INVESTIGATING THE HOST SELECTION BEHAVIOR OF *CEUTORHYNCHUS CARDARIAE*, A POTENTIAL BIOLOGICAL CONTROL AGENT FOR *LEPIDIUM DRABA*

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AUTHORIZATION TO SUBMIT DISSERTATION

This dissertation of Jessica Rendon, submitted for the degree of Doctor of Philosophy with a major in Entomology and titled "Investigating the host selection behavior of *Ceutorhynchus cardariae*, a potential biological control agent for *Lepidium draba*," has been reviewed in final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the College of Graduate Studies for approval.

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ABSTRACT

Lepidium draba (L.) Desv. (Brassicaceae) is a perennial clonal mustard native to Eurasia that was unintentionally introduced into North America in the late 1800s via seed in ship ballast and via contaminated alfalfa seed from central Asia. The leaf petiole gall-forming weevil, *Ceutorhynchus cardariae* (Coleoptera, Curculionidae), is an herbivorous insect species considered for the biological control of this invasive plant. Past host-specificity testing data has revealed that under natural field conditions, *C. cardariae* is a host-specific specialist, but under laboratory, no-choice conditions, its fundamental host range includes two distantly related confamilial species in the genus *Streptanthus: S. anceps* and *S. flavescens*. Pre-release host-specificity testing of weed biological control agents can assess which plants are attacked but not *why* they are attacked. Detailed examination of the pre- and post-alightment behavior on hosts and non-host plants can help determine what plant cues influence host choice by potential agents. To explain the apparent phylogenetically disjunct host range of *C. cardariae*, I investigated the pre- and post- alightment host-selection behavior and underlying physiological responses of *C. cardariae* with regard to the visual, volatile, and surface wax cues of *L. draba* and 13 native North American Brassicaceae genera.

Chapter 1, the introductory chapter, gives detailed background into the study system and rationale for the approach taken in proceeding chapters. Chapter 2 is a literature review on the role of physical and chemical surface wax cues in the host plant selection behavior of herbivore arthropods feeding on Brassicaceae. This review informed me of the importance of surface wax cues in the host selection of several Brassicaceae feeding herbivores, which warranted their examination in my study system. Chapter 3 examines the pre-feeding behavior of *C. cardariae* on *L. draba* and one non-host plant, basil. Distinctive pre-feeding behaviors were observed- weevils would drag their antennae along or just above the leaf surface prior to feeding. This suggests that contact chemoreceptors on the weevils' antennae, and possibly their tarsi, are being used to assess the suitability of the leaf surface. Chapter 4 examines the role of visual, volatile, and contact cues in the host selection behavior of *C. cardariae* on *L. draba.* In this chapter, an array of bioassays which manipulated visual, volatile, and surface wax cues of *L. draba* and plant models were used in a walking arena. The data suggests that *C. cardariae* mainly relies on visual cues to identify a potential host plant prior to contact, while after contact volatile and contact cues are important for final host recognition. Results also indicate that volatile and contact cues are equally important for final host choice. Chapter 5 examines the similarity of the volatile organic compounds (VOCs) from four different *L. draba* populations (closely related genetic populations) and 13 native North American confamilial species. A total of 46 VOCs were identified across all plant species but the volatile blends could not be differentiated among plant species using principal component analyses (PCA). PCA among volatile blends of the four populations of *L. draba, S. anceps,* and *S. flavescens* only, showed slight separation among the populations and species. Follow-up experiments using gas chromatography-flame ionization detector-electroantennogram detection (GC-FID/EAD) to measure antennal responses in female *C. cardariae* when exposed to volatile blends of *L. draba* did not yield any consistent responses.

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DEDICATION

This dissertation is dedicated with love to my grandparents, Efren and Dora Rendon, Karl and Grace Kryter, to my parents, Richard and Kathy Rendon, and to my brother, Richard Rendon II.

But, especially to my Mother, who has read and reread this Dissertation countless times from its very humble beginnings to the finalized product it is now. Her grasp of the English language, grammar, syntax, and editing suggestions have been invaluable on my educational journey since 1st grade.

“Now from his breast into the eyes the ache
of longing mounted, and he wept at last,
his dear wife, clear and faithful, in his arms,
longed for as the sun-warmed earth is longed for by a swimmer
spent in rough water where his ship went down
under Poseidon's blows, gale winds and tons of sea.
Few men can keep alive through a big serf
to crawl, clotted with brine, on kindly beaches
in joy, in joy, knowing the abyss behind...” –Homer’s The Odyssey

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Chapter 1

INTRODUCTION TO THE DISSERTATION

Plant invasions

The term invasive plant refers to plant species of a foreign origin that spread without the direct assistance of human activities in natural or semi-natural habitats and lead to changes in biodiversity composition, structure, or ecosystem processes (Cronk & Fuller, 2014). Negative ecological impacts include outcompeting native plants, depleting soil and water resources, and increasing soil erosion and stream sedimentation (D'Antonio & Vitousek, 1992; DiTomaso, 2000; Lacey et al., 1989; Mack et al., 2000; Pyšek et al., 2012; Pyšek & Richardson, 2010). These negative impacts can directly affect ecosystem services such as water purification, climate regulation, and nutrient cycling (D'Antonio & Vitousek, 1992; Pyšek & Richardson, 2010). Invasive plants also pose costly challenges for effective resource management in rangeland settings (DiTomaso, 2000; Kettenring & Adams, 2011; Masters & Sheley, 2001; Simberloff et al., 2013). In the United States, invasive weeds in rangelands cause an estimated loss of $2 billion annually as they can lower the yield and quality of forage for livestock, poison livestock, interfere with grazing practices, and reduce land value (DiTomaso, 2000).

Several control strategies are utilized to manage invasive plant species: 1) chemical control using herbicides (DiTomaso, 2000), 2) mechanical control such as hand-pulling, tilling, mowing, and bulldozing (Mack et al., 2000), and 3) cultural control means such as prescribed burning, timed grazing, fertilization, reseeding, (DiTomaso, 2000; Masters & Sheley, 2001), and plant competition (Masters & Sheley, 2001). All control means can be effective for given environmental conditions such as accessibility, habitat, infestation size and age, etc. (Mack et al., 2000). All control strategies are also costly, require repeated applications, are not always feasible, e.g. for large infestations or in remote locations, and can have non-target effects (Mack et al., 2000). Classical biological control of weeds offers an alternative control strategy for the management of invasive plants.

Classical biological weed control

Classical biological control of weeds (CBCW) is typically defined as the deliberate release of specialist natural enemies from a weed's native range to reduce the abundance of the weed in its introduced range below an ecological or economic threshold. CBCW aims to mitigate negative impacts of invasive weeds on biodiversity, human welfare, and the economy (Müller-Schärer & Schaffner, 2008). CBCW can provide environmentally benign, long-term control that is cost effective and self-sustaining, and has the potential to spread to large and remote areas invaded by a weed (Culliney, 2005). As of May 2012, a total of 468 biological control agent species have been released against 175 target weeds in 48 plant families in 90 countries, predominantly the United States, Canada, Australia, South Africa and New Zealand (Winston et al., 2014). As with all other weed management strategies, the release of exotic biological control agents for the control of invasive plants is not without risk (Harris, 1988; Howarth, 1991; Louda & Stiling, 2004; Simberloff & Stiling, 1996). Without appropriate environmental safety assessments, the release of a biological control agent may lead to negative non-target effects; where the biological control agent either directly or indirectly negatively impacts native plant species (Louda & Stiling, 2004; Simberloff & Stiling, 1996). CBCW relies on sufficiently host-specific biological control candidates to avoid non-target attack (Heard, 1999; Louda et al., 2003; Sheppard et al., 2005). In a recent review on incidences of non-target attack in CBCW, it was found that non-target attack only rarely occurred. More than 99% of CBCW agents had no known or no significant adverse effects on non-target plants (Suckling and Sforza, 2014). Almost 92% of all direct non-target impacts were minimal or minor, and without long-term impact on non-target plant populations (Suckling & Sforza, 2014). There are only two CBCW cases in which severe direct non-target impacts have occurred: *Cactoblastis cactorum* (Berg) (Lepidoptera: Pyralidae) attacking native *Opuntia* species in the United States and *Rhinocyllus conicus* (Froel.) (Coleoptera: Curculionidae) attacking native thistles in the genus *Cirsium* in the United States (Louda et al., 2003; Louda & Stiling, 2004). In the former case, the insect was inadvertently introduced from the Caribbean. In the latter case the sociopolitical context in the United States in the 1960s did not consider the value of native flora and thus, the insect was considered for release (Suckling & Sforza, 2014). Comprehensive pre-release host-specificity testing of biological control agent candidates is conducted to assess the potential of any non-target attack and to ensure the environmental safety of CBCW candidate species.

Pre-release host-specificity testing of CBCW candidate species

Pre-release host-specificity tests are conducted to determine the range of plant species that could be at risk of non-target attack if the biological control agent candidate were to be released. To determine the host range, the candidate species is offered the target and non-target plant species in several series of tests including no-choice, and multiple-choice designs, and under varying environmental conditions ranging from closely confined to open-field experiments (Heard & Van Klinken, 1998; Schaffner, 2001). In no-choice or starvation tests the candidate species is only presented with one test plant, whereas in dual- and multiple choice tests the agent has the choice between the target weed and at least one test plant species. In open field tests, test plant species are offered to CBCW candidates without confinement, and if that is not possible often larger field cages are used in order to allow candidate agents to express the full range of their host selection behaviors (Marohasy, 1998; Schaffner, 2001). Development, feeding, or oviposition of the CBCW candidate are assessed to determine its fundamental and ecological host range. The fundamental host range comprises the range of plant species that are capable of supporting the full development of the biocontrol candidate (Van Klinken, 1999) and is typically assessed through no-choice tests. The ecological host range is typically a subset of the fundamental host range. It is assessed in a series of choice tests in which biological control agent candidates can choose between plant species and express their host selection behaviors. It therefore comprises plant species that have passed through species-specific behavioral and ecological filters (Nechols et al., 1992; Schaffner, 2001; Schaffner et al., 2018; Van Klinken, 1999; Wapshere, 1989). Open field tests are an important component of the assessment of the ecological host range since the biological control agent candidate is much more likely to display its host selection behavior in an open field environment (Briese, 1999; Clement & Cristofaro, 1995; Schaffner, 2001; Schaffner et al., 2018; Van Klinken, 1999; Wapshere, 1989).

