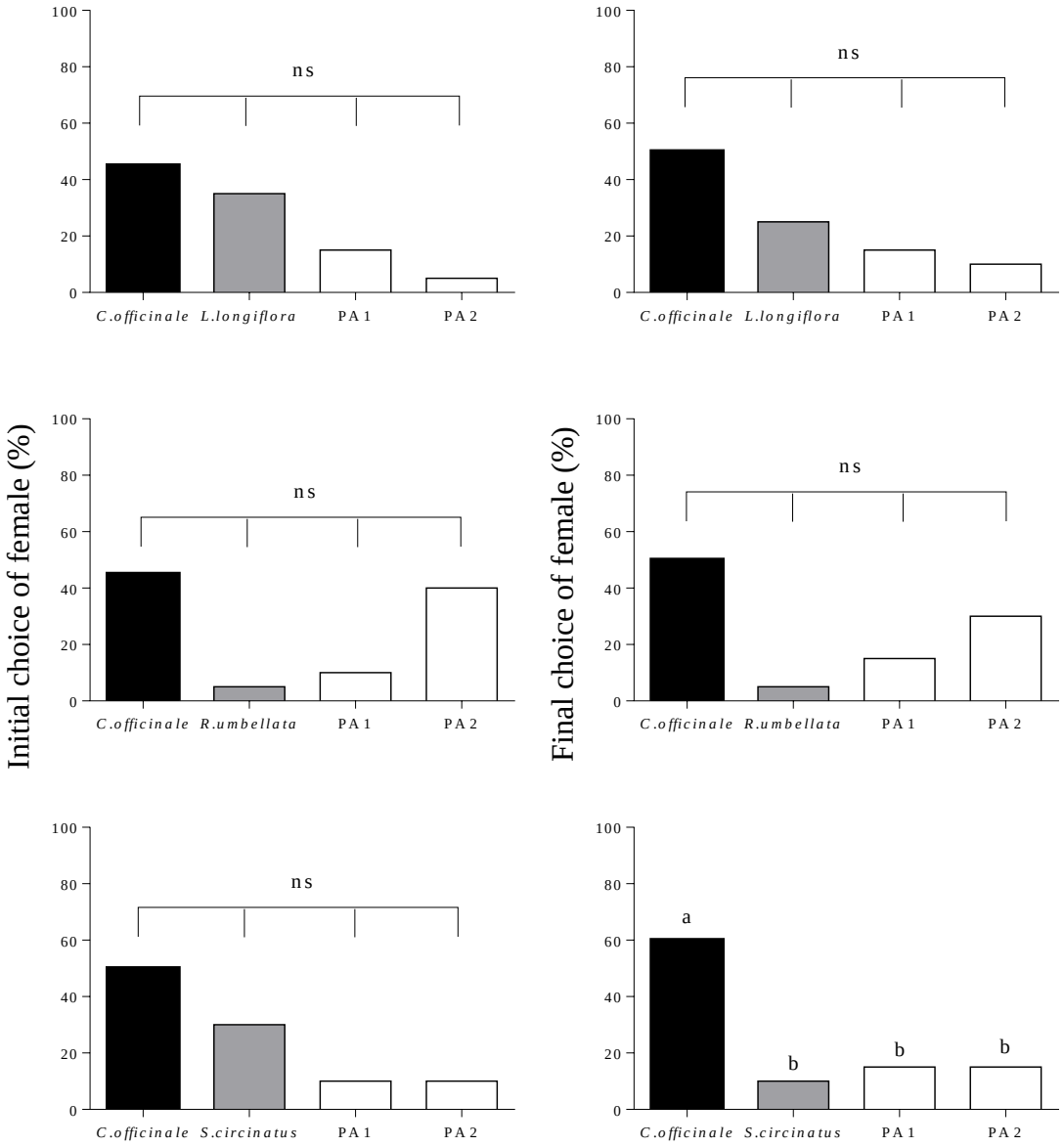


Figure 1.2: Proportion of *Mogulones crucifer* females' initial choice (left column) and final choice (right column) among four quadrants in a four-armed olfactometer arena using volatile headspace of *Cynoglossum officinale*, one test plant species, and purified air in remaining two quadrants (PA1 and PA2). Differing letters on top of bars denote significant differences (χ^2 - test followed by categorical log-linear model $p<0.05$, ns=not significant) (n=20) (see text for details).

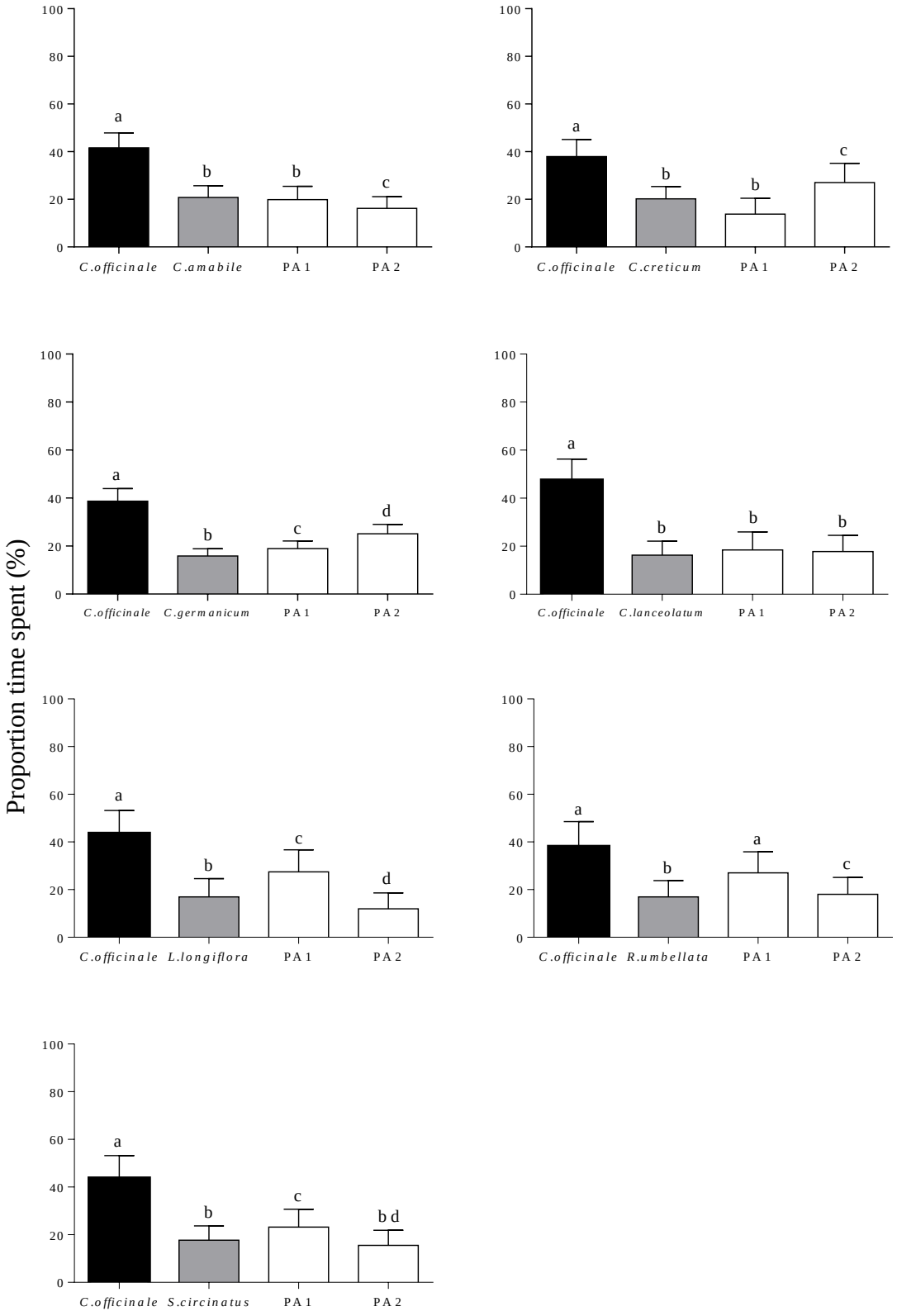
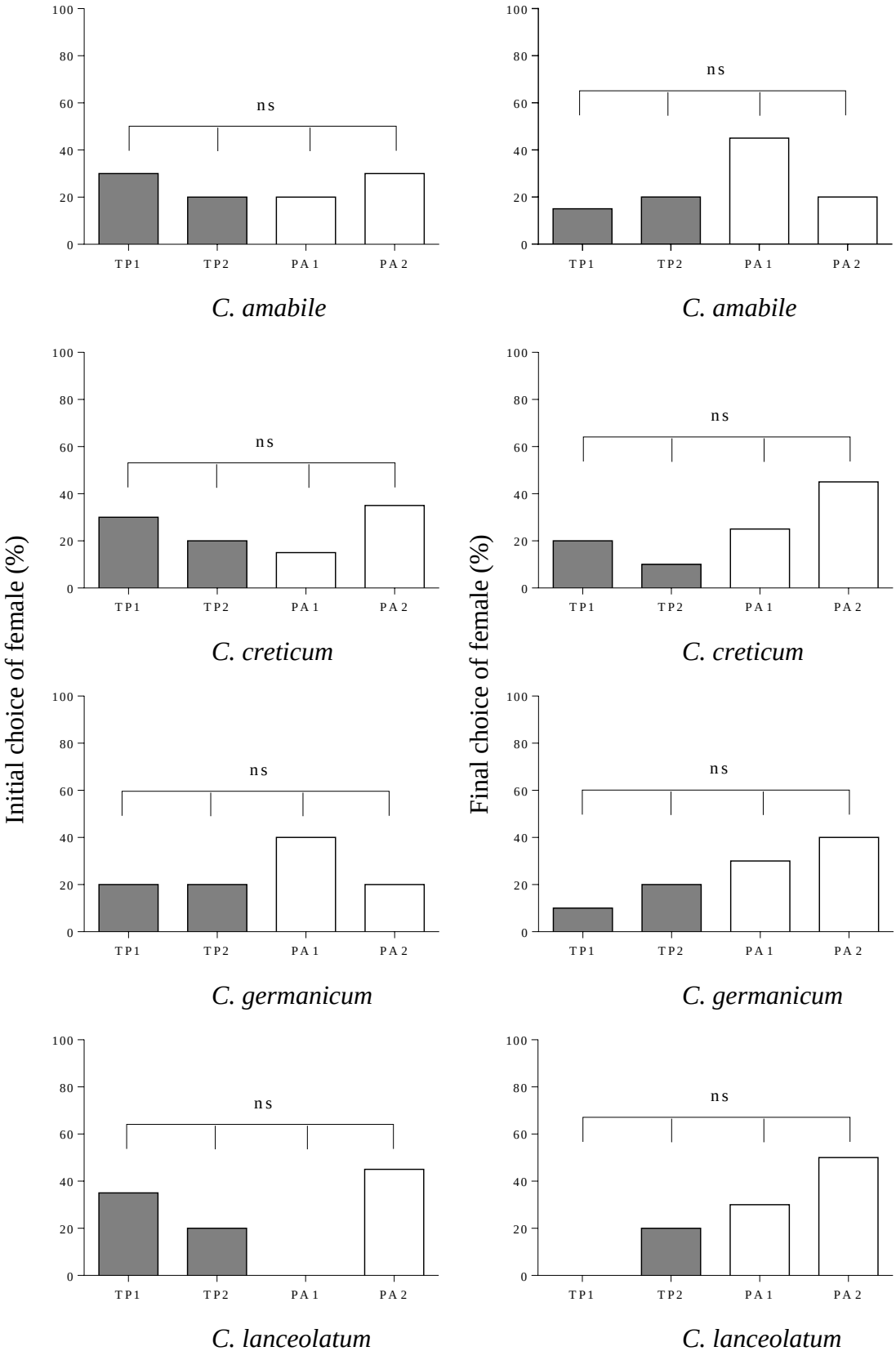

Proportion time spent (%)
100
80
60
40
20
0
a
b
b
c
C.officinale
C.amabile
PA 1
PA 2
a
b
b
c
C.officinale
C.creticum
PA 1
PA 2
a
b
c
d
C.officinale
C.germanicum
PA 1
PA 2
a
b
b
b
C.officinale
C.lanceolatum
PA 1
PA 2
a
b
c
d
C.officinale
L.longiflora
PA 1
PA 2
a
b
a
c
C.officinale
R.umbellata
PA 1
PA 2
a
b
c
b d
C.officinale
S.circinatus
PA 1
PA 2

Figure 1.3: 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 remaining two quadrants (PA1 and PA2). Differing letters on top of bars denote significant differences (categorical log linear model followed by single degree of freedom contrast analysis, $p<0.05$) (n=20) (see text for details).


Initial choice of female (%)
Final choice of female (%)
100
80
60
40
20
0
ns
TP1
TP2
PA 1
PA 2
C. amabile
C. amabile
C. creticum
C. creticum
C. germanicum
C. germanicum
C. lanceolatum
C. lanceolatum

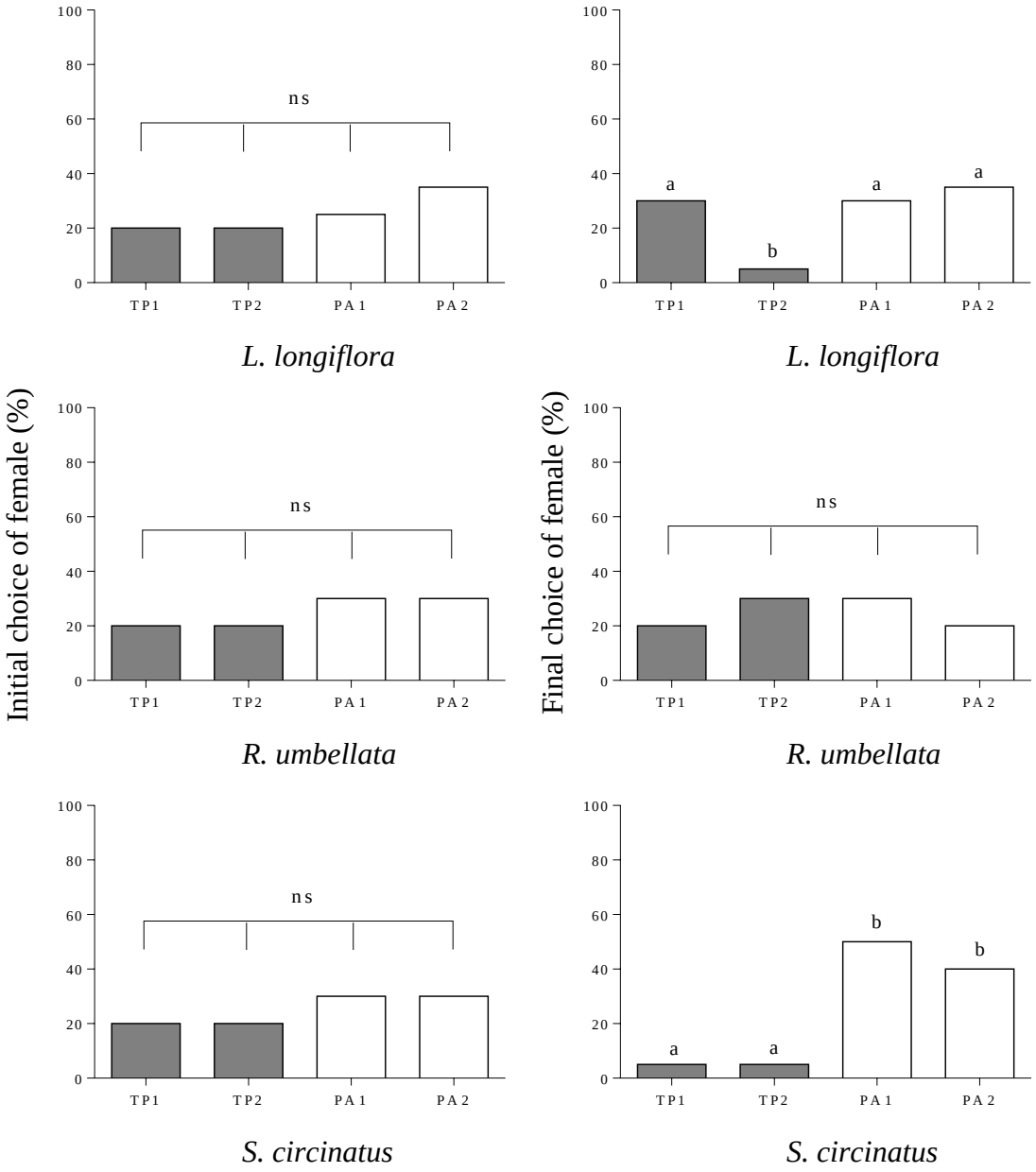


Figure 1.4: Proportion of female *Mogulones crucifer* initial choice (left column) and final choice (right column) among four quadrants in a four-armed olfactometer arena using volatile headspace of one test plant species in two quadrants (TP1 and TP2) and purified air in remaining two quadrants (PA1 and PA2). Differing letters on top of bars denote significant differences (χ^2-test followed by categorical log-linear model, $p<0.05$, ns=not significant) (n=20) (see text for details).

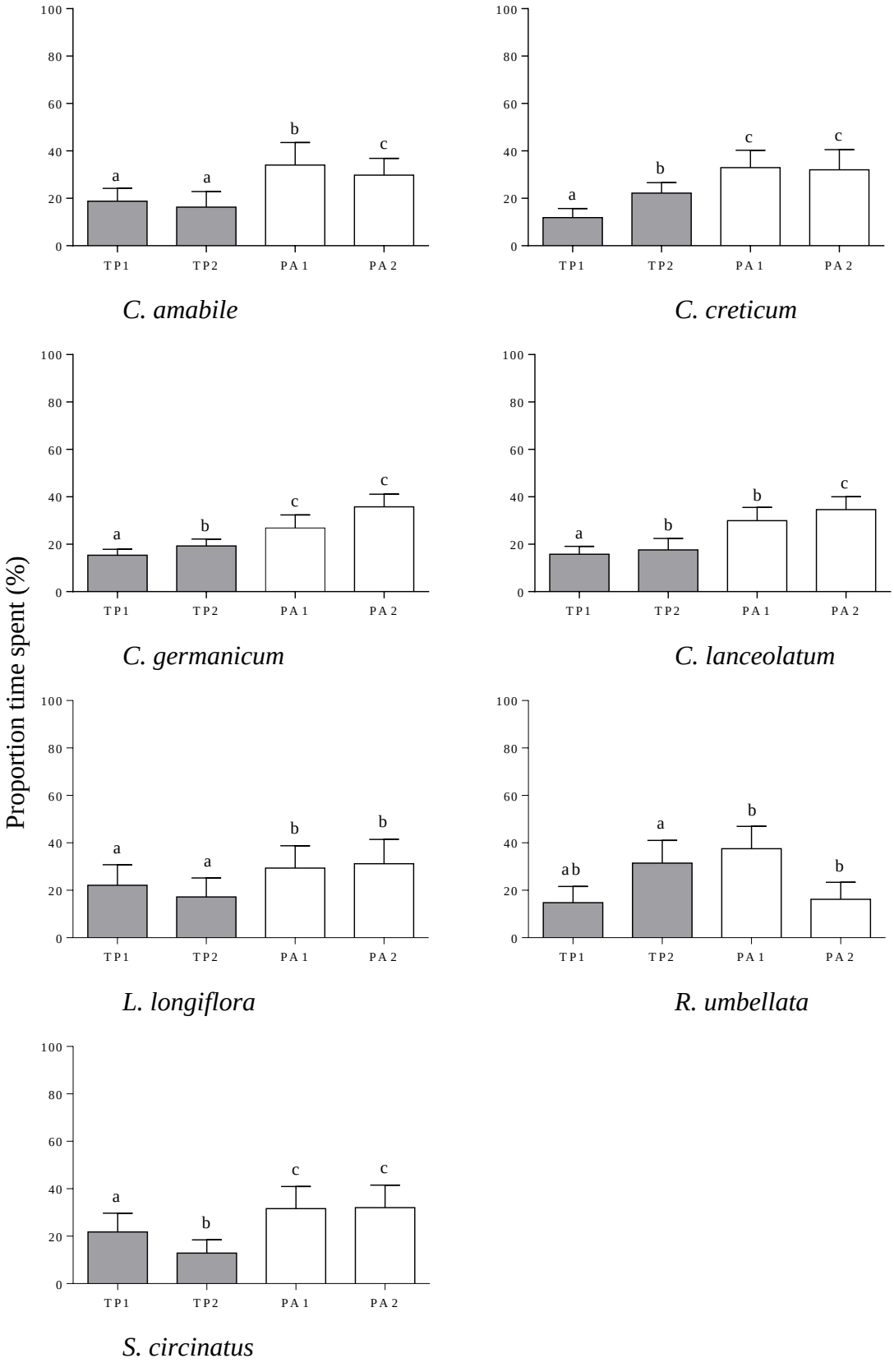

Proportion time spent (%)
100
80
60
40
20
0
TP1
TP2
PA 1
PA 2
a
a
b
c
C. amabile
a
b
c
c
C. creticum
a
b
c
c
C. germanicum
a
b
b
c
C. lanceolatum
a
a
b
b
L. longiflora
a b
a
b
b
R. umbellata
a
b
c
c
S. circinatus

Figure 1.5: 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 remaining two quadrants (PA1 and PA2). Differing letters on top of bars denote significant differences (categorical log linear model followed by single degree of freedom contrast analysis, $p<0.05$) (n=20) (see text for details).

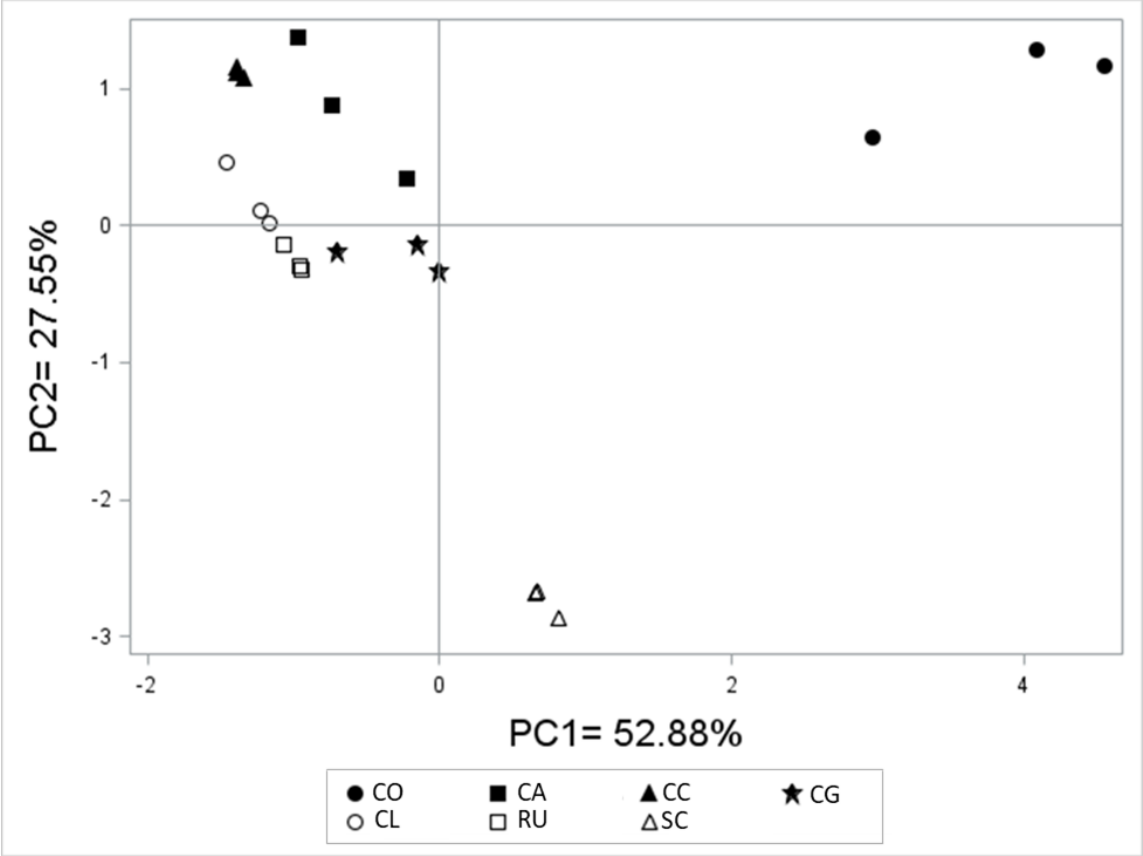


Figure 1.6: Principal component analysis score plot based on relative TIC peak area percentage electrophysiologically active volatile organic compounds identified in *C. officinale*. Plant species: *Cynoglossum officinale* (CO), *Cynoglossum amabile* (CA), *Cynoglossum creticum* (CC), *Cynoglossum germanicum* (CG), *Cynoglossum lanceolatum* (CL), *Rindera umbellate* (RU), *Solenanthus circinatus* (SC).

Chapter 2: BEHAVIORAL AND ELECTROPHYSIOLOGICAL RESPONSES OF *MOGULONES CRUCIFER* TO VOLATILES OF *CYNOGLOSSUM OFFICINALE* AND NORTH AMERICAN CONFAMILIAL PLANT SPECIES

Abstract

In specialist insect herbivores host finding and non-host discrimination is mediated by plant volatile organic compounds (VOCs). In order to appropriately describe the ecological host range of a biocontrol candidate species, the root mining weevil *Mogulones crucifer* Pallas, I investigated whether the weevil could recognize VOC of its Eurasian field host *Cynoglossum officinale* L., which is invasive in western North America and based on VOC, discriminate against five North American confamilial nontarget species during pre-alightment host finding. I conducted behavioral bioassays with *M. crucifer* in a four-armed olfactometer using VOC of *C. officinale,* five confamilial plant species including one species listed as threatened and endangered (T&E) in the United States and purified air as control. In bioassays *M. crucifer* strongly preferred *C. officinale* over all nontarget species and purified air. *M. crucifer* was attracted solely to *C. officinale* and discriminated based on VOC against all confamilial nontargets, responding either with indifference and/or repellence to respective VOC. I then analyzed the foliar VOC headspace of *C. officinale* and seven native North American confamilial nontarget species using gas-chromatography-mass-spectrometry (GC-MS). I found that among the seven nontarget species *Hackelia micrantha* (Eastw.) J.L. Gentry shared the largest number of volatile compounds with *C. officinale* (10) whereas *Oreocarya rugulosa* Payson and *O. celosioides* Eastw. shared the least volatile compounds (3) with *C. officinale*. In addition, I conducted gas chromatography coupled with electroantennographic detection (GC-EAD) experiments with female *M. crucifer* antennae

and VOC of *H. micrantha* and the T&E species *O. crassipes* (I. M. Johnst.) Hasenstab & M.G. Simpson. Five volatile compounds in the foliar headspace of *H. micrantha*, i.e. (z)-3-hexen-1-ol, (z)-3-hexen1-ol acetate, β-ocimene, acetophenone and α-terpineol, of which three were shared with *C. officinale* elicited consistent antennal responses in *M. crucifer* whereas four chemical compounds in the headspace of *O. crassipes*, i.e. m-menthane, 4-cyanocyclohexene, (z)-3-hexen-1-ol acetate and nonanal, of which two were shared with *C. officinale* elicited consistent antennal responses. In behavioral bioassays with *M. crucifer* using three bioactive volatile compounds in these two species that were not shared with *C. officinale*, i.e., acetophenone, α-terpineol, a 1:1 blend of acetophenone and α-terpineol, and 4-cyanocyclohexene all three volatile compounds and the blend of two of the compounds found in *H. micrantha* elicited repelling responses from weevils. Data reported here emphasize the importance of host recognition studies based on olfactory plant cues to advance host range predictions in weed biological control.

Introduction

Classical weed biological control (hereafter weed biocontrol) is defined as the introduction of host-specific natural enemies from the native range of an invasive plant in order to reduce invasive plant densities or the rate of spread permanently in the introduced range (McFayden 1988). The aim of weed biocontrol to re-establish the herbivore-plant relationship in the range where the plant has been introduced and where it is invasive (Clewley et al. 2012; Culliney 2005; Hinz et al. 2019; Hoddle 2004; McFadyen 1998; Messing & Wright 2006; Pemberton 2000; Schwarzländer et al. 2018). If conducted responsibly, weed biocontrol is an environementally benign and safe strategy for the management of invasive weeds (Hinz et al. 2014). However, weed biocontrol has been scrutinized for its environemental safety record by some in the United States since 20 years (Strong 1997). The concerns are a consequence

of reported nontarget attack of the deliberately introduced bicontrol agent *Rhinocyllus conicus* Froehlich (Coeloptera: Curculionidae) on rare native thistles in the genus *Cirsium* in the United States (Louda et al. 2003; Louda et al. 2005; Simberloff 2012). As a consequence, pre-release host-specificity testing of biocontrol candidates for the United States, which was already rigorous, has become even more restrictive to ensure the environemetal safety of agents and minimize the risk for potential nontarget attack (Heard 2002; Hinz et al. 2104; Schaffner 2001; Schwarzlaender et al. 2018b; Wheeler & Schaffner 2013).

Pre-release host-specificity tests are conducted with taxonomically related confamilials of the target weed from their native and introduced range and the selection of test plant species follow the centrifugal phylogenetic approach, i.e., test plant species range from the closest relatives of the target weed to distantly related confamilials (Wapshere 1974). Native confamilial plants in the area of introduction, especiallly those that are rare or endangered and confamilials plants of economic importance (crops and ornamentals) are given emphasis, while selecting nontarget plant species for testing (Schaffner 2001; Wapshere 1974). The fundamental and ecological host range are determined with no-choice and choice tests, respectively. Test types include adult feeding, oviposition, and larval and/or full development experiments under varying laboratory, cage test or open-field conditions (Briese 1999; Heard 2002; Hinz et al. 2014; Marohasy 1998; McEvoy 1996; McFadyen 1998; Kafle 2016; Park et al. 2018; Park et al. 2019; Schaffner 2001; Schaffner et al. 2018; Wapshere 1974). The fundamental host range is determined with no-choice developmental tests and includes those plant species that physiologically support an organism's complete life cycle. However, no-choice tests do not allow the biocontrol candidate to exhibit its normal host selection behavior (Hinz et al. 2014; Schaffner 2001; Sheppard et al. 2005). In contrast, the ecological

host range of a biocontrol candidate is determined using choice tests typically under more natural and sometimes open-field conditions and is designed to identify those plant species that a biocontrol candidate uses as host plants based on both performance and host selection behavior (Schaffner 2001).