Test plant species are traditionally selected according to the centrifugal phylogenetic method, which assumes that the most closely related plant species are also most likely to support development of the biological control agent compared to more distantly related plant species (Wapshere, 1974; 1989). This is in part because it is assumed that more closely related plant species are also more chemically and morphologically similar to the target weed and that specialist herbivores are thought to perceive and respond to these similarities or dissimilarities (Futuyama, 1999; Mitter et al., 1991; Wapshere, 1974; 1989; Wheeler & Schaffner, 2013). In most cases, almost all test plant species are confamilials of the target weed because it is unlikely that a CBCW candidate would be considered environmentally safe if it could to any degree develop on a plant species within a different plant family (Wapshere, 1974). A review of 112 insects, 3 fungi, 1 mite, and 1 nematode CBCW agents established in Hawaii, the continental United States, and the Caribbean, demonstrated that the risk to native flora can be judged reliably from pre-release host-specificity testing and that almost all risk is borne by native plant species closely related to target weeds (Pemberton, 2000).

Host selection behavior of insect herbivores

The host selection behavior of an insect herbivore can be divided in three main phases: finding, examining, and accepting (Bernays & Chapman, 1994; Miller & Strickler, 1984). Finding occurs before the insect has contact with the plant, and examining and acceptance, after contact. Acceptance or rejection of a potential host plant during the finding and examining phases depends on the insects' response to visual, volatile, contact, and gustatory plant cues (Bernays & Chapman, 1994; Dethier, 1982; Heard, 1999; Miller & Strickler, 1984; Wheeler & Schaffner, 2013). These stimuli dictate behavioral and physiological patterns of feeding and oviposition (Beck, 1965; De Wilde & Schoonhoven, 1969; Jermy, 1966; Marohasy, 1998; Miller & Strickler, 1984; Thorsteinson, 1960).

While the usefulness and validity of the centrifugal phylogenetic approach has been reliable in predicting the host-specificity of CBCW agents (Pemberton, 2000; Wapshere, 1974), there are limitations to the phylogenetic method. Often, phylogenies of plant families are still poorly understood as is evidenced by recent realignments of phylogenies based on molecular studies. For example, the Brassicaceae family alignment has long been controversial (Kiefer et al., 2014), and complicated by convergent evolution in nearly every morphological feature (such as fruit and seed morphology), which are used to define tribes and genera (Al-Shehbaz, 2012; Bailey et al., 2006). As a result, past classifications were highly artificial and of limited value in phylogenetic and taxonomic studies of the family (Al-Shehbaz, 2012). Phylogeny is currently based on the chloroplast ndhF gene (Beilstein et al., 2008) and provides the foundation for a comprehensive new tribal classification of the family (Al-Shehbaz, 2012), in which substantial overlap in fruit morphology is seen between the three lineages (Al-Shehbaz, 2012). In addition to poorly understood phylogenies, important plant traits such as plant architecture or a unique secondary metabolite that may play an important role in host recognition of a biological control agent may not necessarily be correlated with plant phylogeny but instead may be found in a diverse number of plant species (Van Klinken, 1999; Wink, 2003; 2008). While the centrifugal phylogenetic approach has been the standard practice for assessing a biological control agent’s likelihood of non-target attack (Harris & Zwölfer, 1968; Wapshere, 1974; 1989), it does not provide a mechanistic explanation as to how and why a biological control agent prefers one plant species over another (Louda et al., 2003; Marohasy, 1998; Rapo, 2012; Smith & Beck, 2013; 2015). If specialist herbivores are relying on specific secondary plant metabolites for their host selection behavior, then chemical similarity may be a better predictor of host utilization than strictly phylogenetic relationships (Becerra & Venable, 1999; Wheeler & Schaffner, 2013).

Furthermore, there are often discrepancies between the no-choice and open field host range testing results (Schaffner et al., 2018). The fundamental host range as determined by no-choice tests is often broader than what is observed in open field tests, which are more indicative of the ecological host range. These differences can lead to uncertainty as to whether certain plant species may or may not be attacked in the case of the release of the agent.

Pre-release risk assessment could be improved by observing and describing the host selection behavior of an agent both pre and post-alightment to determine which cue may be particularly important in its host plant choice (Hinz et al., 2014; Knolhoff & Heckel, 2014; Wheeler & Schaffner, 2013). While there is a large body of literature on the host selection behavior of herbivorous insect species (Bernays & Chapman, 1994; Knolhoff & Heckel, 2014; and references therein), there are few accounts on the host selection behavior of biological weed control candidate species. For example, olfactory cues (Andreas et al., 2008; Kafle, 2017) and visual cues (Reeves et al., 2009) have been investigated on the host selection behavior of biological control agents in only a few studies, and only two studies have examined olfactory and visual cues combined in the context of biological weed control (Müller & Nentwig, 2011; Park et al., 2018).

The Eurasian mustard *Lepidium draba* (L.) Desv. (Brassicaceae), which is invasive in western North America and its biological control candidate, the gall-forming weevil *Ceutorhynchus cardariae* Korotyaev (Coleoptera: Curculionidae), offer an interesting system to study the host selection behavior in a biological weed control system.

Study system

Lepidium draba (L.) Desv. (Brassicaceae) is a perennial clonal mustard native to Eurasia. It is believed to be indigenous to Southwestern and Central Asia (Mulligan & Frankton, 1962), Southern Europe, and the Mediterranean region (Ball, 1964). It can be found in a wide range of climatic conditions (Mulligan & Frankton, 1962) and in a variety of soil types (Corns & Frankton, 1952). It grows 30-60 cm tall and is an obligate outcrosser, relying on insects for pollination (Mulligan & Frankton, 1962). The plant can also reproduce clonally through an extensive rhizomatic system (Francis & Warwick, 2008). Root fragments have high regenerative capabilities. Fragments as small as 1¼ cm can regrow into shoots (Scurfield, 1962). Rosette leaves are greyish-green, scattered to densely pubescent, and are irregularly toothed to entire along their margins (Francis & Warwick, 2008). Stem leaves are grayish-green, with scattered pubescence, and are irregularly toothed to entire along margins (Francis & Warwick, 2008). Flowers are 2-4 mm wide, consist of four white petals, and are compactly arranged in a corymb on the tops of stems (Francis & Warwick, 2008). Seeds are produced in siliques, most of which contain two seeds, and single shoots produce up to 850 siliques (Corns & Frankton, 1952; Mulligan & Findlay, 1974). Seed production can be high, as one study found nearly 17,000 viable seeds of *L. draba* per square ft. (0.09 m2) in Oregon (McInnis et al., 2003). Seeds usually germinate in autumn and early spring and produce rosettes that bolt and flower the following spring from April to June (McInnis et al., 2003). Established plants bolt in early spring, flower from late April to early June, and form seeds in June and July (McKenney, 2005).

Lepidium draba was unintentionally introduced into North America in the late 1800s via seed in ship ballast (Bellue, 1933) and via contaminated alfalfa seed from central Asia

(Groh, 1940b; Mulligan & Findlay, 1974). It was first discovered in Long Island, New York in 1862 (Mulligan & Frankton, 1962) and in Ontario, Canada in 1878 (Mulligan & Findlay, 1974). Since then numerous subsequent introductions have occurred throughout the US (Gaskin et al., 2005; Mulligan & Findlay, 1974). The plant is only found sporadically in the eastern US (Gaskin et al., 2005) but is particularly problematic in the western US (Cripps et al., 2006; Gaskin et al., 2005). The plant can invade open, disturbed, and degraded habitats, such as croplands, pastures, rangelands, and riparian areas (Scurfield, 1962) and can spread both clonally and by seed (Groh, 1940a). It is a declared noxious weed in 15 US states and three Canadian provinces (USDA-NRCS, 2018).

L. draba can form dense stands that decrease plant abundance (McKenney, 2005) and genetic diversity of native competitors (Mealor et al., 2004). In Oregon it has been documented to competitively prevent the seed germination of two federally listed threatened and endangered species: *Limnanthes floccosa* Howell ssp. *grandiflora* Arroyo and *Lomatium cookii* J.S. Kagan (Federal Register E9-17522 2009). Dense stands can also impede important riparian functions such as sediment trapping, bank stabilization and filtration (Francis & Warwick, 2008).

When *L. draba* invades agricultural lands it can reduce crop yields (Corns & Frankton, 1952). It has been documented to inhibit the germination and seedling growth of cabbage (*Brassica oleracea* L.), onion (*Allium cepa* L.), and tomato (*Lycopersicum esculentum* Mill.) (Qasem, 2001). In Oregon alone, *L. draba* is estimated to inflict crop production losses totaling $2.5 million per year (McInnis et al., 2003). In rangeland, *L. draba* can reduce animal forage quality (Puliafico, 2008) and inhibit the germination and seedling growth of alfalfa and forage grasses (Kiemnec & McInnis, 2002). It can also be toxic to livestock, with sulphur levels higher than the reported maximum tolerable level of 0.4% for most grazing animals (McInnis et al., 2003). *Lepidium draba* contains at least 17 glucosinolates (Müller & Martens, 2005; Puliafico, 2008), which are sulfur and nitrogen containing secondary metabolites (Fahey et al., 2001). Glucosinolates and their derivatives have been documented to have several negative effects on grazing animals including gastroenteritis, salivation, diarrhea, thyroid inhibition, liver/kidney lesions, goiter and irritation of the mouth (Francis & Warwick, 2008; McInnis et al., 1993).

Cultural, mechanical, and chemical management strategies have been used to control *L. draba*. Grazing has been used on rangelands (Francis & Warwick, 2008). Despite toxic glucosinolates, it has been proposed that sheep and goats (but not cattle due to toxicity concerns) could safely consume *L. draba* on rangelands provided that these plants were at the pre-flowering stage (Wilson et al., 2006). However, repeat grazing throughout the season would be required to suppress weed populations (Wilson et al., 2006). Additionally, protein and energy levels supplied by this weed are insufficient for grazing sheep (McInnis et al., 1993). Physically removing or damaging the plant is another management strategy, however results are mixed. Mowing alone has proven ineffective (McInnis et al., 2003), and hand pulling or digging can be effective only for small infestations (Francis & Warwick, 2008). Because of the hardy dense root system, complete plant and root system removal is required within 10 days after weed emergence, and this must be performed throughout the growing season for a two- to four-year period (Francis & Warwick, 2008). Flooding has also been effective in small areas, but continuous submersion is necessary and since flooding by its nature can have negative impacts on desired plants, it is not suitable or feasible for most agricultural or rangeland settings (Graves-Medley & Mangold, 2011; Kadrmas & Johnson, 2002). Herbicides to control *L. draba* include metsulfuron, 2,4-D, glyphosate, chlorsulfuron and imazethapyr (Francis & Warwick, 2008). Successful management varies with rates and timing of applications and in most cases repeated applications are required over multiple years in order to effectively manage the weed (Francis & Warwick, 2008). Aside from the fact that herbicides may have direct non-target impacts, chemical control is often also not economically feasible or practical for large or remote infestations that need to be treated for multiple years (Francis & Warwick, 2008). A program to explore classical biological control for *L. draba* was initiated in 2001. Since then several potential biological control candidate species were studied. The leaf petiole gall-forming weevil *Ceutorhynchus cardariae* Korotyaev (Coleoptera, Curculionidae) was identified as the species with the greatest control potential (Hinz & Diaconu, 2015).

Ceutorhynchus cardariae has been found in north- and south-eastern Europe and southern Russia (Korotyaev, 1992; Hinz & Diaconu, 2015). *Lepidium draba* has been described as the sole host plant for *C. cardariae* (Korotyaev, 1992) and has only occasionally been recorded from the closely related *L. campestre* in the field in Europe (Hinz & Diaconu, 2015). *Ceutorhynchus cardariae* has never been reported as a pest on any commercially grown Brassicaceae (Schwarz, 1990). Females lay their eggs in early spring into stems, leaf stalks, and midribs of *L. draba* rosettes and bolting plants, which is thought to cause the formation of galls (Hinz & Diaconu, 2015). Larvae of *C. cardariae* hatch and then feed on the parenchymatic tissue of the developing galls, which can stunt or even kill shoots (Hinz & Diaconu, 2015). Larvae feed for about eight weeks after which time they leave the plant to pupate in the soil. Adults emerge approximately two weeks later and feed on the leaves of *L. draba* for two to three weeks before entering a period of aestivation. The adults become active again in the fall, feeding on the leaves, and some individuals will lay eggs. Most adults overwinter and lay eggs in the following spring (Hinz & Diaconu, 2015). Because *C. cardariae* attacks two life stages of *L. draba* - rosettes as well as bolting plants - it is not dependent on the presence of one particular plant phenostage. These two traits - a long oviposition period (both spring and fall) and the fact that adults attack both phenostages - make it difficult for *L. draba* to escape attack or compensate (Hinz & Diaconu, 2015). The feeding damage caused by larvae and adults is expected to reduce the vigor and the competitive ability of *L. draba* (Hinz & Diaconu, 2015).

Experimental host range of *Ceutorhynchus cardariae*

The host-specificity of *C. cardariae* has been studied at CABI Switzerland since 2003. Testing methodologies included no-choice, multiple-choice, and open field cage tests. A total of 156 test plant species, over half of which are native to North America, and 10 federally listed threatened or endangered (T&E) species, were tested for non-target attack including feeding, gall development, and adult emergence (Weyl et al., 2018). Results of the host-specificity testing indicate a high level of specialization of *C. cardariae* with a fundamental host range that also partially includes species in the tribes Lepidieae, Cardamineae, and Thelypodieae, but with a much narrower ecological host range. Plant species most suitable for development of *C. cardariae* are in genus *Lepidium* (Lepidieae tribe) but also in the distantly related genus, *Streptanthus* (Thelypodieae tribe) (Weyl et al., 2018). The Brassicaceae family is separated into three distinct lineages[1], with the

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[1] Lineages were determined using the chloroplast gene *ndhF*. Using parsimony, likelihood, and Bayesian methods, Beilstein et al., (2006) reconstructed the phylogeny of the gene. The genera were then able to be grouped into monophyletic groups called lineages.

Cardamineae and the Lepidieae tribe belonging to lineage I, and the Thelypodieae belonging to lineage II (Kiefer et al., 2014). Weevil development on *Streptanthus* is therefore unexpected because it does not follow the predictions of the centrifugal phylogenetic approach, which assumes that plant species more closely related to the target are also more likely to be attacked compared to more distantly related plants (Wapshere, 1974). Rather, *C. cardariae* exhibits a 'phylogenetically disjunct' host range. Understanding the mechanisms that are underlying the observed host range pattern and the respective host selection behavior of *C. cardariae* could provide insight into what non-target plant species, if any, may be vulnerable to attack should the weevil be introduced into North America.

Little is known about the different behavioral phases involved in the host selection and acceptance of *C. cardariae*. However, since *C. cardariae* is highly host-specific under natural conditions, it is likely that specific volatile, visual, or surface wax cues, individually or in combination, mediate the host selection process. These cues have been found to mediate the host selection and acceptance behavior of other Brassicaceae feeding beetles [e.g. *Ceutorhynchus assimilis* (Paykull), *Ceutorhynchus pallidactylus* (Marsham), *Meligethes aeneus* (F.), *Phaedon cochleariae* (F.), *Phyllotreta atra* (F.), *Phyllotreta cruciferae* (Goeze), *Phyllotreta striolata* (F.), and *Phyllotreta undulata* (Kutschera)] with regard to volatile cues (Bartlet et al., 1993; Blight et al., 1995; Cook et al., 2007; Free & Williams, 1978; Kühnle & Müller, 2011; Tansey et al., 2010), visual cues (Blight & Smart, 1999; Kühnle & Müller, 2011; Smart & Blight, 1997), and surface wax cues (Bodnaryk, 1992a; Bodnaryk, 1992b; Eigenbrode et al., 2000; Reifenrath et al., 2005; Stoner, 1990; Way & Murdie, 1965). In order to begin testing for which host selection cues may be influencing *C. cardariae*, preliminary tests were performed with volatiles and various olfactometers.

Preliminary olfactometer experiments

The aim of preliminary pre-alightment tests was to investigate the role of volatiles in the host selection behavior of *C. cardariae*. Bioassays included 1) various y-tube setups using different materials such as glass and plastic, different sizes of y-tubes, y-tubes positioned in different angles, and differing duration of tests, 2) various static systems utilizing simple chambered cylinders, and 3) a four-arm olfactometer. Tests using the y-tube and four-arm olfactometer also included an air flow directed into the different apparatuses using a push pull system. A variety of different air flow strengths were used, and the headspace volatiles used were from whole, undamaged *L. draba* plants covered in Teflon bags. Tests using the y-tube and four-arm olfactometer were also performed without airflow. For the static systems, uncovered leaves excised from plants were used.

The simplest static chamber used was a plastic bucket (38 cm high, 22 cm in diameter) that was covered with a mesh lid. One leaf of *L. draba* was placed on the top center of the mesh. Five sets of five weevils that had been starved for 24 hours were placed inside the bucket and their position was recorded after one hour. The mesh allowed the weevils access to volatiles from the leaf, but prevented physical contact. After one hour there was no indication that the weevils were attracted to the leaf, as only two of the 25 weevils were found within 2.5 cm of the leaf. A preliminary test with airflow used a four-arm olfactometer. The olfactometer was 22 cm in diameter, had four equidistant inlet arms, with a central basal outlet in the middle of the arena floor. Two inlet arms were connected via tubing to potted plants whose foliage was hermetically sealed within Teflon bags. The other two inlet arms were connected to Teflon bags containing only air. Four push pumps were used to deliver air to each inlet arm. The airflow in each arm was maintained at approximately 300 ml/minute using flowmeters. Another pump was used to pull air from the basal outlet at the rate of approximately 1200 ml/minute. A total of 38 weevils were observed individually in the arena and their position recorded after 30 minutes. Approximately 45% of the weevils were recorded in the *L. draba* quadrant, approximately 43% in the air quadrant, and approximately 12% of the weevils were not responsive and remained in or returned to the center of the arena. All other olfactometer experiments yielded similar results, i.e. the weevil demonstrated no preference for *L. draba* headspace volatiles over purified air.

In these preliminary tests, *C. cardariae* did not demonstrate a preference for *L. draba* volatiles. However, it is unclear whether the lack of behavioral response may be due to the specific method employed or confirms that the weevil does not rely on volatile olfactory plant cues during host finding. The olfactometers used may not mimic natural conditions sufficiently for the weevil to demonstrate its normal responses to volatiles during host selection. Several other *Ceutorhynchini* species are attracted to olfactory cues. *Ceutorhynchus assimilis* has been observed to be attracted to *Brassica napus* (L.) (Bartlet et al., 1993; Blight et al., 1995; Free & Williams, 1978), and to genotypes of *Sinapis alba* (L.) × *Brassica napus* (Tansey et al., 2010), and *Mogulones borraginis* F. (Park et al., 2018) and *Mogulones crucifer* Pallas (Kafle, 2017) to *Cynoglossum officinale* (L.).

Alternatively, volatiles may be in fact of lesser importance to *C. cardariae* than other plant cues. Other specialist insects rely less on volatile plant cues during host selection. The monophagous leaf beetle, *Altica engstroemi* J. Sahlberg, was not attracted to volatiles from its host plant under various olfactometer tests (Stenberg & Ericson, 2007). In field experiments, it was demonstrated that visual cues alone were sufficient to attract a significant number of starved beetles (Stenberg & Ericson, 2007). Therefore, some specialist insect herbivores seem to rely on plant cue modalities other than olfaction during host finding.

Visual cues can also be critical for host finding. In a study conducted by Prokopy and Owens (1978), the apparently monophagous sawfly, *Hoplocampa testudinea* Klug., and the oliophagous fly, *Rhagoletis pomonella* (Walsh), were found to be more specific in visual orientation to apple, than were the polyphagous Hemipteran, *Lygus lineolaris* (P. de B.). It was proposed that specialist insect herbivores may rely more on visual and less on olfactory cues, while generalist insect herbivores may be visual generalists and rely more on olfactory plant cues (Prokopy & Owens, 1978). This may be because polyphagous species preferred feeding, mating, or oviposition sites on a greater number of plant species, and thus are attracted to a greater array of diverse physical characteristics, than would be monophagous herbivores who have a more limited host range (Prokopy & Owens, 1978). Additionally, it has been proposed that monophagous herbivores with limited dispersal ability that inhabit persistent habitats, that use host plants that dominate plant communities, and that are predictable in time and space, may commonly evolve vision as the main or even exclusive host plant location mechanism (Reeves, 2011; Stenberg & Ericson, 2007). This could also be the case for *C. cardariae* as it is a specialized herbivore with limited dispersal capabilities on a clonal plant that is, at least within patches, dominant. The weevil has never been observed flying and the larvae are endophagous, i.e. cannot change plants.