In the United States, population level nontarget effects by a deliberately introduced biocontrol agent have only been reported for *R. conicus* on native *Cirsium* species (Louda et al. 1997; Louda et al 2005). The cactus moth *Cactoblastis cactorum* (Berg) (Lepidoptera: Pyralidae), also causes population level effects on native North American cacti in the genus *Opuntia*, but the moth was never deliberately introduced into the United States (Johnson and Stiling 1998; Solis et al. 2004). There are a number of nontarget attack incidences, that are classified as 'sustained' attack, meaning that the biocontrol agent has established and sustained populations on nontarget plants post-release (Hinz et al. 2019). However, incidences of sustained nontarget attack have considerably decreased with time (Bowers et al. 1992; Dodge 2005; Hinz et al. 2019; Thomas et al. 1987). Most cases of nontarget attack are defined as collateral, in which biocontrol agents are present at high densities, for example during population outbreaks, following a sharp decline of host weed densities, or both. Collateral nontarget attack occurs on plant species on which it cannot develop but merely growing in the vicinity of the target weed. In contarst, spillover attack occurs on the plant species within the fundamental host range where it can develop fully or to some extend (Blossey et al. 2001; Catton et al. 2015; Fowler et al. 2000; Hinz et al. 2019; Lynch et al. 2002; McFadyen et al. 2002; Suckling and Sforza 2014).

Specialist insect herbivores must be able to locate their host plant(s) in a complex environment. Their ability to locate their host during the host finding stage of host selection

is partially mediated by plant volatiles synthesized as products of the plant metabolism (Nishida 2014; Pophof et al. 2005; Visser 1986). In a diverse environment, insect herbivores use olfactory chemical cues to identify potential mates, food and oviposition sites (Farkas and Shorey 1972; Nishida 2014; Pophof et al. 2005; Visser 1986). Insect herbivores perceive these plant-emitted olfactory cues with their specialized olfactory receptor neurons (ORNs) and utilize them to identify host plants for feeding and oviposition, while discriminating against non-host plants (Anholt 1992; Mustaparta 2002; Vissser 1986). Insects can distiguish between host and non-host plants based on either species-specific volatile compounds or on species-specific ratios of blends of widely occurring and unique volatile olfactory compounds (VOC) (Bruce et al. 2005; Smart and Blight 1997; Visser 1986). Much of the research on the use of olfactory cues by insects has focused on plant volatile organic compounds of agricultural and forestry pests (Bruce and Pickett 2011; Bruce et al. 2005; Germinara et al. 2016; Weissbecker et al. 2004). In weed biocontrol, the role of plant volatile compounds in the context of the host-specificity of a prospective biological candidate has only been recently studied (Beck et al. 2014; Kafle 2016; Park et al. 2018; Park et al. 2019; Piesik et al. 2015; Pophof et al. 2005; Sutton et al. 2017; Wheeler et al. 2014; Wheeler and Schaffner 2013). In some studies, it has been found that specialized potential/released biocontrol agents prefer olfactory cues of their host plant relative to nontarget plant species (Kafle 2016; Park et al. 2018; Park et al. 2019; Sutton et al. 2017).

The identification of VOC from host plants and nontarget plants for example has been associated with respective behavioral responses of biocontrol candidates (Kafle 2016; Park et al. 2018; Park et al. 2019). Bioassays to detect attraction (preference in an olfactometer for host VOC over nonhost VOC or purified air), indifference (no preference for plant VOC over

purified air) or repellence (preference for purified air over plant VOC) by a biological control agent should help allow assessment of whether that agent would seek out nontarget plant species in the field post-release, regardless of whether those species are included in its fundamental or ecological host range (Martini et al. 2015; Vet et al. 1983; Wheeler and Schaffner 2013).

Mogulones crucifer Pallas (previously *M. cruciger* Herbst) (Coleoptera: Curculionidae) is a univoltine root mining weevil native to central Europe (Koch 1992; Schwarzländer 1997) that is host-specific in its native range (Dieckmann 1972; Lohse 1983; Scherf 1964) to the facultative perennial herbaceous *Cynoglossum officinale* L. (Boraginaceae) or houndstongue, which is an invasive rangeland weed in the northwestern United States and southwestern Canada. *Mogulones crucifer* was petitioned for release as a biological control agent against *C. officinale* in North America and Canada in 1996 and it was released for that purpose in Canada in 1997. Since then it has successfully established and controlled *C. officinale* at all release sites throughout that country (Catton et al. 2016; De Clerck-Floate and Wikeem 2009; De Clerck-Floate et al. 2005). The release of the weevil is however, prohibited in the United States due to concerns by the United States Fish and Wildlife Service (USFWS) about potential risks of nontarget attack on federally listed threatened and endangered (hereafter T&E) confamilial species of *C. officinale.* In the United States, there are six T&E species in the Boraginaceae family, and 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., *P. strictus* (Greene) I.M. Johnst and the subtropical *Eriodictyon altissimum* P. V. Wells (USFWS, 2019).

During host-specificity testing of *M. crucifer,* it was found that the weevil has a broad fundamental host range, which formed the basis for the concerns of the USFWS and prevented the release of *M. crucifer* in the United States. The weevil was able to develop on 16 out of 29 native North American Boraginaceae species tested during no-choice tests and these nontarget species included the T&E species *A. grandiflora*, *P. hirtus* and to a certain extent (one mature larvae found alive in one root) *H. venusta* (Andreas 2004; De Clerck-Floate and Schwarzländer 2002; Jordan et al. 1993; Schwarzländer 1996). In post-release nontarget monitoring in Canada, sporadic attack of *M. crucifer* was observed on exotic and native confamilial species sympatrically occurring with *C. officinale* at field sites. Native nontargets attacked included *H. micrantha* (Eastw.) J.L. Gentry, *Lithospermum ruderale* Douglas ex Lehm. and *Oreocarya spiculifera* Piper (syn: *Cryptantha spiculifera* (Piper) Payson) but attack rates were always low and attributed to spillover (Andreas et al. 2008). Additional post-release nontarget monitoring at the same field sites during 2011 found that *C. officinale* was nearly or completely extinct at the respective field sites and nontarget attack of the aforementioned species had completely subsided (pers. communication Rosemarie De Clerck-Floate). Moreover, in a recent post-release analysis of nontarget attack on *H. micrantha*, a congener of the T&E species *H. venusta*, which occurs sympatrically with *C. officinale* in Canada, it was clearly demonstrated that attack is transitory and limited to spillover (Catton et al. 2015; Catton et al. 2016).

In a previous study, Kafle (2016), found that *M. crucifer* preferred volatiles of its host plant *C. officinale* over those from ten confamilial nontarget species, i.e., nine North American confamilials including four of the five herbaceous T&E species and the European *Borago officinalis* L. When female *M. crucifer* were presented with olfactory cues of confamilial

nontarget plant species vs purified air, the weevils responded either with indifference or were repelled with the nontarget plant species tested (Kafle 2016).

Here, I aimed to add additional information on the pre-alightment host selection of *M. crucifer* by assessing behavioral responses of *M. crucifer* with regard to the remaining herbaceous T&E species, *Oreocarya crassipes*, which was previously unavailable for testing, and other previously untested native North American confamilial species in the genera *Cryptantha* and *Oreocarya*, the most species-rich genera in the United States. The goal of this study was to provide behavioral and electrophysiological explanations for the apparent discrepancy between the broad fundamental host range of *M. crucifer* and its narrower ecological host range observed in the field.

Materials and methods

Materials

During early spring in late April 2018 and 2019, respectively, adult *M. crucifer* were collected from a *C. officinale* infestation near Bonners Ferry, ID (N48. 42373˚, W116. 10759˚) from plants that just began to develop new leaves following overwintering. Weevils were then transported to the laboratory at the University of Idaho in Moscow, ID (N46.7288˚, W117.0126˚). Their gender was visually determined by the presence of a ventral abdominal depression observable in males (Jordan et al. 1993) and 10 to 15 pairs were moved to individual cylindrical plastic containers (diameter: 11 cm, height: 15 cm) lined with a moistened paper towel and covered with a gauze cloth lid. To stimulate natural conditions, cylinders were kept in an environmental chamber (I-35VL, Percival Mfg. Co., Boone, Iowa) at 12:12 (L:D) at 7˚C day: 2˚C night during winter and 16:8 (L:D) at 17˚C day: 8˚C night during spring. Every second day, weevils were fed fresh *C. officinale* leaves during summer

(from May to September) and once a week (from October to April) during winter. Weevils were used throughout the year for bioassays as described below.

North American native confamilial plant species used for this study were selected based on their relatedness to *C. officinale* (Chacón et al. 2016), whether the taxa were previously adequately included in testing or endangerment status, and the availability of propagules (Table 2.1). A recent phylogenetic realignment of the Boraginaceae family separated the genus *Cryptantha sensu. lato*. Most species of the former genus were realigned in the genera *Cryptantha sensu stricto* and *Oreocarya* Greene, in addition three new genera were created (Hasenstab-Lehman and Simpson 2012). Both *Cryptantha* and *Oreocarya* remain the most species-rich genera in the United States (Hasenstab-Lehman and Simpson 2012; Kartesz 1999). Selected species included the following native North American Boraginaceae: *Oreocarya rugulosa* Payson (=*Cryptantha rugulosa* (Payson) Payson) because a congener of this species supported development of *M. crucifer* and it is within one of the species-richest genera endemic to North America (De Clerck-Floate and Schwarzländer 2002), *O. celosioides* Eastw. (=*Cryptantha celosioides* (Eastw.) Payson supported development of *M. crucifer* (De Clerck-Floate and Schwarzländer 2002), *O. crassipes* (I.M. Johnst.) Hasenstab & M.G. Simpson (=*Cryptantha crassipes* I.M. Johnst.) is a federally listed T&E species in the United States, *Cryptantha kelseyana* Greene supported larval development of *M. crucifer* in no-choice experiments (De Clerck-Floate and Schwarzländer 2002; USFWS 2019) and *Cryptantha ambigua* (A. gray) Greene was chosen as a representative of the genus *Cyptantha* (De Clerck-Floate & Schwarzländer 2002). *Hackelia californica* (A. Gray) I. M. Johnst. and *H. micrantha* (Eastw.) J. L. Gentry were added to the list of species selected for this study because *M. crucifer* were repelled by foliar VOC of these species in previous behavioral

bioassays (Kafle 2016) and *H. micrantha* was attacked post-release by *M. crucifer* in the field (Catton et al. 2014; 2015; 2016).

Plants for experiments were propagated from seed. Seeds of the T&E species *O. crassipes* were kindly provided by Karen Little (Sul Ross State University, Department of Biology, Geology and Physical Sciences, Alpine, Texas). *Oreocarya rugulosa, O. celosioides, C. ambigua* and *C. kelseyana* were kindly provided by Dr. Dick Scott (Central Wyoming College, Riverton, Wyoming). *H. californica* plants were collected in the Deschutes National Forest, near Camp Sherman, Oregon (N44.47011˚, W121.6282˚). *Hackelia micrantha* seeds were acquired from Klamath-Siskiyou Native Seeds, Jacksonville, Oregon.

Seeds of all plant species were first hydrated on wet filter paper in Petri dishes (90 mm diameter) for 24 hours. Hydrated seeds were then sown into Sunshine Mix potting soil (Sun Gro Horticulture Canada Ltd., Vancouver, Canada) in seedling starter trays (15 cm x 12 cm x 5 cm) at the University of Idaho. After three to four weeks, seedlings were transplanted into tree pots (12.7 cm x 30.5 cm x 5 L) (Stuewe & Sons, Inc. Tangent, Oregon) in a 1:3 mixture of sand and Sunshine Mix No. 2 (Sun Gro Horticulture Canada Ltd., Vancouver, Canada) along with 2.5 g trace elements (FRIT Industries, Inc., Ozark, Alabama), 1.25 g chelated iron (Grow More Inc., Gardena, California), and 47.5 g limestone (Grow More Inc., Gardena, California), 47.5 g triple super phosphate (Bonide Products Inc., Oriskany, New York), and 187.5 g Osmocote® (The Scotts Company LLC., Marysville, Ohio) per 12 kg of sand and Sunshine Mix No. 2 blend. Transplanted plants were watered lightly every third day and maintained at the University of Idaho's greenhouses at the Manis Entomological Laboratory and Parker Research Farm in Moscow, Idaho at 16:8 (L:D) and 25˚C day and 18˚C night.

Due to the inability to propagate *H. californica* plants in the lab from seed, rootstocks of

these plants were dug up in the field in the Deschutes National Forest and transferred in tree pots with soil from the field site to minimize transplant shock. Plants were maintained in an environmentally controlled greenhouse and watered every second day at the Parker Research Farm at 16:8 (L:D) and 25˚C day and 18˚C night.

Behavioral olfactometer bioassays

We followed the protocols for behavioral bioassays using a four-armed olfactometer as described in detail in Chapter 1. A four-armed olfactometer (Syntech Ltd., Hilversum, The Netherlands) as described by Vet et al. (1983) was used to assess behavioral responses of female *M. crucifer* to volatiles from the host plant *C. officinale* and nontarget North American confamilial plants (Fig. 1.1). We used methods previously developed for four-armed olfactometer tests with *M. crucifer* (Kafle 2016). In brief, the olfactometer allows four different odors to be pushed through four-inlet arms into a central rhomboid-shaped experimental arena (diameter: 22 cm) with a basal outlet, and covered with a heavy clear glass plate (thickness: 10mm), within the glass plate an individual insect can freely move (Vet et al. 1983). To separate the arena into four quadrants, perpendicular lines were drawn on the glass plate meeting in the center and dividing the experimental area in four 37.50 cm^2 quadrants. Tygon ® tube (8 mm internal diameter, Fisher Scientific Co., Pittsburgh, Pennsylvania) was used to connect each of the four inlet arms to volatile sources: foliage of potted plants, which were placed inside sealed, sterilized polyvinyl acetate bags (20 cm x 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 into the olfactometer air that was purified using activated charcoal (Sigma-Aldrich Co. LLC, St. Louis, Missouri) and humidified by passing through distilled water in a 500 ml gas-washing

bottle (Chemglass Life Sciences LLC, Vineland, New Jersey). Air flow rate was set to 300 ml/minute, measured and maintained with flow meters (King Instrument Company, Ins., Garden Grove, California). From the basal outlet of the olfactometer, air was drawn at the rate of 1200 ml/minute using a Rena ® Air 400 pump. Plants used as volatile sources were kept diagonally in two opposite directions and purified air (control treatments) in two remaining quadrants. For dual-choice experiments, volatiles from a single plant species were delivered to the opposing quadrants. For multiple-choice experiments, volatiles from *C. officinale* were delivered opposite to volatiles of a different plant species, while the remaining quadrants received purified air. Details of these two bioassay designs are provided below. White polyethylene vinyl acetate (PEVA) sheets were used to eliminate visual cues distraction to *M. crucifer*. For uniform light in the olfactometer, a single full spectrum LED light source (Jansjö ® LED lamp, Inter Ikea Syatem B. V., Delft, The Netherlands) was used. We used female *M. crucifer* with previous contact with *C. officinale* in experiments assuming that females are more responsive than males to suitable hosts because they must find hosts for oviposition. We determined in previous tests that female *M. crucifer* were reactive in the olfactometer bioassays and responses did not differ from those of males or naïve females (Kafle 2016). Weevils used during experiments 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 arena from the outlet using a fine paintbrush. The hose was reconnected, and the behavior of the weevil was recorded for 30 min. with a video camera (Contour Roam 2, Contour Ins., and Seattle, Washington) fitted above the olfactometer arena. Weevils were recorded as ‘unresponsive’ if they did not make any choices after 5 min. of exposure and

were discarded from the experiments. Each weevil was used only once. After every five replicates, the odor sources were replaced and the olfactometer was rotated 90 degrees to reduce positional effects. After 10 replicates, all the tubes were washed with distilled water and 70 % ethyl alcohol. The initial choice of a weevil was determined when it entered a 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 conducted between 0900 hrs. and 1600 hrs. The video recordings containing movement and positions of weevils were analyzed using the behavioral software program Noldus Observer XT 11 (Noldus Information Technology BV, Wageningen, The Netherlands).

The following response variables were measured in bioassays: initial choice, final choice and the proportion time spent (TS) by *M. crucifer* females in each quadrant. The initial choice is defined as the quadrant first entered by weevil for a minimum of 30 sec. and was used as a measure to evaluate *M. crucifer*'s ability to discriminate between different odors quickly. The final choice, i.e., the quadrant in which a weevil was found at the end of the 30 min. reporting period was because it was assumed to be *M. crucifer's* ultimate preferred odor source. The time proportion in each quadrant of the olfactometer arena was considered a measure for the strength of preference of an odor.

Dual-choice bioassays

We conducted dual-choice bioassays in which the weevil was given a choice between headspace volatiles of a confamilial plant species and purified air to determine whether *M. crucifer* females were able to identify these plant species as potential hosts in the absence of *C. officinale*. These bioassays allowed for three possible behavioral outcomes: attraction,

when the plant volatiles were preferred over purified air; indifference, when plant volatiles were not preferred over purified air; and repellence, when purified air was preferred over plant volatiles (Martini et al. 2015; Kafle 2016). For this, two opposing arms of the olfactometer were provided with headspace volatiles from one plant species and the two perpendicular arms of the olfactometer were provided with only purified air as control. The responses of weevils in relation to the control (purified air) were measured as described above. For each plant species tested, there were 20 replicates.

Multiple-choice bioassays

We conducted bioassays in which female *M. crucifer* could choose between odors of *C. officinale* and of one nontarget plant species to determine the relative attraction of nontarget confamilial plant species to the weevil in the presence of its preferred field host. For these tests, volatile headspace from *C. officinale* was provided in one arm and volatile headspace from one non-target confamilial was provided in the opposing arm of the olfactometer. Purified air was provided in the other two perpendicular arms. The responses of weevils to confamilial plant volatiles in relation to *C. officinale* volatiles and purified air were assessed. As for dual-choice tests, for each *C. officinale* vs. nontarget species test, 20 replicates were conducted, and the response variables were recorded for each weevil as described above.

Collection and analyses of plant volatiles

VOCs were collected in 2018 at the Manis Entomological Laboratory and Parker Research Farm greenhouses using a portable volatile collection system (PVCS) (Park et al. 2019). Volatiles were collected from *C. officinale* and individual potted plants of the following plant species that were used in behavioral bioassays: *C. ambigua, C. kelseyana, O. celosioides, O. rugulosa*. For the T&E *O. crassipes*, volatiles were collected in the field in Alpine, Texas

(N29.548108˚, W103.586249˚) in early March 2018. Volatiles from *H. californica* and *H. micrantha* which were only used for volatile analysis were collected in a greenhouse at the Parker Research Farm. VOC collection and storage were identical for *C. officinale* and seven North American plants. For VOC collections, we followed the protocols described in detail in Chapter 1. Pre-sterilized (140℃ for 1 hr.) polyvinyl acetate bags (20 cm x 15 cm, Reynolds Consumer Products LLC., Richmond, Virginia) were used for the collection of foliar volatiles from individual plants. While collecting foliar volatiles, the end of the bags was gently sealed using pre-sterilized cotton balls and cable ties. Rena ® Air 400 pumps (Mars Fishcare North America, Inc., Chalfont, Pennsylvania) were modified to create push-pull pumps by switching the direction of the diaphragm within the pump assemblage to create a uniform airflow. Air was purified using activated charcoal filters (Orbo ™, Sulpelco, Sigma-Aldrich Co. LLC, St. Louis, Missouri), and pushed into the bag at a rate of 300 ml/min. through a perforation (5 mm) made at the upper corner of the bag. A modified Rena ® Air 400 pump was used to draw foliar volatile headspace air out of the bag at a rate of 300ml/min. and VOC were collected in volatile collection traps (VCTs hereafter) containing 40 mg of 80-100 mesh Porapak-Q adsorbent (Southern Scientific Inc. Micanopy, Florida). Prior to collection, VCTs were rinsed with 1000 µL of dichloromethane (EMD Chemicals Inc., Gibbstown, New Jersey) to remove contamination in the adsorbent. Four pairs of flowmeters (King Instrument Company Inc., Garden Grove, California) were used to maintain airflow through the bags. Based on the number of volatile peaks obtained from gas chromatography-mass spectrometry analysis of collected volatile samples and previous research (Kafle 2016), collection time was set to 6 hrs. (0900 h to 1500 h). Volatiles were collected from 3 plant individuals along with 1 control (surrounding air) simultaneously.

After each collection, the VOC in the VCTs were extracted by eluting with 200 μL of dichloromethane into a glass vial (National C5000-180, Thermo Fisher Scientific Inc., Rockwood, Tennessee) and stored in a freezer (-80˚C) for later use.

A Hewlett-Packard 7890 Gas Chromatograph (Agilent Technologies Inc., Palo Alto, California) equipped with a fused silica HP-5MS capillary column (30 cm x 0.25 mm x 0.25 μm, Agilent Technologies Inc., Palo Alto, California), which is coupled with a Hewlett-Packard 5973 Mass Selective Detector (Agilent Technologies Inc., Palo Alto, California) was used for identification and analysis of headspace volatile organic compounds. Temperature of the injection port was set to 250˚C. The initial oven temperature was set to 40˚C and the temperature was held 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., then held isothermally for 2 min. Helium was used as a carrier gas at 3.0 mL/min. Volatile extract (1 μL) was injected into the gas chromatograph using splitless mode. Mass spectra were obtained using electron impact (EI, 70 eV). The relative amount of each identified component was determined based on peak area normalization of the total ion concentration (Puttick et al. 1988). Those compounds with a relative peak area of 1% or more of the total chromatogram in any one of three samples were included. Peaks detected in both plant volatile samples and purified air control samples were regarded as contaminants and subtracted from the total peak area. 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 addition to *C. officinale*, headspace VOC of *H. micrantha* and *O. crassipes* were subjected to coupled gas chromatography-flame ionization detector/electroantennogram analysis with female *M. crucifer* to detect electrophysiologically active compounds in the volatile blend of those plant species. *The same protocols as described in Chapter 1 were used for the GC-FID/EAD experiments.* GC-EAD recordings were performed with *C. officinale, H. micrantha* and *O. crassipes* VOC using five different female *M. crucifer* antennal preparations for each plant species. Peaks detected by FID GC were determined to be electrophysiologically active if they elicited antennal responses i.e. the voltage changes in the antenna was distinguishable from background noise which arise from antennal preparations and the GC-EAD associated hardware in at least three of the five antennal preparations used. Synchronous voltage changes by both FID and antennae to the respective compounds indicate olfactory sensitivity by the insect to the compound eluting at that particular retention time and any contributions from potential impurities (as identified by GC-MS analysis of respective volatile samples) were discarded.

Behavioral bioassays with specific volatile compounds from nontarget confamilial plant species

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 *H. micrantha*-emitted volatile compounds α-terpineol (Sigma-Aldrich Co. LLC, St.

Louis, Missouri) and acetophenone (Sigma-Aldrich Co. LLC, St. Louis, Missouri) and the *O. crassipes*-emitted volatile compound 4-cyanocyclohexene (Sigma-Aldrich Co. LLC, St. Louis, Missouri). Remaining pure compounds from both plant species were not commercially available at the time of experiments. Two inlet arms were 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 α-terpineol, acetophenone, 4-cyanocyclohexene and a 1:1 blend of 5 µL of 10ng/µL of α-terpineol and acetophenone dissolved in mineral oil (Paraffin oil, light, Thermo Fisher Scientific Inc., Fair Lawn, New Jersey), and the remaining two to purified air. Volatile compounds were individually tested in two quadrants of the olfactometer and purified air was provided in the remaining quadrants and considered as control. Four Rena ® Air 400 push pumps (Mars Fishcare North America, Inc., Chalfont, Pennsylvania) 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 500 ml gas-washing bottles (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 create a pull pump 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 % of *M. crucifer*). A weevil was considered to have made an initial choice (IC) for an odor when it entered 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 (FC) of that weevil. Bioassays were only conducted between 0900 h and 1600 h. All 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, final choice and proportional time spent in each quadrant of the olfactometer arena.

Statistical analysis

First choice quadrant, final choice quadrant, and time spent each quadrant were treated as categorical responses. The proportion of initial choices and final choices of female *M. crucifer* in bioassays were initially assessed using χ-square tests of homogeneity. A log-linear

categorical model was subsequently used to assess pair-wise comparisons among quadrants. The strength of preference for each choice was measured with the time (min. and sec.) spent in each quadrant of the four-armed olfactometer. Differences among the four quadrants were assessed using a log-linear categorical model assuming the time to be a discrete count of the total accumulated seconds spent in each quadrant. Within this model, single degree-of-freedom contrasts were used to compare quadrants count (secs). For all analyses, p-values <0.05 were regarded as significant.

Relative concentrations of VOC compounds identified through GC-MS were based on peak area normalization of the total ion concentration. Electrophysiologically active compounds that are present in *C. officinale*, as identified by GC-FID/EAD in percentage of total ion concentration and listed in results and listed in results, 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). All analyses were conducted using the statistical software SAS Version 9.4 (SAS Institute Inc., 2013).

Results

Multiple-choice bioassays with C. officinale*, confamilial species and purified air*

In multiple-choice bioassays, *M. crucifer* females did not prefer *C. officinale* VOC over any North American confamilial species tested here based on their initial quadrant choice (IC) (Fig. 2.1, Table 2.2). *Mogulones crucifer* preferred *C. officinale* VOC over *C. ambigua* ($p<0.0125$), *O. crassipes* ($p<0.0363$), *O. celosioides* ($p<0.0338$) and *O. rugulosa* ($p<0.0136$) for their final choice (FC) but not *C. kelseyana* ($p=0.1501$) (Fig. 2.1, Table 2.2). *Mogulones crucifer* spent more time in quadrants with *C. officinale* VOC when compared to all confamilial plant species tested or purified air ($p<0.0001$ for all species) (Fig. 2.2, Table 2.2).

Dual-choice bioassays

Previous study (Kafle 2016), found that *M. crucifer* consistently attracted to *C. officinale* volatiles in dual-choice bioassays, spending more time in those quadrants than in purified quadrants. In my dual-choice bioassays, *M. crucifer* responded with indifference or were repelled to VOCs of the five confamilial plant species when compared to purified air. *M. crucifer* females preferred purified air over VOC of *O. crassipes* ($p<0.0337$) and *O. rugulosa* ($p<0.0321$) for their initial choice (IC). There was a tendency towards repellence in the initial choice for *C. kelseyana* ($p=0.0557$) (Fig. 2.3, Table 2.3). *M. crucifer* females responded with indifference in their final choice to all plant species tested although there was a tendency towards preference of purified air over VOC for *C. kelseyana, C. ambigua* and *O. crassipes* (for all $p=0.0557$) (Fig. 2.3, Table 2.3). Female weevils spent more time in quadrants with purified air than in quadrants with VOC of any or the tested confamilial species ($p<0.0001$ for all species) (Fig 2.4, Table 2.3).

Plant species VOC

The volatile headspace of *C. officinale* comprised 25 volatile chemical compounds. The total number of identified compounds obtained from the seven confamilial species ranged from 3 to 23 with the highest number found in *C. ambigua* (Table 2.8). (Table 2.8). *Hackelia micrantha* shared the largest number of compounds (10) with *C. officinale* and *O. crassipes* and *O. rugulosa* shared the least number (3) of compounds with *C. officinale* (Table 2.8).

GC-EAD

Based on six electrophysiologically active compounds from *C. officinale* (Table 2.5), principle component analyses separated *C. officinale* from all seven North American species tested. The first principal component (PC1) explained 41.59% of the variability and separates *C. officinale* from *C. ambigua*, *C. kelseyana*, *H. micrantha*, *O. celosioides*, *O. crassipes* and

O. rugulosa (Fig. 2.7). The second principal component (PC2) explained 30.81% of variability and separates *C. officinale* from *O. crassipes*, *H. californica* and *H. micrantha* (Fig. 2.7).

Electrophysiological analysis with C. officinale, H. micrantha *and* O. crassipes

M. crucifer responded to six chemical compounds in *C. officinale*: methyl isovalerate, (z)-3-hexen-1-ol, benzaldehyde, 6-methyl-5-hepten-2-one, (z)-3-hexen-1-ol acetate, and (z)-β-ocimene (Table 2.5). Five chemical compounds in the VOC headspace of *H. micrantha*, i.e. (z)-3-hexen-1-ol, (z)-3-hexen1-ol acetate, β-ocimene, acetophenone and α-terpineol elicited consistent antennal responses in female *M. crucifer* (Table 2.6). Similarly, four chemical compounds in the VOC headspace of *O. crassipes*, i.e., m-menthane, 4-cyanocyclohexene, (z)-3-hexen-1-ol acetate and nonanal elicited consistent responses in female *M. crucifer* (Table 2.7).

No-choice bioassays with pure compounds

In behavioral bioassays with acetophenone, α-terpineol, or a 1:1 blend of acetophenone and α-terpineol and 4-cyanocyclohexene, *M. crucifer* females preferred purified air quadrants over pure VOC compound quadrants for their initial choice with the exception of the 1:1 compound blend to which the weevils responded with indifference ($p=0.2615$) (Fig. 2.5, Table 2.4), *M. crucifer* preferred purified air quadrants over all VOC tested in their pure form or blended for their final choice (FC) (Fig. 2.5, Table 2.4). *M. crucifer* females also spent more time in quadrants with purified air when compared to the VOC compounds tested including 1:1 blend quadrant ($p<0.0001$) (Fig. 2.6, Table 2.4).