Objectives

The specific objectives of this dissertation are to 1) determine the relative importance of visual, volatile, and surface wax cues in the host selection of *C. cardariae*, both singularly and in combination; and 2) utilize this information to determine native confamilial plant species that could be at risk of attack by *C. cardariae* should the insect be released in North America. Specifically, I investigated *C. cardariae's* host selection behavior in a series of pre- and post-alightment studies to test for specific sensory cues that may be influencing host plant choice. The overall goal of this research was to contribute to the environmental safety assessment of *C. cardariae* as a biological control agent for *L. draba*. I hypothesize that female weevils use a combination of volatile, visual, and/or surface wax cues to discriminate *L. draba* from non-host plants. With a total of 156 test plants used in host-specificity testing experiments, a smaller and more manageable list of test plants species was selected for my study. This list included the target plant species, *L. draba*, and 13 native North American species within the Thelypodieae and Lepidieae tribes (Table 1.1). These species were selected to represent two levels of plant attack found within each tribe and were observed during host-specificity testing. A high level of attack (supported gall or adult development) was designated as 'potential alternative host' and a low level of attack (none to limited feeding) was designated as 'non-host.'

The subsequent chapters in this dissertation are presented as follows: Chapter Two is a literature review on the role of physical and chemical surface wax cues in the host plant selection behavior of herbivore arthropods feeding on Brassicaceae. This review informed me of the importance of surface wax cues in the host selection of several Brassicaceae feeding herbivores, which warranted their examination in my study system. Chapter Three examines the pre-feeding behavior of *C. cardariae* on *L. draba* and one non-host plant, basil. Basil was chosen for its ease of propagation, absence of glucosinolates, and because of its phylogenetic distance to *L. draba* (Byng et al., 2016). Distinctive pre-feeding behaviors performed prior to the onset of adult feeding and its potential implications for *C. cardariae's* host selection behavior is presented. Chapter Four examines the role of volatile, visual, and contact cues in the host selection behavior of *C. cardariae* on *L. draba.* In this chapter, an array of bioassays - which manipulated volatile, visual, and surface wax cues of *L. draba* and plant models - were used in a walking arena. Chapter Five examines the volatile compounds emitted by *L. draba* and 13 native North American confamilial species.

Table 1.1: A list of the 13 native North American species that have been used in this research, along with the reason for their inclusion.

Tribe	Species	Reasons for inclusion
Lepidieae	*Lepidium crenatum* (Greene) Rydb.	Non-host: limited feeding.[2]
	Lepidium latipes Hook.	Possible host: supported gall formation, but limited adult emergence. [2]
	Lepidium oblongum Small	Possible host: supported adult development. [1]
	Lepidium papilliferum (L.F. Hend.) A. Nelson & J.F. Macbr.	Non-host: hardly any feeding. [2]
Thelypodieae	*Stanleya pinnata* (Pursh) Britton	Possible host: supported gall formation. [2]
	Stanleya viridiflora Nutt.	Non-host: limited feeding. [2]
	Streptanthus anceps (Payson) Hoover	Possible host: supported adult development. [1]
	Streptanthus crassicaulis Torr.	Non-host: no feeding at all. [2]
	Streptanthus farnsworthianus J.T. Howell	Non-host: limited feeding. [2]
	Streptanthus flavescens Hook.	Possible host: supported adult development. [1]
	Streptanthus glandulosus ssp. glandulosus Hook.	Possible host: supported adult development. [1]
	Streptanthus glandulosus ssp. niger Hook.	Possible host: supported gall formation. [2]
	Streptanthus inflatus Greene	Possible host: supported adult development. [2]

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Chapter 2

THE ROLE OF PHYSICAL AND CHEMICAL SURFACE WAX CUES IN THE HOST PLANT SELECTION BEHAVIOR OF HERBIVORES FEEDING ON BRASSICACEAE

Abstract

Physical and chemical cues of surface waxes of Brassicaceae plants have been shown to influence the host plant selection behavior of insect herbivores. However, the fragmented body of knowledge on this subject has not been reviewed to date. Here we provide a synthesis of the literature on the role of physical (structure, amount, and chemical makeup of aliphatic hydrocarbons) and chemical (biologically active metabolites and chemicals present on the leaf surface) aspects of surface wax cues as they pertain to the host plant selection behavior of Brassicaceae-feeding insect herbivores. We focus on the chemoreception of tarsal and antennal chemoreceptors and excluded the following articles from this review: those that only indirectly examined the role of surface wax cues, those focused on visual cue aspects of surface waxes, and those that focused only on trichomes, predators and tri tropic interactions, and gustatory chemoreception.

A literature search identified 55 original studies including 19 insect species within four orders. Of these, 13 species were classified as oligophagous and six were classified polyphagous. Eighty-seven percent of articles that investigated physical aspects, and 96% of articles that investigated chemical aspects of surface waxes of Brassicaceae plants, reported an effect on the respective insects' host selection behavior. Most articles focused on agricultural crops and respective pests. Studies differed in the type of growing conditions and

insect life stages used. Similarly, experimental techniques varied greatly: larval behavior or female oviposition behavior on plants were studied depending on the organism, plant models were used, in some cases sprayed with leaf surface extracts, electrophysiological studies were conducted, and surfaces waxes were manipulated mechanically or chemically. Depending on their systematic position, insects had a preference for glossy or waxy surface waxes. Notable chemicals influencing insect behavior were glucosinolates (GS) and thia-triaza-fluorene compounds, also known as cabbage identification factors (CIF). Differences in the host selection behavior was observed within two species depending on what life stage was used, and three insect species demonstrated differences in their host selection behavior depending on experimental growing locations. Feeding guilds represented in the studies (chewing, sap sucking, and boring) did not seem to have any effect on the host selection behavior. Likewise, feeding specialization (oligophagous or polyphagous) or plant propagation method (field grown or laboratory setting) did not alter responses of surface waxes on the host selection behavior of the respective insects studied.

Introduction

The plant cuticle serves many diverse ecological functions: waterproofing, reflecting radiation, protecting against plant pathogens, and being a medium for communicating semiochemicals (Eigenbrode, 2004). It is covered by lipophilic materials commonly referred to as surface waxes, or epicuticular waxes. These waxes mainly consist of long chain aliphatic lipids, fatty acids, esters, alkanes, and other hydrocarbons. The quality and quantity of waxes can vary tremendously between plant species as well as between abaxial and adaxial leaf surfaces of individual leaves (Justus et al., 2000). They can also contain extractable secondary metabolites including: sugars, amino acids, sesquiterpenes, phenolics, and glucosinolates (GS) (Eigenbrode & Espelie, 1995). Epicuticular lipids can influence how secondary metabolites are perceived by insects and have synergistic effects (Spencer et al., 1999). These contact chemicals can be present within or on the surface, or be very close-range volatiles associated with the surface of the plant and can be perceived by insects through the tarsi or antennae. The differences in amount, composition, and chemical makeup of various plant surface waxes often explain the attractiveness of host plants, as these characteristic blends of surface waxes can be used by insects for host plant selection (Bernays & Chapman, 1994).

Insects use chemoreceptors on their tarsi, mouthparts, antennae, and ovipositor to assess specific compounds or combinations of compounds present on the leaf surface, or as close-range volatilized metabolites (Bernays & Chapman, 1994 and references therein). Insects often "sample" the leaf surface by drumming with tarsi or antennae, walking up and down the leaf surface, and test bite, presumably to enhance contact between sensory organs and stimulants, nutrients, and deterrents (Renwick, 1989). Contact chemoreception is dependent on physical contact with the stimulus and has the form of a hair that consists of an opening at the top (pore) and a dendrite that extends down into the body of the insect where a sensory neuron interrupts the chemicals sensed. The entire chemoreceptors structure is called a sensillum and can be sensitive to only one type of chemical, or a group of different chemicals (Chapman, 2003). With respect to gustatory chemoreceptors on mouthparts, these receptors generally come into contact with chemicals in an aqueous solution (i.e. masticated plant material and insect saliva). For this review, we focus on "dry" chemoreception from tarsal and antennal chemoreceptors as they are generally the first receptors to operate when an insect initially encounters the leaf surface. The chemicals present on the dry leaf surface, or volatilized very near the leaf surface, are presumed to be taken up by a diffusion pathway via the lateral pores to the dendrites. The insect sensory neuron interprets these chemicals and a behavioral response is elicited from the insect. Specialized insects that feed on a limited number of plants within a plant family have been shown to use the specific classes of compounds that are characteristic of particular plant families for host recognition and acceptance (Renwick, 1989).

Host selection by insects within the family Brassicaceae has been of particular interest to researchers. Brassicaceae includes many valuable economic crops which are fed upon by pestiferous crop insects. These pests are commonly controlled with insecticides, but breeding new crop lineages that are naturally resistant to insect crop pests is seen as preferable to widespread insecticide applications on crops meant for human and animal consumption (Eigenbrode et al., 2000). If the use of plant surface wax cues may differ among Brassicaceae insect pests or with insect traits such as life-history characteristics, feeding guild, systematic position, or feeding specialization level, these patterns may be exploited for host plant resistance breeding or integrated pest management strategies. Secondarily, all Brassicaceae plants contain GS. This makes them an interesting system in which to study the effects of specialization on unique plant chemistry (Renwick & Radke, 1988).

Reviewing the literature on how Brassicaceae surface waxes mediate host selection of Brassicaceae-feeding herbivores could increase the understanding of surface wax cues in post-alightment host-finding. For example, chemical ecological research involving volatiles is dominating the research on host finding in insects (Reeves, 2011), including Brassicaceae feeding herbivores. There is much less research on the role of chemical-contact surface wax cues once an insect makes contact with the plant. A search of the terms “Brassicaceae host finding volatile” in the Google Scholar search engine generated 3260 listed articles, while in comparison, a search of the term “Brassicaceae host finding surface wax chemical” rendered 932 listed articles. This current preference for pre-alightment host finding rather than post-alightment contact cues may or may not be reflective of the relative importance of contact cues in nature.

A review of the role of surface wax cues and their role in host finding could also aid biological weed control efforts to develop candidates for Brassicaceae target weeds. Biological weed control requires understanding the potential for non-target effects of a biological control candidate (Sheppard et al., 2005). Information on phylogenetic relationships between the target weed and related confamilial plant species is generally used in host range testing experiments with the assumption that species more closely related to the target weed are at greater risk of attack than more distantly related species (Wapshere, 1974). However, other important behavioral plant traits, for example secondary metabolites, may not necessarily be correlated or associated with plant phylogeny (van Klinken, 2000). The role of plant chemistry with regard to host range testing of biological control candidates is not well understood despite the suggestion that shared plant secondary metabolites might be an as good indicator for host utilization as are phylogenetic relationships (Wheeler & Schaffner, 2013). Investigations of the chemical factors mediating the interactions between the biological control candidate and the host plant, in this case surface wax chemical cues, could improve the predictability of potential non-target risks (Wheeler & Schaffner, 2013).