Discussion

Behavioral responses of Mogulones crucifer *to volatile headspace of nontargets and* C. officinale

Similar to Kafle (2016), *M. crucifer* females preferred their Eurasian field host *C. officinale* over all tested North American native confamilial plant species. Specifically, the weevil was only attracted to VOC of *C. officinale* while females responded with indifference or were repelled to VOC from the tested nontargets. Based on time spent, *M. crucifer* females were repelled by all three *Oreocarya* spp. (*O. celosioides, O. crassipes* and *O. rugulosa*) and both *Cryptantha* spp. (*C. ambigua* and *C. kelseyana*) tested. *M. crucifer* were also repelled by two *Oreocarya* species (*O. crassipes* and *O. rugulosa*) in their initial choice. In previous host-specificity studies it was found that *M. crucifer* successfully developed on six *Cryptantha* species, including *C. kelseyana* and the former *C. celosioides* (now *O. celosioides*) though at lower rates when compared to *C. officinale* (De Clerck-Floate and Schwarzländer 2002). *Oreocarya spiculifera* was found attacked at very low and varying degrees at *C. officinale* field sites in Canada but this nontarget attack was considered to be spillover attack, i.e., plants were attacked only because *C. officinale* densities were declining and/or *M. crucifer* populations were large or at outbreak densities (Andreas et al. 2008; Hinz et al. 2019). The fact that plants are not or only very marginally attacked in the field can be explained by the responses of *M. crucifer* females in our bioassays to VOC of species in these two important Boraginaceae genera as described above. Moreover, we found that *Mogulones crucifer* females were repelled in all measured response variables (initial choice, final choice and time spent) by a volatile compound unique in this study to *O. crassipes,* 4-cyanocyclohexene, which elicited an electrophysiological response in *M. crucifer.* Unfortunately, we were not able to acquire a second bioactive volatile compound found in the headspace of *O. crassipes,* m-menthane, but based on our consistent results testing pure volatile compounds, we speculate that this volatile compound would also have triggered repelling responses by *M.*

crucifer. Most *Oreocarya* and *Cryptantha* species have a southwestern distribution and occur primarily in dry and desert habitats in the United States (Hasenstab-Lehman and Simpson 2012; Higgins 1969; USDA, NRCS 2019). In contrast, *C. officinale* has a more northwestern distribution and requires ample winter and spring moisture (De Jong & Klinkhamer 1988). In addition, there are few reports of sympatric occurrence of species in the genera *Oreocarya* and *Cryptantha* with *C. officinale* (Kartesz 1999). Based on the observed indifference/repellence and the lack of overlapping distribution of the tested T&E *O. crassipes*, the probability of encounter with *M. crucifer* is vanishingly small for this species and should render *O. crassipes* safe and free from attack (De Clerck-Floate and Schwarzländer 2002).

Electrophysiological and behavioral responses to non-host plant volatiles

In this study, the volatile profile of *C. officinale* and native confamilial plant species comprised terpenoids, alcohols, aromatic compounds, aldehydes, esters, ketones and mono- and sesquiterpenes. Some volatile compounds present in *C. officinale* which are heptanal, 2(5H)-furanone, 1-heptanol, 1-octanol and β-sesquiphellandrene and confamilial plant species i.e., 4-cyanocyclohexene, acetic acid in *O. crassipes*, β-myrcene and caryophyllene in *H. micrantha* are species-specific.

M. crucifer antennae responded to six chemical compounds in the volatile headspace of *C. officinale*: methyl isovalerate, (z)-3-hexen-1-ol, benzaldehyde, 6-methyl-5-hepten-2-one, (z)-3-hexen-1-ol acetate, and (z)-β-ocimene in previous (Kafle 2016) and in our GC-FID/EAD experiments. Principle component analysis separated *C. officinale* and all the tested plant species which indicates that headspace volatiles of *C. officinale* is different from other selected confamilial plant species. Antennae of *M. crucifer* females responded to five volatile

compounds emitted by *H. micrantha*, three of which were shared with *C. officinale.* Although the volatile α-Terpineol has been reported (hydrodistilled) from at least six different plant families (Abdallah et al. 2013; Ariño et al. 1997; Bachrouch et al. 2010; Bilia et al. 2014; Nasi et al. 2008; Nickavar et al. 2002; Rota et al. 2008; Sacco and Chialva 1988; Zhang et al. 2015). α-Terpineol has been studied for its potential as an insecticide against two stored grain insect pests, the locust bean moth *Ectomyelosi ceratoniae* Zeller and the Mediterranean flour moth *Ephestia kuehniella* Zeller (both Lepidoptera: Pyralidae), where it decreased fecundity and hatching rates of the storage pests when applied as a fumigant (Bachrouch et al. 2010). α-Terpineol may repel *M. crucifer* whenever it is part of a plant VOC blend, because female weevils responded also with repellence to a congener of *H. micrantha*, *H. californica*, which had α-Terpineol in its foliar VOC headspace (Kafle 2016). *M. crucifer* antennae also responded to acetophenone, a volatile compound present in *H. micrantha* and *C. amabile*, both plant species that triggered repelling behavioral responses of *M. crucifer* during olfactometer bioassays. Different derivatives of acetophenone have been extracted from at least six different plant families (Bruce and Pickett 2011; Gupta and Singh 1989; Mageroy et al. 2017; Singh et al. 1997; Spencer and Towers 1991; Zhang et al. 2010). In one study, the presence of acetophenone in white spruce, *Picea glauca* (Moench) Voss was associated with resistance of the conifer against the Eastern spruce budworm, *Choristoneura fumiferiana* Clemens (Lepidoptera: Torticidae) (Mageroy et al. 2017). Repellence of *M. crucifer* to α-terpineol and acetophenone in behavioral bioassays suggests that this weevil uses this specific volatile compound to recognize nonhosts during host finding. The repellence in our study would explain the reduced use of *H. micrantha* in the

field by the weevil over time and lack of nontarget attack in the absence of *C. officinale* (Catton et al. 2014; 2015; 2016)

When a 1:1 blend of α-terpineol and acetophenone was tested, female *M. crucifer* were repelled to the blend based on FC and TS but responded with indifference their initial choice. Behavioral responses of insects may differ according to quality and quantity of plant volatile compounds (Birkett et al. 2004; Bruce and Pickett 2011; Quiroz et al. 1997). For example, the orange wheat blossom midge, *Sitodiplosis mosellana* (Géhin) (Diptera: Cecidomyiidae), responded differently to differing bends of volatiles in a four-armed olfactometer. When a natural ratio of the blend (7 ng α-pinene, 5 ng 6-methyl-5-hepten-2-one, 10 ng 3-carene, 4 ng acetophenone and 4 ng 2-dodecanone) was offered, midges were attracted to the blend. However, when an unnatural ratio of the blend (7 ng α-pinene, 15 ng 6-methyl-5-hepten-2-one, 10 ng 3-carene, 4 ng acetophenone and 4 ng 2-dodecanone) was tested, insects were no longer attracted blend of host volatiles to the (Birkett et al. 2004; Quiroz et al. 1997). While there are numerous studies on specific blends of ubiquitous chemical compounds in mediating host plant recognition by specialist herbivores (Bruce and Pickett 2011; Bruce et al. 2005; Cunningham 2012; Visser 1986), very few have discussed the role of specific volatile compound blends in mediating nontarget plant discrimination (Zhang et al. 2015). One of the few studies that exists tested male and female *Ectropis obliqua* Prout (Lepidoptera: Geometridae) in a y-tube olfactometer and found that they were repelled by the following volatile compounds from the non-host plant *Rosmarinus officinalis* L. (Lamiaceae): myrcene, γ-terpinene, camphor, cis-verbenol, verbenone at concentration of 10μL (Zhang et al. 2015).

We chose *O. crassipes* for GC-EAD/FID experiments, because of the repelling behavioral responses of *M. crucifer* and the plant's T&E status in the United States. In GC-EAD/FID, experiments antennal preparations of female *M. crucifer* elicited responses to four volatile compounds emitted by *O. crassipes*. Two of these are shared with *C. officinale* and the other two are 4-cyanocyclohexene, which was unique to *O. crassipes* in our study, and m-menthane. To our knowledge, this is the first time that 4-cyanocyclohexene has been identified in a plant within the Boraginaceae or analyzed for its repelling attributes. We were unfortunately unable to acquire m-menthane for GC-EAD/FID experimentation but our data suggest that 4-cyanocyclohexene is involved in the discrimination of *O. crassipes* by *M. crucifer*.

VOCs and herbivore-induced plant volatiles (HIPVs) are important components of plant's defense system which can either attract carnivores or repel herbivores (Degen et al. 2004; Arimura et al. 2005). Most of the compounds present in plant includes repellents, feeding deterrents, toxins and growth regulators (Maia and Moore 2011). Repellent plants are those plants which deter herbivores with the presence of toxins and odors that can cause herbivores to either reject or unable to find host plants in the field (Agrawal et al. 2006). Some of the specific VOCs from nontarget plants can mask the host's odour and phytophagous insect pests might get repelled from nontarget VOCs (Bruce et al. 2003). There are some studies which shows that EAD active non-host volatiles are repellent to target insects (Zhang and Schlyter 2004; Mauchline et al. 2008; Schlyter et al. 2000; Zhang et al. 2007; Zhang et al. 2014). For example, pollen beetle *Meligethes aeneus* Fab. (Coleoptera, Nitidulidae) were repelled when tested in a 4 arm olfactometer with non-host plant *Lavandula angustifolia* (Lamiaceae) volatile compounds linalool and linalyl acetate (Mauchline et al. 2008). When

the response of female *Cryptorrhynchus lapathi* L. (Coleoptera: Curculionidae) was tested to some of the non-host volatile α-pinene, insects were repelled by these volatiles in Y-tube olfactometer (Cao et al. 2015). To our knowledge, there are no studies in biocontrol of weeds that focus on the role of repellent compounds and how these compounds are sensed by the insect antennae. Our study provides information on how a specialist insect used in biocontrol of weed can be studied to assess repelled behavior with relative to nontarget plants.

Implications for Mogulones crucifer *attack of nontargets in the field*

Hackelia micrantha has been attacked by *M. crucifer* at *C. officinale* field sites in Canada where it sympatrically occurred with *C. officinale* , but *Mogulones crucifer* populations were not sustained on populations of *H. micrantha* when *C. officinale* was absent (Andreas et al. 2008; Catton et al. 2015; 2016). Even when *C. officinale* was present, performance of the weevil on *H. micrantha* was always lower than on *C. officinale*, which was explained as spillover attack (Catton et al. 2015; 2016; Hinz et al. 2019). *Oreocarya spiculifera* was also attacked by *M. crucifer* in the field but only at very low and varying rates and attributable to spillover (Andreas et al. 2008). Spillover occurs when densities of a biocontrol agent are high relative to densities of its target, and alternative native host plants are available. For biological weed control the definition of spillover has been clarified to include only attack on nontarget species on which the agent can develop, (in contrast to attack on unrelated plants on which the agent can not completely develop, which is defined as collateral attack) (Hinz et al. 2019). *M. crucifer* has not been attracted to any confamilial nontarget plant species in our study and our study adds more information on repelling compounds that allow *M. crucifer* to effectively discriminate against these species at least as long as *C. officinale* is not present. In the absence of any attraction and the presence of indifference or repellence, it is unclear to

foresee how any of the species tested here or previously by Kafle (2016) should be at risk of attack other than temporary spillover, especially if their distribution does not or only marginally overlaps with that of *C. officinale* (Kartesz 1999).

Our study suggests that *M. crucifer* can locate its host using plant volatile cues during host finding, and it is attracted to *C. officinale* while discriminating against all nontarget species tested. We identified electrophysiologically active compounds from related confamilial nontarget species of *C. officinale* that represent species rich North American genera using GC-EAD/FID, including one T&E species and observed weevil repellence to some of these bioactive compounds in behavioral bioassays. In pre-release host-specificity testing, phytochemical information has not been much studied although it has been identified as important (Schaffner 2001; Wheeler and Schaffner 2013, Hinz et al. 2014; Hinz et al. 2019). These findings highlight the importance of attractants and repellents in host selection. Additionally, a possible mechanism has been provided for how specialist insects discriminate their host and nontarget plants and maintain host fidelity under natural conditions, which to our knowledge has not been explored in biocontrol of weeds.

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Table 2.1: Confamilial North American plant species selected for pre-alightment host selection studies with *Mogulones crucifer*.

Plant species	**Synonyms**	**Life history**	**Rationale for testing**	**Native Range**	**Previous host range data**
Cryptantha ambigua (A. gray) Greene		Annual[5]	Congener now *Oreocarya spiculifera* Piper (=*Cryptantha spiculifera* (Piper) Payson) was attacked in the field[1]	Western North America[5]	Previously not included in host range testing
Cryptantha kelseyana Greene		Annual[5]	Supported *M. crucifer* development in previous host range testing[2]	Western North America[5]	Was tested[2, 3] Supported development of *M. crucifer*[2]
Hackelia californica (Gray) I.M. Johnst.		Perennial[5]	Congener of T&E, *H. venusta*	Mountain ranges of Northern California and Southern Oregon, United States[5]	*M. crucifer* were repelled by this plant volatile in previous behavioral bioassays[6]
Hackelia micrantha (Eastw.) J.L. Gentry		Perennial[5]	North American confamilial recently attacked by *M. crucifer*	West North America to Southeast United States[5]	*M. crucifer* were repelled by this plant volatile in previous behavioral bioassays[6] Nontarget attack in the field[1, 7, 8]
Oreocarya celosioides Eastw.	*Cryptantha celosioides* (Eastw.) Payson	Biennial to perennial[5]	Supported *M. crucifer* development in previous host range testing[2]	Western North America[5]	Was tested[2]. Supported development of *M. crucifer*[2]
Oreocarya crassipes (I. M. Johnst.)	*Cryptantha crassipes* I. M. Johnst	Perennial[5]	T&E listed confamilial species[4]. Close	Southwestern United States[5]	Previously not included

Hasenstab & M.G. Simpson			congener was found attacked in field[1]		in host range testing
Oreocarya rugulosa Payson	*Cryptantha rugulosa* (Payson) Payson	Biennial to perennial[5]	Close congener was found attacked in field[1]	Northwestern United States[5]	Previously not included in host range testing

[1](Andreas et al. 2008); [2](De Clerck-Floate and Schwarzländer 2002); [3](Schwarzländer et al 1999); [4](Williams et al. 2011); [5](USDA, NRCS 2019) [6](Kafle 2016); [7](Andreas 2004); [8](Catton et al. 2016).

Table 2.2: Summary statistics for behavioral responses of *Mogulones crucifer* females in multiple-choice bioassays with volatile headspace of *Cynoglossum officinale*, five North American confamilial species and purified air. Test statistics are log-linear categorical models. Within the model, single-degree-of-freedom contrasts were used for pair-wise comparison between four choices and total time spent in each odor quadrant (n = 20 for all bioassays; see text for details).

	Initial choice		**Final choice**		**Percent time spent in quadrants of olfactometer**	
Plant species	**χ^2_a**	**p-value**	**χ^2_a**	**p-value**	**χ^2_a**	**p-value**
Cryptantha ambigua	0.00	1.0000	10.87	0.0125	463.68	<0.0001
Cryptantha kelseyana	7.57	0.0557	5.32	0.1501	306.67	<0.0001
Oreocarya celosioides	3.62	0.3057	8.68	0.0338	201.16	<0.0001
Oreocarya crassipes	6.26	0.0996	8.52	0.0363	80.37	<0.0001
Oreocarya rugulosa	6.26	0.0996	10.68	0.0136	248.19	<0.0001

a= Logistic regression analysis; df= 3 for all statistics, $p<0.05$

Table 2.3: Summary statistics for behavioral responses of *Mogulones crucifer* females in dual-choice bioassays with volatile headspace of confamilial North American species of *Cynoglossum officinale* and purified air. Test statistics are log-linear categorical models. Within the model, single-degree-of-freedom contrasts were used for pair-wise comparison between four choices and total time spent in each odor quadrant (n = 20 for all bioassays; see text for details).

	Initial choice		**Final choice**		**Percent time spent in quadrants of olfactometer**	
North American species	χ^{2}_{a}	**p-value**	χ^{2}_{a}	**p-value**	χ^{2}_{a}	**p-value**
Cryptantha ambigua	1.92	0.5889	7.57	0.0557	74.83	<0.0001
Cryptantha kelseyana	7.57	0.0557	7.57	0.0557	31.00	<0.0001
Oreocarya celosioides	4.64	0.1998	4.66	0.1985	265.90	<0.0001
Oreocarya crassipes	8.69	0.0337	7.57	0.0557	128.86	<0.0001
Oreocarya rugulosa	8.80	0.0321	5.20	0.1577	179.05	<0.0001

a= Logistic regression analysis; df= 3 for all statistics, $p<0.05$

Table 2.4: Summary statistics for behavioral responses of *Mogulones crucifer* females in dual-choice bioassays using the *Hackelia micrantha* volatile compounds acetophenone and α-terpineol and the *Oreocarya crassipes*-specific volatile compound 4-cyanocyclohexane. Test statistics are log-linear categorical models. Within the model, single-degree-of-freedom contrasts were used for pair-wise comparison between four choices and total time spent in each odor quadrant (n=20 for all bioassays; see text for details).

	Initial choice		**Final choice**		**Percent time spent in quadrants of olfactometer**	
Pure compounds	**χ^2_a**	**p-value**	**χ^2_a**	**p-value**	**χ^2_a**	**p-value**
Acetophenone	26.00	<0.0001	10.00	0.0186	771.53	<0.0001
α-terpineol	24.00	<0.0001	20.00	0.0002	340.73	<0.0001
Blend (Acetophenone and α-terpineol)	4.00	0.2615	20.00	0.0002	271.44	<0.0001
4-Cyanocyclohexane	30.75	<0.0001	23.30	0.0600	402.55	<0.0001

a= Logistic regression analysis; df= 3 for all statistics, $p<0.05$

Table 2.5: Relative total ion concentration (TIC) peak area percentage of six electrophysiologically bioactive compounds in *Cynoglossum officinale* and selected North American confamilial Boraginaceae species. Compound identity was confirmed by comparing mass-spectra and retention time using data in the NIST library (National Institute of Standards and Technology) and comparing calculated retention indices.

	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)	3.35	4.76	7.26	8.43	9.35	9.72
Cynoglossum officinale	5.79	2.52	1.66	2.55	16.69	3.14
Cryptantha ambigua	-	-	-	-	-	9.72
Cryptantha kelseyana	-	3.53	-	-	-	6.05
Hackelia californica	-	3.72	6.88	-	14.00	21.50
Hackelia micrantha	-	2.50	5.40	-	1.90	22.00
Oreocarya celosioides	-	1.80	-	-	8.00	-
Oreocarya crassipes	-	2.64	5.13	-	1.06	-
Oreocarya rugulosa	-	2.40	-	-	6.60	-

Table 2.6: Relative total ion concentration (TIC) peak area percentage of electrophysiologically active compounds in *Hackelia micrantha.* Tentative identification of compound is based on comparison of their mass-spectra and retention time with data in the NIST library (National Institute of Standards and Technology) and comparing calculated retention indices.

	Compounds				
	(z)-3-hexen-1-ol	**(z)-3-hexen-1-ol acetate**	**β-Ocimene**	**Acetophenone**	**α-Terpineol**
Retention time (min)	4.76	8.58	9.72	10.17	13.19
Hackelia micrantha	2.50	1.90	22.00	2.00	1.90

Table 2.7: Relative Total Ion Concentration (TIC) peak area percentage of electrophysiologically active compounds in *Oreocarya crassipes*. Tentative identification of compound is based on comparison of their mass-spectra and retention time with data in the NIST library (National Institute of Standards and Technology) and comparing calculated retention indices.

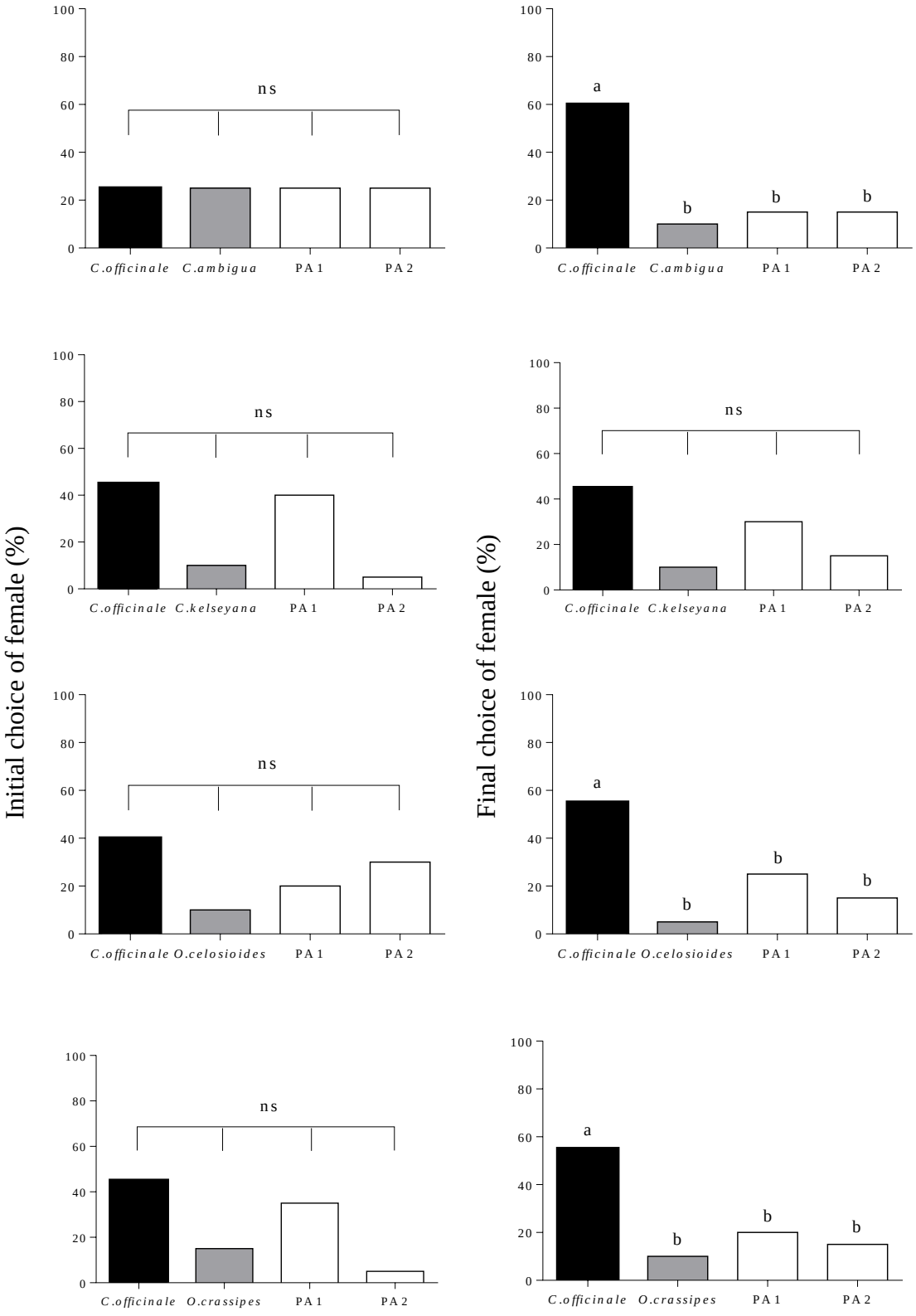
	Compounds			
	m-menthane	**4-cyanocyclohexene**	**(z)-3-hexen-1-ol acetate**	**Nonanal**
Retention time (min)	6.91	8.25	8.58	11.32
Oreocarya crassipes	4.44	9.54	1.06	6.88

Table 2.8: Relative total ion concentration (TIC) peak area percentage of volatile organic compounds collected in the headspace of plant species used in this study: *Cynoglossum officinale* (CO), *Cryptantha ambigua* (CA), *Cryptantha kelseyana* (CK), *Hackelia Californica* (HC), *Hackelia micrantha* (HM), *Oreocarya celosioides* (OC), *Oreocarya crassipes* (OCR) and *Oreocarya rugulosa* (OR). RT: Retention time. Tentative identification of compound 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.

Compound	**RT (min)**	**CO**	**CA**	**CK**	**HC**	**HM**	**OC**	**OCR**	**OR**
Butanenitrile, 2-methyl	2.69	-	2.35	-	-	-	-	-	-
Cyanic acid, 2,2-dimethylpropyl ester	2.26	-	1.64	-	-	-	-	-	-
Methyl isovalerate	3.36	5.80	-	-	-	-	-	-	-
Aziridinone, 1,3-bis(1,1-dimethylethyl)	3.58	-	-	-	-	-	3.10	-	3.10
Octane	3.62	1.44	2.64	-	-	-	-	-	-
Hexane, 3-ethyl	3.62	-	2.57	-	-	-	-	-	-
N-Ethoxyisobuten-3-imine	3.71	-	1.80	1.78	-	-	-	-	-
Propanenitrile, 3-(5-diethylamino-1-methyl-3-pentyynyloxy)	3.75	-	-	1.77	-	-	-	-	-
Cyclopropane, 1,1,2,3-tetramethyl	3.70	-	1.91	-	-	-	-	-	-
1,2-Benzisothiazol-3-amine	4.01	-	1.56	2.51	-	-	-	-	-
2,4-Dimethyl-1-heptene	4.41	-	2.18	-	-	-	-	-	-
3-Hexen-1-ol, (Z)-	4.76	2.52	-	3.53	3.72	2.50	1.80	2.64	2.40
3(5H)-Furanone	4.88	-	-	2.83	-	-	-	-	4.00
p-Xylene	5.01	-	2.13	-	-	-	-	-	-
2-Heptanone	5.57	-	-	-	-	-	-	1.79	-
Heptanal	5.80	2.81	-	-	-	-	-	-	-
2(5H)-Furanone	6.08	5.03	-	-	-	-	-	-	-
Cyclopentane, (3-methylbutyl)	6.12	-	3.18	-	-	-	-	-	-
Cyclopentane, 1,2,3,4,5-pentamethyl	6.14	-	-	-	-	-	3.30	-	-
Ethanone, 1-(1-methylcyclohexyl)	6.20	2.97	-	3.38	-	-	-	-	-
2,6,6-Trimethylbicyclo [3.1.1] hept-2-ene	6.52	-	3.19	-	-	-	-	-	3.90
1-Hexene, 4,5-dimethyl	6.85	1.22	-	-	-	-	-	1.96	-

Cyclohexanol, 2-methyl-5-(1-methylethenyl)	6.90	-	1.11	-	-	-	-	-	-
Cyclohexane, 1-methyl-2-propyl	6.90	-	-	-	-	-	1.80	4.57	-
3-Heptene, 4-propyl	6.90	-	-	-	-	-	-	2.04	-
m-Menthane	6.91	-	-	2.24	-	-	1.80	4.44	1.50
Heptane, 2,5-dimethyl	7.26	-	-	1.46	-	-	-	-	-
Benzaldehyde	7.26	1.70	-	-	6.88	5.40	-	5.13	-
1-Heptanol	7.57	1.29	-	-	-	-	-	-	-
β-Phellandrene	7.60	-	-	-	-	-	2.50	-	3.00
1,8-Nonadien-3-ol	7.61	-	-	1.89	-	-	-	-	-
1-octen-3-ol	7.82	-	-	-	1.20	-	-	-	-
β-myrcene	8.10	-	-	-	-	1.30	-	-	-
4-Cyanocyclohexene	8.25	-	-	-	-	-	-	9.54	-
6-methyl-5-heptene-2-one	8.43	2.60	-	-	1.98	-	-	-	-
Octanal	8.45	3.64	-	-	2.05	-	-	4.78	-
3-Hexen-1-ol, acetate, (Z)-	8.58	17.00	-	-	14.00	1.90	8.00	1.06	6.60
4-Hexen-1-ol, acetate, (Z)-	8.60	-	35.25	-	4.40	-	-	-	-
Butanoic acid, 4-hexenyl ester	8.61	-	-	2.76	-	-	-	-	-
Hexyl ester acetic acid	8.78	2.51	-	1.82	1.07	1.70	-	-	-
Benzyl alcohol	9.11	-	-	-	1.54	1.40	-	-	-
Limonene	9.11	-	3.01	-	-	-	-	-	-
2-Azido-2,4,4,6,6-pentamethylheptane	9.19	-	3.22	1.55	-	-	-	-	-
Eucalyptol	9.20	-	-	3.21	-	-	-	-	-
Octane, 2,3,6,7-tetramethyl	9.28	-	3.07	-	-	-	-	-	-
Cyclohexanone, 2,2,6-trimethyl	9.32	2.14	-	-	-	-	-	-	-
d-limonene	9.47	-	-	-	2.73	-	-	-	-
β-Ocimene	9.72	3.10	9.72	6.05	21.50	22.00	-	-	-
3-Carene	9.73	-	2.95	-	-	-	-	-	-
Octane, 2,6-dimethyl	9.89	-	-	3.08	-	-	-	-	-
Y-Terpinene	9.98	-	-	3.58	-	-	-	-	-

Tetradecane	9.99	-	-	3.69	-	-	-	-	-
Acetophenone	10.17	-	-	-	-	2.00	-	-	-
1-octanol	10.38	3.84	-	-	-	-	-	-	-
Dodecane, 4-methyl	10.46	-	1.36	2.90	-	-	-	-	-
Nonanal	11.33	9.50	3.90	-	5.19	3.60	2.70	6.88	7.70
Linalool	11.45	5.83	-	-	-	7.80	-	-	-
Phenylethyl Alcohol	11.75	-	-	-	1.14	-	-	-	-
Methyl salicylate	12.89	3.34	-	-	4.81	2.70	-	-	-
α-Terpineol	13.20	-	-	-	1.18	1.90	-	-	-
Butanoic acid, 3-hexenyl ester	13.67	-	2.97	-	-	-	-	-	-
Decanal	14.19	2.16	1.45	-	2.37	2.00	-	-	-
1,4-benzenedicarbio	14.78	-	-	-	-	4.20	-	-	-
isopathaldehyde	14.81	-	-	-	-	4.50	-	-	-
p-Cymen-7-ol	15.50	-	-	-	-	2.40	-	15.50	-
Cyclohexanone, 2-(2-butynyl)	15.50	4.23	-	-	-	-	-	-	-
2-Decenal	14.19	1.96	-	-	-	-	-	-	-
Ethanone, 1-(4-ethyphenyl)	15.72	-	-	-	-	-	-	-	1.10
Acetic acid	17.63	-	-	-	-	-	-	1.57	-
caryophyllene	18.88	-	-	-	-	2.70	-	-	-
β-sesquiphellandrene	19.88	2.59	-	-	-	-	-	-	-
Pentadecane	21.82	-	-	-	-	-	-	-	1.70
α-Farnesene	21.85	2.63	-	-	16.40	17.00	-	-	-
β-Cedrene	22.53	-	-	-	2.71	-	-	-	-
Butylated Hydroxytoluene	22.13	-	7.79	-	-	-	-	-	3.80
3-Hexen-1-ol, benzoate, (Z)-	22.52	-	-	-	1.64	-	-	-	-
Number of volatile compounds (Shared compounds with *C. officinale*)		25	23 (4)	18 (4)	19 (9)	19 (10)	8 (3)	13 (6)	11 (3)