Here we identified and examined the literature on the physical aspects (structure, amount, and chemical makeup of aliphatic hydrocarbons) and chemical aspects (biologically active metabolites and chemicals) of surface waxes in Brassicaceae and how they may mediate host selection behavior of Brassicaceae feeding herbivores. We examined each research article in smaller compartmentalized categories that would allow for facilitation of discussion of larger topics. For example, by organizing articles based on where the test plants were grown, we can discuss what impact this may have on how surface waxes influence insect herbivores. Our goal was to detect any general patterns between certain insects and these traits, which explain the behavioral and use patterns of herbivores to plant surface waxes.

Materials and methods

Research studies were queried through an internet search using Google Scholar and Web of Science. The following search terms were used: ‘host finding,’ ‘cue,’ ‘surface waxes,’ ‘crucifer,’ ‘Cruciferae,’ ‘Brassicaceae,’ ‘host plant recognition,’ ‘leaf surface extracts,’ ‘host plant acceptance,’ and ‘surface wax chemicals.’ Terms were entered into the search engines both individually and in varying combinations such as ‘Brassicaceae host finding surface waxes’ and ‘Cruciferae leaf surface extracts.’ For each article found, its references were cross-checked in order to find additional studies. An internet search using Google Scholar was also conducted to determine the level of feeding specialization for each insect studied (i.e. monophagous, oligophagous, or polyphagous). Search terms included the scientific species name, ‘feeding,’ and ‘host range.’

A series of mutations can change the normal waxy bloom of plants into glossy wax blooms that have a reduced quantity of wax, and/or different chemical composition of the wax (Jeffree et al., 1976; Stoner, 1990). In this review we will hereon refer to plants with reduced surface waxes as ‘glossy plants’ and plants with normal or waxy leaf surfaces as ‘waxy plants.’ Physical aspects of the leaf surface refer to structure, amount, and chemical makeup of aliphatic hydrocarbons. Chemical aspects of the leaf surface refer to biologically active metabolites and chemicals present on the surface. The articles found on the role of surface wax cues in the host selection of Brassicaceae-feeding insect herbivores varied greatly in their experimental approaches. We categorically decided to exclude the following article types: those that only indirectly investigated the role of chemical cues in surface waxes (e.g. electrophysiological studies on tarsi using purchased chemical compounds that were assumed to be present or part of surface waxes, but that had not been extracted directly from plant surface waxes). Dethier (1982) states that recording activity of individual chemoreceptor neurons in response to pure compounds may have limited predictive value for the activity of the same neurons responding to a complex mixture. Also excluded were articles investigating visual effects of surface waxes on insects. A heavy wax bloom increases reflectance in other wavelengths and makes the plants appear whiter (Prokopy et al., 1983). Some studies have shown that *Delia radicum* (L.) (Diptera, Anthomyiidae) prefers darker green glossy cabbages over their whitish-blue waxy counterparts due to the difference in the spectral preferences of the flies (Prokopy et al., 1983). Visual cues are typically more important during the pre-alightment phase prior to an insect making contact with the plant and thus, respective studies were excluded. Review articles that investigated the influence of plant trichomes without mention of the chemical and/or physical aspects of the associated surface waxes were also excluded. Similarly, we excluded studies that focused on predators or tri-trophic interactions as the relationship between herbivores and the leaf surface is the focus of this review. We also did not consider articles that focused solely on insect gustatory chemoreception of the leaf surface.

All articles matching the search criteria were examined and the following information was recorded: 1) systematic classification of the organism(s), 2) feeding specialization, 3) life stage studied, 4) feeding guild of study organism, 5) host plant species and location of plant propagation, 6) experimental approach, and 7) results and conclusions. This categorization allowed for graphing and analyzing results into smaller tables.

Statistical analysis included χ^2 tests of homogeneity to test 1) whether insect feeding specialization impacted the role of surface waxes on host selection behavior and 2) whether plant propagation locales impacted the role of surface waxes on host selection behavior. The tests were conducted using the statistical software package SAS (Version 9.4; SAS Institute Inc., 2013).

Results

Number of articles and systematic classification of studied organisms

Seventy-three published studies were identified that investigated the role of physical and/or chemical surface wax cues in the host selection of insect herbivores feeding on Brassicaceae. Eighteen of these were review articles. Many of these provided overviews of the role of epicuticular lipids on insect-plant interactions, albeit broader in focus than discussed here. They did not include in-depth information specifically on Brassicaceae-feeding herbivores, and where such herbivores are mentioned, there was a lack of information on the chemicals involved in interactions or the experimental approach taken (Chapman & Bernays,1989). Because of this, there were not included in this review as original research articles. The remaining 55 articles comprised four insect orders and a total of 19 insect species in eight families (Table 2.1). These included: *Brevicoryne brassicae* (L.) (Hemiptera: Aphididae), *Ceutorhynchus pallidactylus* (Marsh.) (Coleoptera, Curculionidae), *Delia floralis* (Fallen) (Diptera, Anthomyiidae), *Delia radicum* (L.) (Diptera, Anthomyiidae), *Lipaphis erysimi* (Kaltenbach) (Hemiptera, Aphididae), *Phaedon cochleariae* (F.) (Coleoptera, Chrysomelidae), *Phyllotreta atra* (F.) (Coleoptera, Chrysomelidae), *Phyllotreta cruciferae* (Goeze) (Coleoptera, Chrysomelidae), *Phyllotreta striolata* (F.) (Coleoptera, Chrysomelidae), *Phyllotreta undulata* (Kutz.) (Coleoptera, Chrysomelidae), *Pieris brassicae* (L.) (Lepidoptera, Pieridae), *Pieris rapae* (L.) (Lepidoptera, Pieridae), *Plutella xylostella* (L.) (Lepidoptera, Plutellidae), *Aleyrodes proletella* (L.) (Hemiptera, Aleyrodidae), *Bemisia tabaci* (Gennadius) (Hemiptera, Aleyrodidae), *Bemisia argentifolii* (Bellows and Perring) (Hemiptera, Aleyrodidae), *Mamestra brassicae* (L.) (Lepidoptera, Noctuidae), *Myzus persicae* (Sulzer) (Hemiptera, Aphididae), and *Trichoplusia ni* (Hübner) (Lepidoptera, Noctuidae).

Within the Coleoptera, there was a trend towards the family Chrysomelidae with five of the six beetles studied belonging to it, and one beetle belonging to the Curculionidae family. The two fly species were very closely related; both belonged to the genus Delia. Three of the hemipterans were whiteflies, and three were aphids. During the time of publication of several of the articles, *B. argentifolii* and *B. tabaci* were designated as a separate species. However, according to de Barro et al., (2005), there is insufficient data to raise *B. argentifolii* to species status. Though *B. argentifolii* is currently considered a "race" or "strain" of *B. tabaci*, we will continue to refer to them as separate species for consistency and to indicate their ecological differences for the purposes of this review. More than half of the original research included lepidopterans (53%, n=55), especially *Plutella Xylostella* (diamondback moth), which was the study object or one study object in 40% (n=55) of the studies.

Herbivore preference for glossy and waxy plants

Glossy genotypes' morphology showed reduced wax densities of crystallites, with sparsely distributed short rods; waxy genotypes had dense mats of rods and filaments, (Eigenbrode et al., 1991b; Stoner, 1992a). For 15 of the species studied (79%, n=19), a preference for either glossy or waxy plants was observed across studies (Table 2.2). Six beetle species preferred glossy plants, one fly preferred waxy plants, three hemipterans preferred waxy plants while one preferred glossy plants. Finally, four lepidopteran species preferred waxy plants while one preferred glossy plants (Table 2.2).

Level of feeding specialization and feeding guilds

Based on the analysis of the host range literature search (Table 2.3), we categorized 13 herbivore species (68%) as oligophagous, and six (32%) as polyphagous (Table 2.3). We defined oligophagous as feeding within the Brassicaceae family and on closely related plant families containing GS (Capparidaceae, Tropaeolaceae, and Limnanthaceae). We defined polyphagous as feeding within the Brassicaceae family and on plant families that do not containing GS. All beetles and flies studied were oligophagous. One lepidopteran was polyphagous and four were oligophagous. Two of the Hemipteran were oligophagous and four were polyphagous. None of the 19 herbivores studied were strict specialists on plant species in the Brassicaceae family. While there are differing levels of oligophagy, we felt further categorization would not be helpful.

All 19 insect species studied belonged to one of three feeding guilds. Two fly species, *D. floralis* and *D. radicum*, and one beetle, *C. pallidactylus*, were borers; six hemipterans, *A. proletella, B. argentifolii, B. tabaci, B. brassicae, L. erysimi,* and *M. persicae* were sap suckers; five beetle species, *P. cochleariae, P. atra, P. cruciferae, P. striolata,* and *P. undulata* and four lepidopterans, *P. brassicae, P. rapae, P. xylostella,* and *T. ni* were chewers (Table 2.4). Feeding guild did not readily correlate with whether or not the physical or chemical properties of surface waxes would influence host selection behavior of insects (Table 2.4). Boring insects' host selection behavior was influenced by the physical properties of surface waxes in 100% of the articles in which they were studied (n=3); sap sucking insects' host selection behavior was influenced by the physical properties of surface waxes in 83% of the articles in which they were studied (n=18); chewing insects' host selection behavior was influenced by the physical properties of surface waxes in 88% of the articles in which they were studied (n=48) (Table 2.4). Boring insects' host selection behavior was influenced by the chemical properties of surface waxes in 95% of the articles in which they were studied (n=19); chewing insects' host selection behavior was influenced by the chemical properties of surface waxes in 100% of the articles in which they were studied (n=5) (Table 2.4).

Differences in host selection behavior based on insect life stage

Mamestra brassicae and *P. xylostella* differed in host plant preference based on either past larval experience or which life stage was used (Table 2.5). *Mamestra brassicae* adults preferred to lay eggs on the plant variety (waxy or glossy) on which they had been reared as larvae (Way & Murdie, 1965). Preference for glossy or waxy plants differed between the adult and larval stage of *P. xylostella.* Glossy phenotypes were more attractive than waxy ones for oviposition by the moth, but herbivory was reduced on glossy plants and larval dispersal was increased (Eigenbrode et al., 1991b; Ulmer et al., 2002) (Table 2.5).