Initial choice of female (%)
Final choice of female (%)
100
80
60
40
20
0
ns
a
b
C.officinale
C.ambigua
C.kelseyana
O.celosioides
O.crassipes
PA 1
PA 2

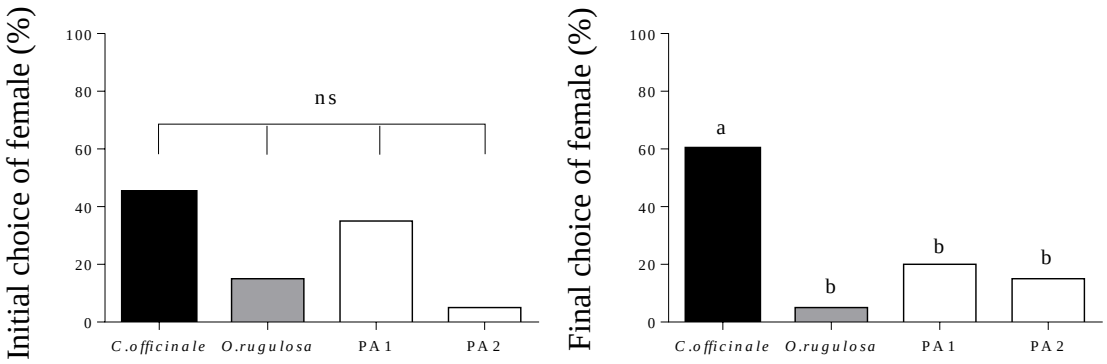


Figure 2.1: Proportion of *Mogulones crucifer* females' initial choice (left column) and final choice (right column) among four quadrants of a four-armed olfactometer arena using volatile headspace of *Cynoglossum officinale*, one test plant species, and purified air in remaining two quadrants (PA1 and PA2). Differing letters on top of bars denote significant differences (χ^2-test followed by categorical log-linear model $p<0.05$, ns=not significant) (n=20; see text for details).

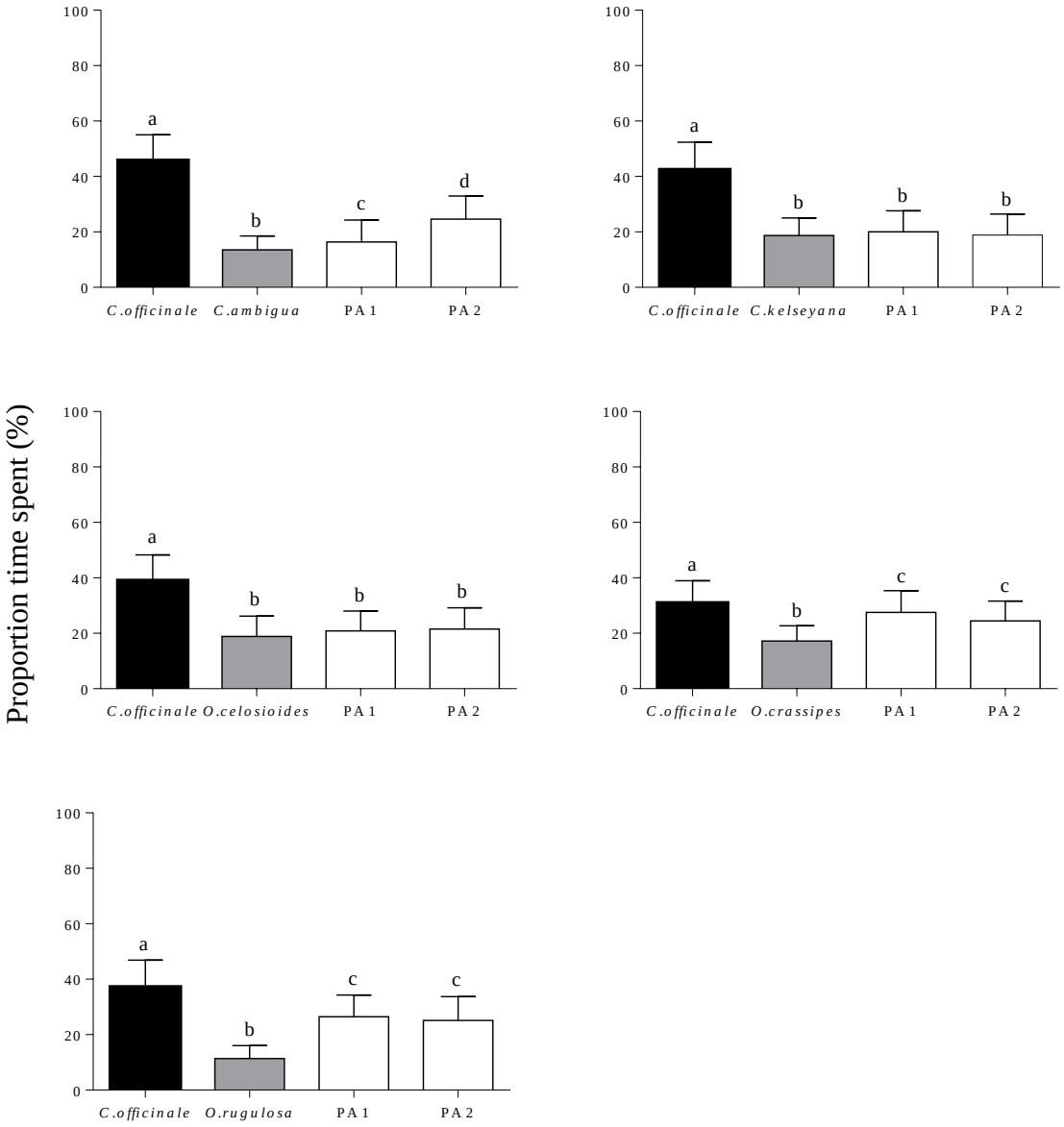
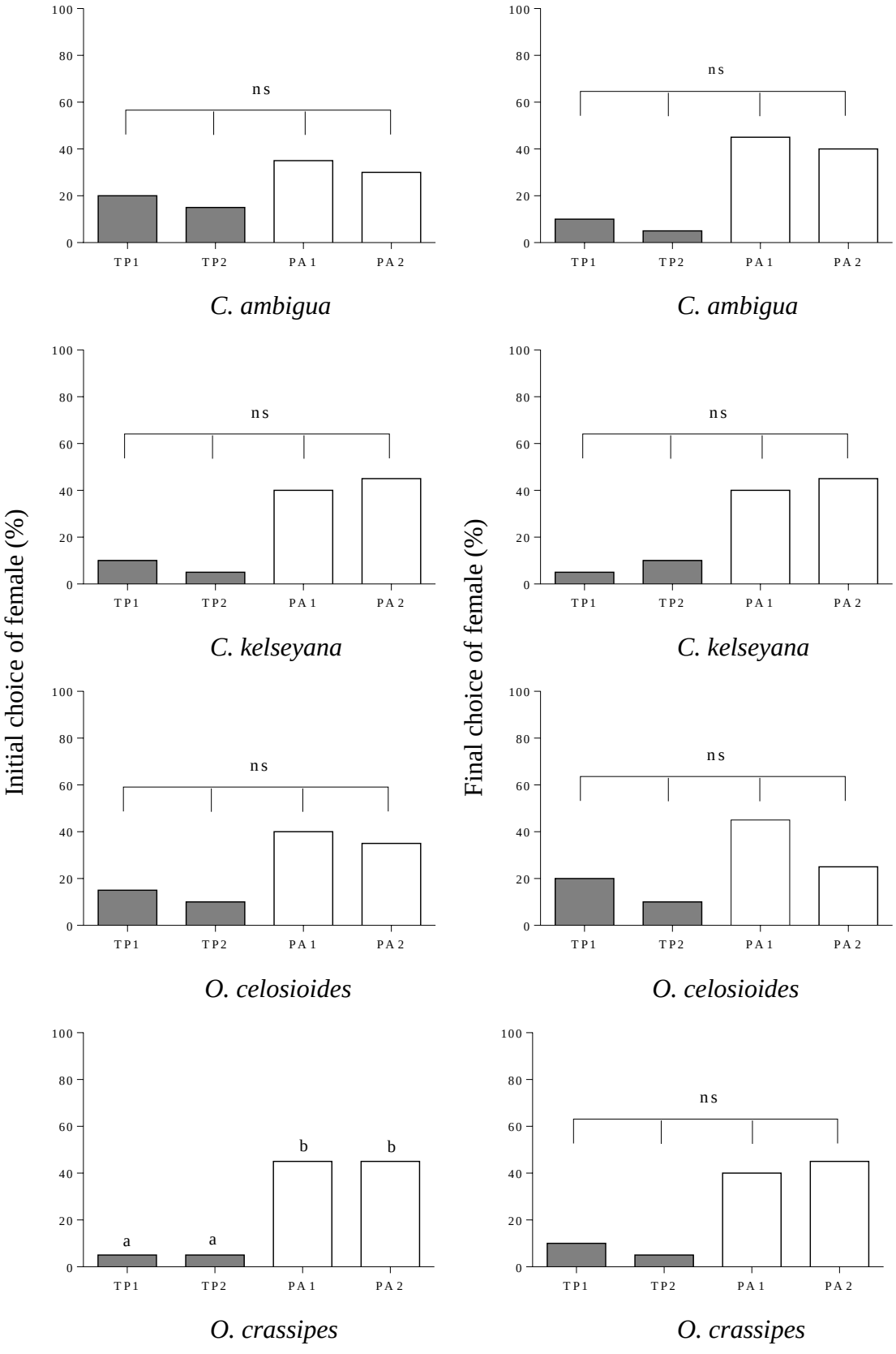


Figure 2.2: Proportion of time spent by female *Mogulones crucifer* in each of four quadrants of a four-armed olfactometer arena using volatile headspace of *Cynoglossum officinale*, one test plant species, and purified air in remaining two quadrants (PA1 and PA2). Differing letters on top of bars denote significant differences (Categorical log linear model followed by single degree of freedom contrast analysis, $p<0.05$) (n=20; see text for details).


Initial choice of female (%)
Final choice of female (%)
100
80
60
40
20
0
ns
a
b
TP1
TP2
PA 1
PA 2
C. ambigua
C. kelseyana
O. celosioides
O. crassipes

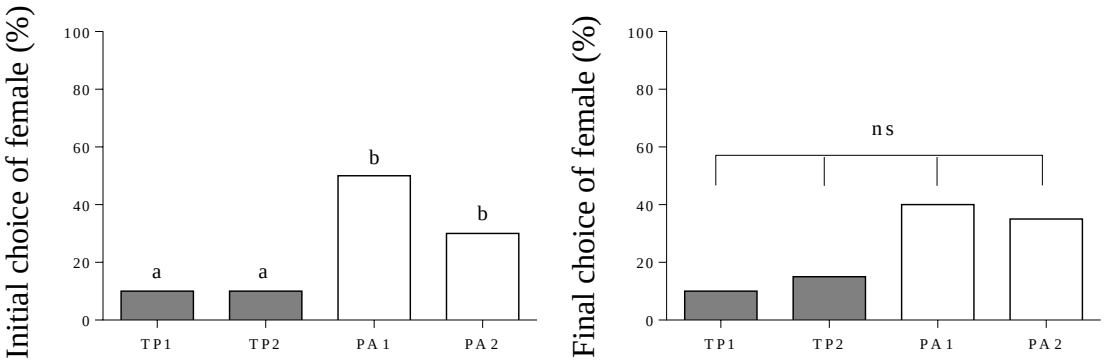


Figure 2.3: Proportion of *Mogulones crucifer* females' initial choice (left column) and final choice (right column) among four quadrants in a four-armed olfactometer arena using volatile headspace of one test plant species in two quadrants (TP1 and TP2) and purified air in remaining two quadrants (PA1 and PA2). Differing letters on top of bars denote significant differences (χ^2-test followed by categorical log-linear model, $p<0.05$, ns=not significant) (n=20; see text for details).

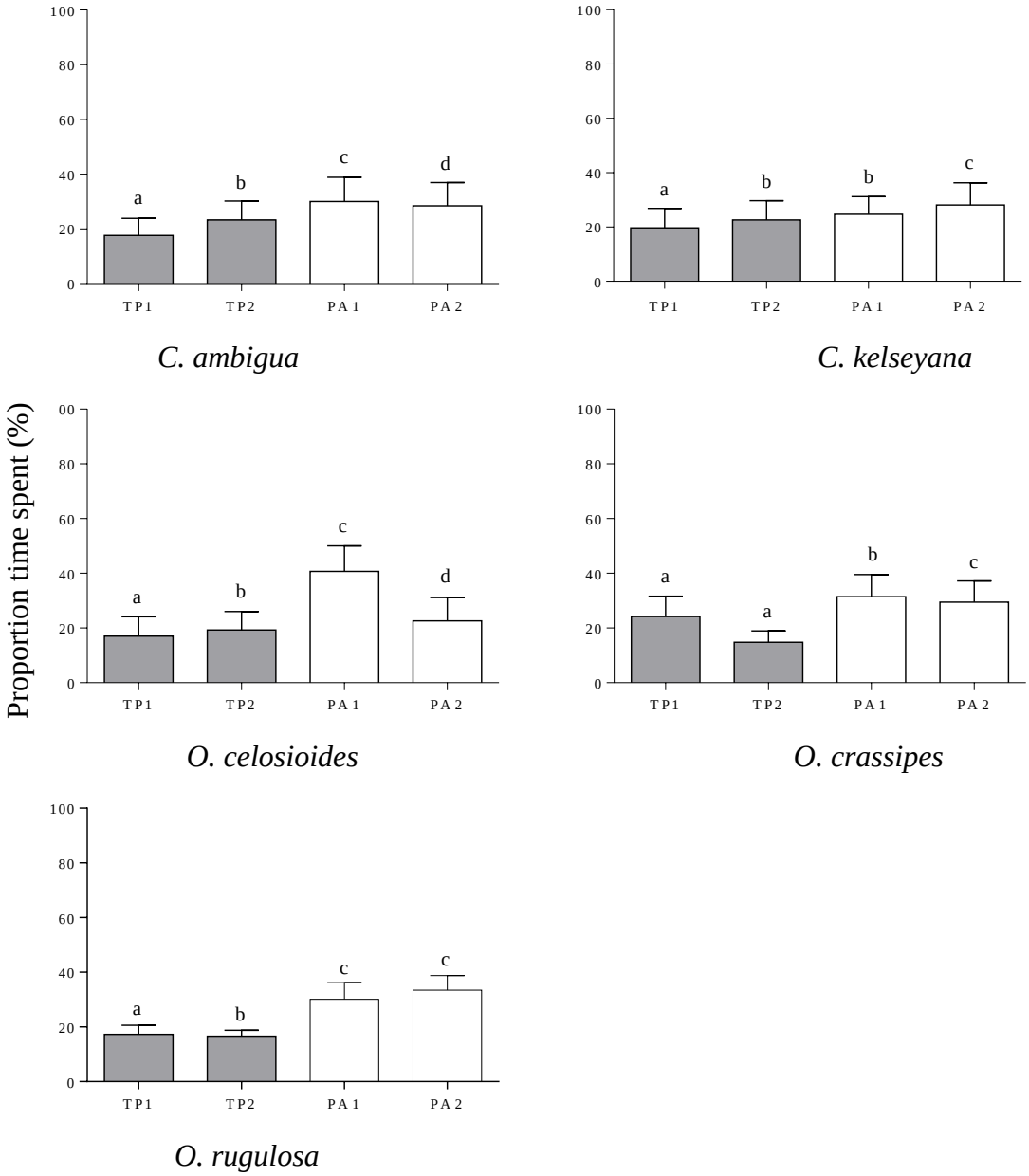


Figure 2.4: Proportion of time spent by female *Mogulones crucifer* in each of four quadrants of a four-armed olfactometer arena using volatile headspace of one test plant species in two quadrants (TP1 and TP2) and purified air in remaining two quadrants (PA1 and PA2). Differing letters on top of bars denote significant differences (Categorical log linear model followed by single degree of freedom contrast analysis, $p<0.05$) (n=20; see text for details).

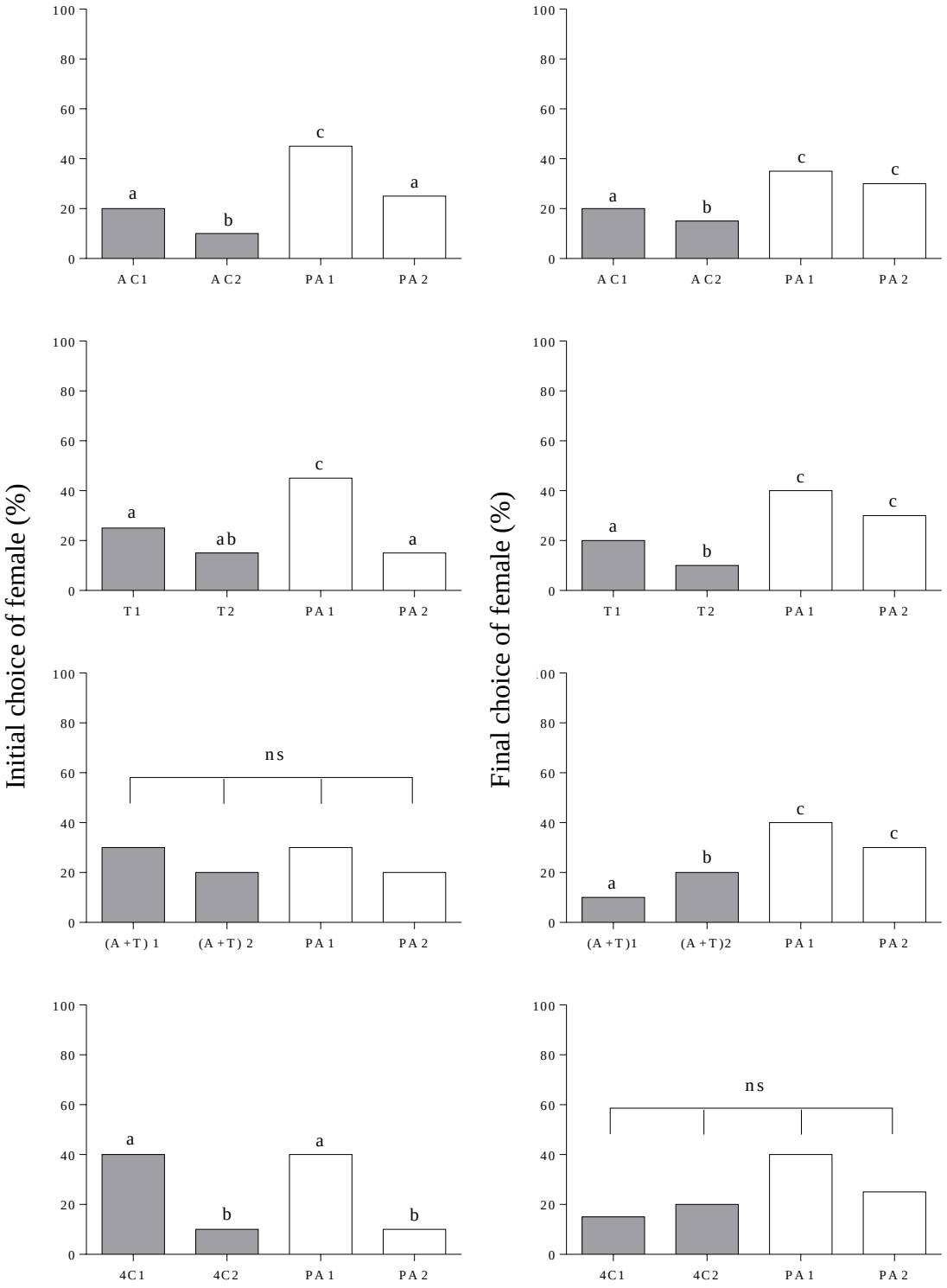


Figure 2.5: Proportion of *Mogulones crucifer* females' initial choice (left column) and final choice (right column) among four quadrants in a four-armed olfactometer arena using the pure volatile compounds acetophenone, α-terpineol, a 1:1 blend of the aforementioned two compounds and 4-cyanocyclohexene in two quadrants (AC1 and AC2 = acetophenone, T1 and T2 = α-terpineol, (A+T)1 and (A+T)2= blend of

acetophenone and α-terpineol, and 4C1 and 4C2 = 4-cyanocyclohexene) and purified air (PA1 and PA2) in the remaining two quadrants. Differing letters on top of bars denote significant differences (χ^2 -test followed by a categorical log-linear model, $p<0.05$, ns=not significant) (n=20; see text for details).

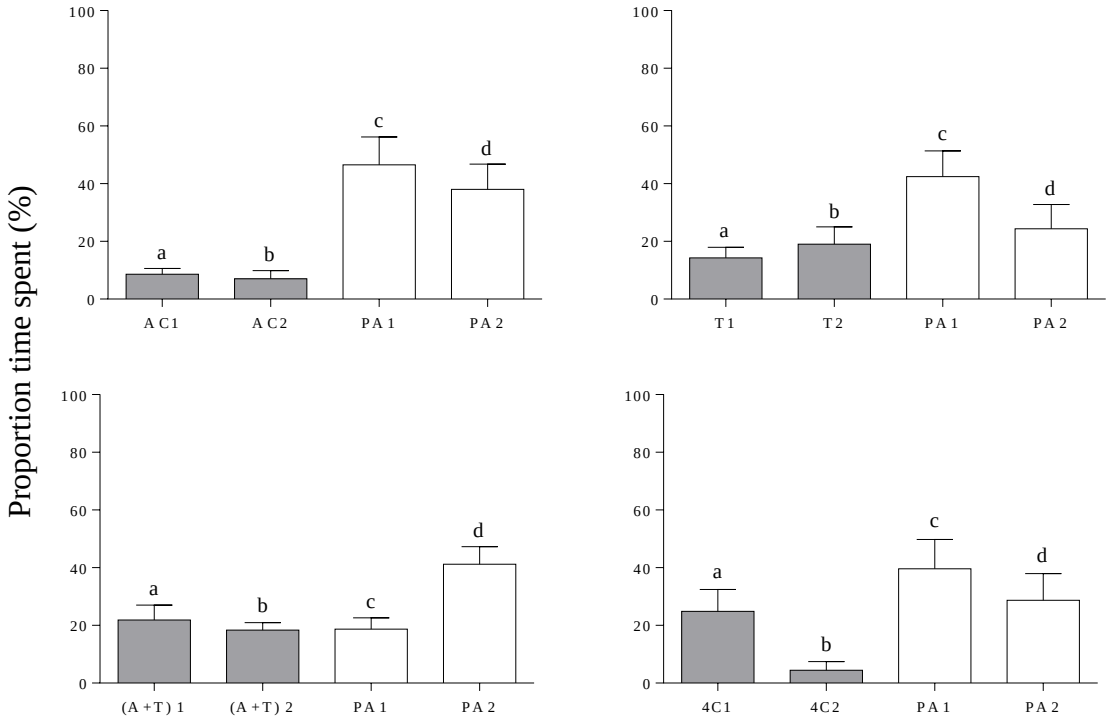


Figure 2.6: Proportion of time spent by female *Mogulones crucifer* in each of four quadrants of a four-armed olfactometer arena using pure volatile compounds acetophenone, α-terpineol, a 1:1 blend of the aforementioned two compounds, and 4-cyanocyclohexene in two quadrants (AC1 and AC2 = acetophenone, T1 and T2 = α-terpineol, (A+T)1 and (A+T)2= blend of acetophenone and α-terpineol, and 4C1 and 4C2 = 4-cynocyclohexene) and purified air (PA1 and PA2) in the remaining two quadrants as control. Differing letters on top of bars denote significant differences (Categorical log linear model followed by single degree of freedom contrast analyses, $p<0.05$) (n=20; see text for details).

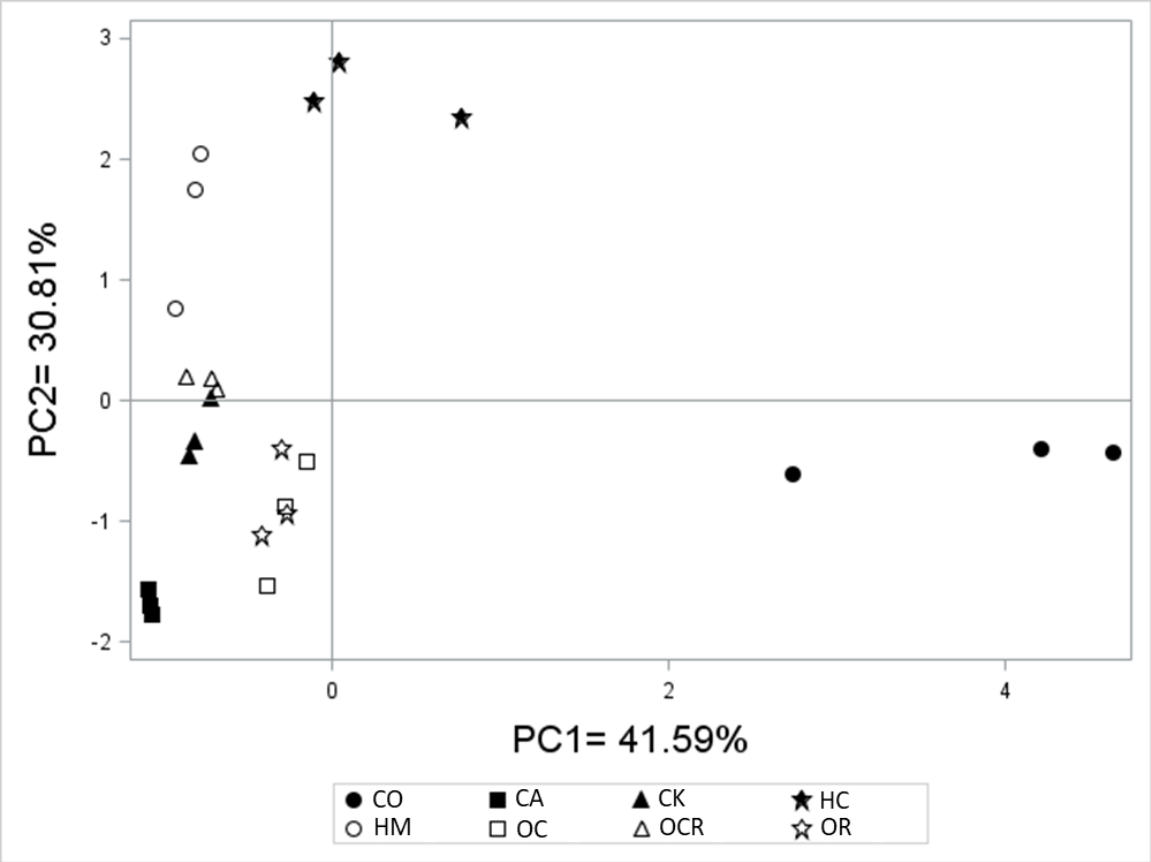


Figure 2.7: Principal component analysis score plot based on relative TIC peak are percentage electrophysiologically active volatile organic compounds identified in *C. officinale*. Plant species: *Cynoglossum officinale* (CO), *Cryptantha ambigua* (CA), *Cryptantha kelseyana* (CK), *Hackelia californica* (HC), *Hackelia micrantha* (HM), *Oreocarya celosioides* (OC), *Oreocarya crassipes* (OCR), *Oreocarya ruguosa* (OR).

SUMMARY AND CONCLUSION

The overall goal of this study is to assess the pre-alightment host selection behavior of *M. crucifer* through behavioral and electrophysiological experiments. For the better prediction of the ecological host range of biological control organisms, pre-release host specificity testing with behavioral bioassays along with electrophysiological and chemical basis of host plant selection is important (Wheeler & Schaffner, 2013). Host finding in herbivorous insects is mediated by olfactory cues before feeding and oviposition, such data should be given importance in decision-making processes for the introduction of biological control organisms where the biocontrol agent has broader fundamental host range than its ecological host range (Hinz et al., 2014). These two chapters provide data that are relevant as *M. crucifer* has broad fundamental host range and considered as a risk to many native nontarget confamilials (Andreas 2004; De Clerck-Floate & Schwarzländer 2002; Jordan et al. 1993; Schwarzländer 1996; USDA 2010).

Mogulones crucifer can locate its field host and discriminate all tested Eurasian, Asian and North American confamilial plant volatiles in behavioral bioassays. *M. crucifer* strongly preferred the headspace VOCs of *C. officinale* over those of four Eurasian, three Asian and five North American confamilial non-target species. Most tested nontargets, including the last T&E species that needed to be tested, elicited a repellent response; it is therefore unlikely that the weevil would discover or seek out any of these species during host finding. *M. crucifer* may be less repelled by VOCs of Eurasian confamilials than those of NA confamilials, although many Eurasian species still elicited repelling responses.

Electrophysiological experiments using GCMS and GCEAD/FID helped in identifying those compounds which were responsible for the attraction and/or repellence

behavior of *M. crucifer* towards host and non-host plants. This result suggests that knowledge on plant chemistry that determines host utilization of prospective biocontrol agent could help in pre-release host specificity testing. Our data suggests that presence of α-Terpineol, acetophenone and 4-cyanocyclohexene in North American confamilials, diminish the probability of non-target attack in the field assuring safer environment. Our findings reconfirm previously collected data on behavioral bioassays in our lab that *M. crucifer* is a near-monophagous specialist on *C. officinale* (Kafle 2016), that can discriminate even the closest Eurasian relatives of *C. officinale* during the pre-alightment host selection phase despite being able to develop on those plants that are within fundamental host range. Our findings contribute to detail understanding of the ecological host range of biological control candidates.

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