Effect of plant propagation location on surface wax mediated host selection and usage

Three of the insect responses differed depending on whether plants were grown in the field or in a greenhouse or laboratory (Cole & Riggall, 1992; Jackson et al., 2000; Ulmer et al., 2002) (Table 2.6). *Bemisia argentifolii* preferentially selected waxy over glossy collard phenotypes in the field, but survives, develops, and reproduces as well on glossy collard phenotypes as it does on waxy phenotypes in no-choice greenhouse experiments (Jackson et al., 2000). In the field, *B. brassicae* populations were much smaller on glossy *B. oleracea* lines, but glossy lines grown in the greenhouse were as susceptible to aphid populations as the waxy line (Cole & Riggall, 1992). Glossy *Brassica rapa* (L.) lines showed resistance to *P. xylostella* under field conditions, while in greenhouse experiments no resistance was observed (Ulmer et al., 2002).

Physical aspects affecting host selection behavior

Approximately 87% (n=60) of the instances in which an insect was investigated for the physical aspects of surface waxes (structure, amount, and chemical makeup of aliphatic hydrocarbons), there was a reported impact on insect host selection (Table 2.4). *P. xylostella* larvae moved more rapidly, spent more time walking, and engaged in searching behaviors more often on glossy cabbage leaves and extracts of glossy leaves, than on waxy cabbage leaves and extracts of waxy leaves (Eigenbrode et al., 1991a; Eigenbrode & Pillai, 1998). *Phaedon cochleariae* adhered better to glossy leaves than to waxy leaves of *B. oleracea*, and their tarsal setae were observed to be covered with clumps of wax after being on the waxy leaves (Stork, 1980).

Several studies involved the mechanical removal of the surface waxes of waxy leaves in a non-destructive manner using either cotton swabs, camel hair brushes, soft paper towels, or by the application of gum Arabic (Åhman, 1990; Blua et al., 1995; Eigenbrode & Shelton, 1990; Lin et al., 1984; Reifenrath et al., 2005) (Table 2.9). In all cases, except for *B. brassicae*, the mechanical removal of waxes caused insects *(B. tabaci, L. erysimi, P. cochleariae, and P. xylostella),* to utilize the mechanically de-waxed plants the same as naturally glossy plants.

Several studies chemically removed the surface waxes of waxy leaves in a non-destructive manner using either the carbamate herbicide S-ethyldipropylthiocarbamate (EPTC), surfactant Latron CS-7, or quick dips in chloroform, ether, dichloromethane, or synthetic detergent (Badenes-Pérez et al., 2004; Bodnaryk, 1992b; Eigenbrode & Shelton, 1992; Eigenbrode et al., 1993; Eigenbrode et al., 2000; Justus et al., 2000; Lin et al., 1984; Reifenrath et al., 2005; Riggin-Bucci et al., 1998; Uematsu & Sakanoshita, 1989). In almost all cases, chemical removal of waxes from *B. oleracea* resulted in insects (*P. cochleariae, P. rapae, P. xylostella,* and *T. ni*) utilizing the chemically de-waxed plants at the same rate as naturally glossy plants. However, applying EPTC to *B. napus* effected mixed results. Altering waxy *B. napus* to exhibit glossy wax characteristics had no effect on *B. brassicae* and *P. cruciferae* plant preference (Eigenbrode et al., 2000), while *P. xylostella* did treat induced glossy plants the same as naturally glossy plants (Justus et al., 2000).

Chemical aspects affecting host selection behavior

Approximately 96% (n=25) of the instances in which an insect was investigated for the chemical aspects of the surface waxes (biologically active metabolites and chemicals present on the leaf surface), there was a reported effect on insect host selection (Table 2.4). Specific chemical compounds isolated from leaf surface waxes influenced host selection in six insect species (Table 2.7). These chemicals included GS and cabbage identification factors (CIF) compounds. In oviposition bioassays, the GS glucobrassicin stimulated oviposition in *Delia floralis* (Gouinguene & Städler, 2006), and glucobrassicin and gluconasturtiin stimulated oviposition in *Delia radicum* (Baur et al., 1996a; Gouinguene & Städler, 2006). In electrophysiological studies, the D_2 and D_3- sensilla of *D. floralis* reacted to glucosinolate fractions (Baur et al., 1996a, b) and the D_3 and D_4- sensilla of *D. radicum* reacted to glucosinolate fractions and were especially sensitive to the GS gluconasturtiin, glucobrasssicin, and glucobrassicanapin (Roessingh et al., 1992). The D_3 and D_4- sensilla present on the prothoracic tarsi of *D. floralis* and *D. radicum* reacted to CIF compounds in electrophysiological studies (Baur et al., 1996a; Gouinguene & Städler, 2006; Roessingh et al., 1997). In oviposition bioassays, glucobrassicin (3-indolyl-methyl-glucosinolate) stimulated oviposition for *P. brassica* (van Loon et al., 1992). Glucobarbarin and gluconasturtin acted as strong oviposition stimulants for *P. xylostella* (Badenes-Pérez et al., 2011).

Insight into the chemical aspects of surface waxes was obtained by using sulphur and jasmonic acid treated plants. For *P. brassicae and P. rapae,* it was found that fewer eggs were laid on leaves of jasmonic acid treated plants compared to control plants (Bruinsma et al., 2007). When offered a choice between the purified glucosinolate fraction isolated from leaf surface extracts of jasmonic acid treated plants and that from control plants, no discrimination was detected (Bruinsma et al., 2007). The effects of sulphur fertilization was examined for *D. radicum* and *P. xylostella* (Marazzi et al., 2004a, b). *Brassica napus* (L.) plants were grown under three differ sulphur regimes: sulphur-free (S_0), normal sulphur (S_n, normal field concentration), and sulphur-rich (S_+, 2 × concentration of S_n). Oviposition behavior for both insects was observed in response to plants grown under these regimes and to methanolic leaf-surface extracts from plants grown under these regimes. *D. radicum* preferred to lay eggs on both the plants and the extracts from the S_n and S_+ treatments (Marazzi et al., 2004a). *Plutella xylostella* laid more eggs on surrogate leaves that were treated with S_n extracts than on S_0 plants (Marazzi et al., 2004b). Electrophysiological experiments were also performed. Sulphur treated plants yielded higher EAG amplitudes than the S_0 extracts (Marazzi et al., 2004b). The total glucosinolate content sharply increased from S_0 to S_n plants, whereas it was slightly lower in S_n versus S_+ plants (Marazzi et al., 2004b).

When mechanically removing the lower and upper leaf surface waxes of *B. napus* and *Nasturtium officinale* (W.T. Aiton.) with gum Arabic, no GS were detectable. Leaf surface extracts of *N. officinale* applied onto *Pisum sativum* (L.) leaf discs did not evoke feeding in *P. cochleariae* (Reifenrath et al., 2005). When using gum Arabic surface wax peelings, GS were found to be present on the leaf surface of *Barbarea* spp. and no GS were found on the leaf surface of *B. napus* or *N. officinale* plants (Badenes-Pérez et al., 2011).

Methodologies for testing physical and chemical cues

Physical cues were tested by comparing glossy and waxy plants using five differing approaches: infestation assessments, oviposition studies, movement/behavior studies, feeding behavioral studies, and adhesion experiments (Eigenbrode et al., 2000; Bodnaryk 1992a; Jackson et al., 2000; Lamb et al., 1993; Stork, 1980; Uematsu & Sakanoshita, 1989) (Table 2.9). Three different experimental approaches were used to test chemical cues: oviposition, electrophysiology, and feeding behavior/movement studies (Badenes-Pérez et al., 2011; Bruinsma et al., 2007; Gouinguene & Städler, 2006; Reifenrath et al., 2005; Städler & Reifenrath, 2009) (Table 2.8). Oviposition studies used real plants, leaf surface extracts applied to plant models, and mechanically damaged plants. Different types of solvents were used for leaf surface extraction and included cold, hot, or boiling water, methanol, hexane, chloroform, ether, or dichloromethane. Electrophysiological studies utilized methanolic, dichloromethane, chloroform, and water leaf surface extracts (Badenes-Pérez et al., 2011; Baur et al., 1996a, b; Gouinguene & Städler, 2006; Griffiths et al., 2001; Hopkins et al., 1997). Feeding behavior and movement studies were done with leaf surface extracts (chloroform, methanol, and water) applied to *P. sativum* (Reifenrath et al., 2005). Infestation levels were tested in the field as well as greenhouse conditions (Hopkins et al., 1992; Roessingh et al., 1992).

No significant differences were observed for the χ^2 test of homogeneity between the oligophagous and polyphagous levels of specialization ($Chi^2 = 0.1082$; pr > $Chi^2 = 0.7422$), nor between plant growth locations (Field or Artificial) (Chi2 = 0.1907; pr > Chi2= 0.6623).

Discussion

In general, beetles studied showed a preference for glossy plants while Lepidoptera preferred waxy plants (Table 2.2). This may be in part due to the feeding life stages of the study organisms investigated. Most of the beetles examined feed as adults whereas all lepidopterans feed as larvae. Morphological structures that allow for attachment to plants for adult beetles and larval lepidopteran can impact their feeding choice. For example, *P. cochleariae* adheres much better to glossy cultivars of *B. oleracea* (Stork, 1980). The setae on the tarsi are thought to be hindered by the vertical rods and dendritic plates on waxy leaves (Stork, 1980). Insects utilize different adaptive morphological means to attach and adhere to plant surfaces: claws, swollen tarsal pads, and modified tarsal setae. These different structural adaptations allow for improved mobility on waxy or glossy plants, respectively (Stork, 1986). Numerous insect species, including several flea beetle species (*P. cochleariae, P. cruciferae, M. persicae,* coccinellids, and anthocorids) have been shown to prefer glossy leaved cultivars of *B. oleracea* over waxy ones (Anstey & Moore, 1954; Eigenbrode & Espelie, 1995; Stork, 1980; Way & Murdie, 1965). All of the aforementioned species except *M. persicae* use adhesive setae to hold on to plants (Stork, 1980). In contrast, waxy *B. oleracea* plants are more susceptible than glossy cultivar to *B. brassica* and *A. proletella* (Thompson, 1963; Way & Murdie, 1965). Both these insects grip with fleshy pads rather than adhesive setae (Stork, 1980). *Plutella xylostella* larval preference for waxy plants over glossy and de-waxed plants could also be explained by attachment. If this insect is better at attaching to waxy than glossy plants, then by removing surface waxes, we expect them to also not adhere as well to them; which is indeed what was observed in the experiments conducted on de-waxed plants and *P. xylostella* larvae.

P. xylostella prefers to oviposit on glossy plants (Badenes-Pérez et al., 2004; Justus et al., 2000; Uematsu & Sakanoshita, 1989). Waxy or glossy surfaces may influence successful egg attachment. For example, eggs deposited by *P. xylostella* on glossy waxes were flatter than those on waxy plants, perhaps because glossy waxes allowed for better adhesion of eggs due to greater surface area of attachment (Uematsu & Sakanoshita, 1989). It has also been suggested that oviposition site preference is not related to factors influencing egg attachment, and that factors that influence oviposition site preference act before egg attachment (Justus et al., 2000). For example, the removal of the surface waxes may allow insects better access to underlying chemical stimulants (Justus et al., 2000). This would explain why *B. tabaci, P. cochleariae,* and *P. xylostella* adults preferred naturally glossy plants to mechanically de-waxed plants. De-waxing the plants should result in any stimulants being more available to the insects and increase the plants' attractiveness, and this result was seen in the behavior of these insects (Justus et al., 2000). If there are chemical stimulants present, it follows that removing the surface wax barrier to these stimulants would increase their attractiveness.

The research summarized in this review suggests that differing feeding modes do not affect whether or not surface waxes influence the host selection of respective insect herbivores. However, with the exception of the beetles, which included five chewers and one borer, other insect orders were not represented by more than one feeding guild. Because of this and the fact that examples for some guilds were only studied scarcely (e.g. *C.*

pallidactylus), while representatives of other guilds were studied very often (e.g. *P. xylostella*), it is difficult to draw conclusions between feeding guilds.

Most of the studies were conducted with oligophagous insect herbivores. With a lack of polyphagous or near-monophagous insects examined, it is problematic to compare and contrast the different feeding modes (Table 2.3). Only within the hemipterans was there a balance between the two levels of host use specialization in the literature reviewed here. In all other insect orders represented in this review, one feeding specialization level typically dominated, which did not allow conclusions concerning how feeding specialization may affect host finding within orders.

The larval feeding experience of *M. brassicae* (reared on glossy or waxy plants) affected adult oviposition preference of females for glossy or waxy plants. The moths may acquire their oviposition preferences from the larval feeding experience through the host selection principal (HHSP) or through chemical legacy (Corbet, 1985; Rietdorf & Steidle, 2002). The host selection principle states that herbivores feed on the same plants they experience as larvae, and assumes that a memory of the feeding substrate is formed during the larval stage and transferred across metamorphosis to the adult stage (Corbet, 1985; Rietdorf & Steidle, 2002). Alternatively, the chemical legacy hypothesis assumes that minute amounts of chemicals inside the insect body impact the central nervous system or the chemoreceptors, and that this will influence the adult insect’s preference (Corbet, 1985). The latter hypothesis does not require learning or memory transfer between life stages (Rietdorf & Steidle, 2002). *Plutella xylostella* adults prefer to oviposit on glossy plants, while the larvae - especially first instars - prefer waxy plants. For lepidopteran larvae, host plant choice is greatly influenced by ovipositing females since they select oviposition sites to optimize larval survival (Renwick, 2001). The discrepancy between preferences of *P. xylostella* life stages may be a result of female moths assessing the leaf surface between glossy and waxy plants and of how well larvae can to attach to and form mines on glossy and waxy plants, respectively. Because adults preferred to oviposit on glossy plants, they may have easier access to underlying chemical components than they do on waxy plants, on which the chemical components may be covered (Uematsu & Sakanoshita, 1989).

Environmental conditions under which plants are grown can influence their susceptibility to insect attack (Eigenbrode, 2004). For example, insect resistance in glossy crops cannot be reliably expressed when plants are propagated in greenhouses or laboratories (Eigenbrode, 2004). This was the case for three insect species: *B. argentifolii* (Jackson et al., 2000), *B. brassicae* (Cole & Riggall, 1992; Lamb et al., 1993), and *P. xylostella* (Eigenbrode et al., 1990; Ulmer et al., 2002). One possible explanation may be that reduced surface waxes make plants more vulnerable to water stress, leading to an increase in stress-induced defenses (Eigenbrode, 2004). Alternatively, under field conditions, insects may be exposed to climatic conditions and natural enemies affecting their behavior and success (Eigenbrode et al., 1991a, 1995). Because first instars of *P. xylostella* feed in leaf mines, the sooner they begin to feed, the sooner they are protected from mortality factors such as desiccation and predation (Eigenbrode et al., 1991b). In a series of studies, Eigenbrode (2004) and Eigenbrode et al. (1995) showed that the presence of predators greatly enhanced glossy plants’ resistance to *P. xylostella* in the field and in the greenhouse. In many studies, *B. brassicae* was shown to prefer waxy over glossy plants in open field infestation level experiments (Stoner, 1990, 1992a, b; Way & Murdie, 1965). Yet Åhman (1990) found no preference for waxy plants over glossy ones, or any decreased preference for waxy plants that had been de-waxed. This discrepancy may be explained by Åhman's (1990) experiments using greenhouse plants and confining insects to clip cages.

In another example, *P. xylostella* herbivory and survival was reduced on glossy plants and larval dispersal was increased in the field. However, in the lab, survival was greater on glossy plant lineages when larvae were confined to leaf disks (Eigenbrode et al., 1990). Ulmer et al., (2002) reported there were no significant differences in fourth instar larval survival on any of the lineages tested, but pupal weight was lower on the glossy line of *Brassica rapa* (L.) than on its waxy counterpart. Thus, glossy-leafed *Brassica* resistance to *P. xylostella* was due to non-preference by neonates in response to quantity, chemistry, and structure of leaf surface waxes (Eigenbrode & Shelton, 1990; Eigenbrode et al., 1991a, b). These behavioral differences may lead to increased larval mortality on glossy plants due to starvation, desiccation, drowning, and predation (Eigenbrode et al., 1991b, 1995).

The two most well-known chemicals associated with Brassicaceae plant defenses and insect host selection are glucosinolates and the thia-triaza-fluorene compounds also known as CIF. Glucosinolates are polar secondary plant metabolites unique to the plant order Brassicales and found in the plant families Brassicaceae, Capparidaceae, and Caricaceae (Fahey et al., 2001). More than 120 different glucosinolates have been identified (Fahey et al., 2001). Glucosinolates are well known to influence the host selection behavior of insect herbivores (Hopkins et al., 2009). CIF is a non-glucosinolate polar compound that stimulates oviposition in some Brassicaceae feeding insects and is present on the leaf surface in extremely small amounts - estimated to be approximately 1 ng/g cabbage leaf (Roessingh et al., 1997). This is considerably less than glucosinolates which are estimated to be 60 ug/g cabbage leaf (Roessingh et al., 1992). However, even at such low concentrations, CIF can be more stimulatory for oviposition than the more abundant glucosinolates (Baur et al., 1996a; Hopkins et al., 1997; Roessingh et al., 1997). Receptor neurons in the tarsal C_5 sensilla of *D. radicum* are extremely sensitive to CIF that has a threshold of about 4 X 10^{-11} to 10^{-12} M (Hurter et al., 1999). It has been suggested that because *D. floralis* and *D. radicum* are extremely sensitive to CIF, for which they have specialized receptors, they have evolved under a strong selection pressure and that the perception of CIF is a crucial step in their oviposition site selection (De Jong & Städler, 2002). A different study, however, found that about 15% of all the C_5 sensilla tested were completely insensitive to CIF (Roessingh et al., 1997).

It is thought that insects scratch the leaf surface with their tarsal claws to make sensory contact with glucosinolates (Barker et al., 2006). Insects may mechanically remove some of the waxes with their tarsal structures (spines) and grant themselves access to the deeper layers below the wax (Barker et al., 2006). The tarsal sensilla of the *Delia* flies and *Pieris* butterflies may reach into the stomata opening and contact glucosinolates present within the leaf. The stomata of *B. oleracea* are about 1–5 lm wide and 10–12 lm long (Zobayed et al., 2001). The tip of the tarsal sensilla of *D. radicum* measure approximately 1.25–2 lm long (Isidoro et al., 1994). Therefore, penetration of the sensilla into stomata is theoretically possible (Städler & Reifenrath, 2009). When treating plants with jasmonic acid, the resulting changes in leaf surface glucosinolates profiles did not seem to explain the change in oviposition preference of *P. brassicae* and *P. rapae*, suggesting that as yet unidentified chemicals may be involved (Bruinsma et al., 2007). When sulphur was used, experiments indicate that chemical compounds other than isothiocyanates found on the leaf surface mediate the oviposition preference of *P. xylostella* (Marazzi et al., 2004b).

Mechanical and chemical manipulation experiments of surface waxes indicate that waxy plants can suppressed oviposition (Uematsu & Sakanoshita, 1989), and be an antixenotic factor that affects the rate of feeding (Bodnaryk, 1992b). One explanation for these results may be that because of smaller amounts of epicuticular lipids in glossy plants, stimulants and deterrents beneath or within the lipids may be more readily accessible to insects on the leaf surface. This enhanced accessibility could explain resistance or susceptibility of glossy plants to insect attack and it was postulated that this may be an adaptive response of the plant against specialist herbivores (Eigenbrode & Espelie, 1995). Müller (2006). Waxes can cover up the feeding stimulus, thus rendering it less detectable to specialists (Müller, 2006). Surface waxes can also impede attachment to the leaf surface (Stork, 1980). Drumming behavior (defined as moving of tarsi and/or antennae up and down rapidly over the top of the leaf surface) has been hypothesized to provide tactile information as well as to dislodge wax crystals so that the polar stimulants for oviposition can be more easily detected (Städler, 1994).

Eigenbrode et al., (1991a) and Eigenbrode & Pillai (1998) concluded that preference for glossy or waxy plants was due at least in part to the physical structure or amount of lipids. Leaf surface wax compounds were strongly implicated in eliciting reduced acceptance of the glossy cabbage by neonate *P. xylostella* (Eigenbrode et al., 1991a; Eigenbrode & Pillai, 1998). By adding amyrins from glossy cabbage to waxy cabbage, acceptance was reduced. The amyrins appear to be principal deterrents influencing larval acceptance of these plants (Eigenbrode & Pillai, 1998) and that waxy plants contain stimulants that increase biting and palpating and decrease walking, while the waxes of glossy plants may contain deterrents that reduce acceptance behavior by the larvae (Eigenbrode & Pillai, 1998). It is not always clear if the active factors are the primary wax components, or plant secondary compounds associated with the waxes. There may have been other compounds not identified in the surface wax extracts that also contributed to insect preference.

Isolating the chemicals present on the leaf surface without penetrating the leaf tissue can be challenging and requires specific considerations. For example, prolonged subsequent dips in different organic solvents (Griffiths et al., 2001) increase the chance that compounds are leached out from within the plant tissue, and not solely from the leaf surface (Jetter et al., 2000; Roessingh et al., 1992). Dipping leaves into chloroform or dichloromethane several times is thought to dissolve the cuticular wax layer, and subsequent dipping in methanol solvents for glucosinolate extraction may not only collect surface wax chemicals, but also chemicals from damaged epidermal cells, resulting in the extraction of the mesophyll (Roessingh et al., 1992; Hopkins et al., 1997; Hurter et al., 1999). Gum Arabic is used to remove plant epicuticular waxes without damaging the lower epidermal layers (Jetter et al., 2000; Müller & Riederer, 2005). This method cannot be used on plant species whose leaves are not hard enough or do not have sufficient epicuticular waxes. For mechanical removal of surface waxes, leaves can be treated for one hour with an aqueous solution of gum Arabic (Jetter & Schäffer, 2001). Then, epicuticular waxes can be removed with a dry film of gum Arabic, leaving the leaves physically intact and without damaging epidermal and mesophyll tissue. The polymer films are subsequently analyzed for the presence of glucosinolates (Müller & Riederer, 2005).

Whether glucosinolates are present directly on the leaf surface, sheltered by them, or are within lower levels of the leaf surface is not clear yet (Städler & Reifenrath, 2009). If glucosinolates are present in the surface waxes, they may only be present in minute amounts, and may occur naturally only as part of highly complex mixtures, tightly bound to the leaf surface (Chew & Renwick, 1995). Based on the current literature, the location, types, and amounts of glucosinolates present vary greatly between plant species, and whether or not they are present on the leaf surface. Glucosinolates are on the leaf surface of some plant species, but not others. For example, no glucosinolates were found on the leaf surface of *B. napus* and *N. officinale* (Badenes-Pérez et al., 2011; Reifenrath et al., 2005). In contrast, glucosinolates were found on *Barbarea* spp. (Badenes-Pérez et al., 2011).

The location and origin of CIF is also unclear (Städler & Reifenrath, 2009). Because CIF has a smaller molecular volume, the transportation of it through the intact cuticle is at least more likely than that of glucosinolates (Städler & Reifenrath, 2009). This is also likely since the CIF concentrations did not differ between extracts of leaves exposed to light and dark, respectively. Due to the fact that glucosinolate concentrations were correlated with when stomata were open, it was concluded that glucosinolates in the extracts originate from inner leaf tissue and are washed through the open stomata (Reifenrath et al., 2005; Städler & Reifenrath, 2009).

Attempting to separate the roles of surface waxes into strictly physical and chemical components is challenging. Both components can be intertwined with chemicals embedded in the physical structures of the epicuticular lipids (Jeffree et al., 1975), directly below the lipids, or in otherwise not entirely known locations (Städler & Reifenrath, 2009). This leads to methodological barriers to determine the specific mode of action of surface wax-based insect-plant interactions (Eigenbrode & Shelton, 1992). The use of nondestructive methods to mechanically remove surface waxes, and then test them for the presence of chemical compounds, have also been employed (Reifenrath et al., 2005). Scanning electron micrographs and thin layer chromatography (TLC) can also be used to observe changes in wax crystallite morphology and surface wax load and composition (Riggin-Bucci et al., 1998). It is not always clear whether the primary wax components, or plant secondary compounds associated with the waxes, are the active factors (Eigenbrode & Pillai, 1998).

Summary and conclusions

Surface waxes influence the host selection behavior of Brassicaceae feeding herbivores both through their physical aspects (structure, amount, and chemical makeup of aliphatic hydrocarbons), and their chemical aspects (biologically active metabolites and chemicals). Physical aspects can impede insect attachment depending on the morphology of their tarsi, possibly influencing why certain insects prefer glossy over waxy plants and vice versa. They may also alter insect perception of chemical stimulants and deterrents present on or near the leaf surface - as evident from experiments performed with mechanical and chemical manipulations of surface waxes (Bodnaryk, 1992a, b; Blua et al., 1995). The chemical compositions of the epicuticular lipids were also shown to influence insects' host selection (Eigenbrode & Pillai, 1998; Eigenbrode et al., 1991a). Notable feeding stimulants were glucosinolates and CIF compounds. These compounds were shown to stimulate feeding, oviposition, and electrophysiological recordings in several insect species (Baur et al., 1996a; Reifenrath et al., 2005; Stoner, 1997).

There was a strong tendency towards examination of Brassica crops and their pests, but these agriculture systems may or may not be representative of all Brassicaceae feeding herbivore systems. And while nearly 97% of the reviewed articles reported surface waxes having an influence on the host selection behavior of the insect(s) under study, this may or may not be a true reflection of their ecological importance in nature, or if this is skewed due to a tendency in the literature to focus on these systems.

Attempts to separate out the respective roles of physical and chemical surface wax cues is difficult, as physical and chemicals cues are often intertwined. Most research focused on examining only one factor. For example, those studies which reported infestation levels on glossy and waxy plants usually did not test whether physical, chemical, or both aspects of surface waxes was influencing the host preference. However, a few studies did attempt to examine the effects of both factors (e.g. Reifenrath et al., 2005).

It is noted that surface waxes have the potential to alter the entire ecology of the plant and affect tritrophic interactions between herbivores and predators (that also must maneuver on waxy or glossy surfaces) (Eigenbrode, 2004; Eigenbrode & Jetter, 2002; Stoner, 1992a). Differing chemical profiles present on both the undamaged and damaged leaf surface are also available to predators, which in turn can impact the behavior of herbivores (Eigenbrode & Jetter, 2002). While this review specifically did not include articles focused on these topics, they do play a role in herbivore host selection behavior and do not operate in isolation from the physical and chemical aspects of leaf surface waxes.

Table 2.1. Number and proportion of articles investigating the role of surface wax cues in the host selection behavior of herbivores by insect species.

Order: Family	**Insect species**	**Total articles***	**% of articles reviewed**
Coleoptera: Chrysomelidae	*Phaedon cochleariae*	2	4%
Coleoptera: Chrysomelidae	*Phyllotreta atra*	1	2%
Coleoptera: Chrysomelidae	*Phyllotreta cruciferae*	7	13%
Coleoptera: Chrysomelidae	*Phyllotreta striolata*	2	4%
Coleoptera: Chrysomelidae	*Phyllotreta undulata*	1	2%
Coleoptera: Curculionidae	*Ceutorhynchus pallidactylus*	1	2%
Diptera: Anthomyiidae	*Delia floralis*	5	9%
Diptera: Anthomyiidae	*Delia radicum*	15	27%
Hemiptera: Aleyrodidae	*Aleyrodes proletella*	2	4%
Hemiptera: Aleyrodidae	*Bemisia argentifolii*	3	5%
Hemiptera: Aleyrodidae	*Bemisia tabaci*	1	2%
Hemiptera: Aphididae	*Brevicoryne brassicae*	8	15%
Hemiptera: Aphididae	*Lipaphis erysimi*	2	4%
Hemiptera: Aphididae	*Myzus persicae*	2	4%
Lepidoptera: Noctuidae	*Mamestra brassicae*	2	4%
Lepidoptera: Noctuidae	*Trichoplusia ni*	3	5%
Lepidoptera: Pieridae	*Pieris brassicae*	3	5%
Lepidoptera: Pieridae	*Pieris rapae*	10	18%
Lepidoptera: Plutellidae	*Plutella xylostella*	22	40%

* The Total Articles column does not total 55 because some researchers evaluated more than one species in their experiments. Likewise, % of Literature does not total 100%.

Table 2.2: Preference for glossy or waxy plants by order and species

Order: Family: Species	**Preference of surface wax type**	**References**
Coleoptera: Chrysomelidae *Phaedon cochleariae*	Glossy	Reifenrath et al., 2005, Stork, 1980
Coleoptera: Chrysomelidae *Phyllotreta atra*	Glossy	Way & Murdie, 1965
Coleoptera: Chrysomelidae *Phyllotreta cruciferae*	Glossy	Bodnaryk, 1992a, b, Eigenbrode et al., 2000, Stoner, 1990; Stoner, 1992a, b, Way & Murdie, 1965
Coleoptera: Chrysomelidae *Phyllotreta striolata*	Glossy	Stoner, 1992ab
Coleoptera: Chrysomelidae *Phyllotreta undulata*	Glossy	Way & Murdie, 1965
Coleoptera: Curculionidae *Ceutorhynchus pallidactylus*	Glossy	Way & Murdie, 1965
Diptera: Anthomyiidae *Delia radicum*	Waxy	Roessingh & Städler, 1990, Way & Murdie, 1965
Hemiptera: Aleyrodidae *Aleyrodes proletella*	Waxy	Thompson, 1963, Way & Murdie, 1965
Hemiptera: Aleyrodidae *Bemisia argentifolii*	Waxy	Blua et al., 1995, Farnham & Elsey, 1995, Jackson et al., 2000
Hemiptera: Aleyrodidae *Bemisia tabaci*	Glossy	Blua et al., 1995
Hemiptera: Aphididae *Brevicoryne brassicae*	Waxy	Cole & Riggall, 1992, Eigenbrode et al., 2000, Stoner, 1990, Stoner, 1992a, b, Thompson, 1963, Way & Murdie, 1965
Lepidoptera: Noctuidae *Mamestra brassicae*	Waxy	Picoaga et al., 2003, Way & Murdie, 1965
Lepidoptera: Noctuidae *Trichoplusia ni*	Waxy	Dickson & Eckenrode,1980, Dickson et al., 1984, Eigenbrode et al., 1993
Lepidoptera: Pieridae *Pieris rapae*	Waxy	Dickson & Eckenrode, 1980, Dickson et al., 1984, Eigenbrode et al., 1993, Picoaga et al., 2003, Stoner, 1990, Stoner, 1992a, b, Stoner, 1997, Way & Murdie, 1965
Lepidoptera: Plutellidae *Plutella xylostella*	Glossy (adults) Waxy (larvae)	Badenes-Pérez et al., 2004, Dickson et al., 1984, Eigenbrode & Pillai, 1998, Eigenbrode & Shelton, 1990, 1992, Eigenbrode et al., 1990, 1991a, b, 1993, 2000, Lin et al., 1983, Ramachandran et al., 1998, Riggin-Bucci et al., 1998, Stoner, 1990, 1992a, Uematsu & Sakanoshita, 1989, Ulmer et al., 2002

Table 2.3. Level of specialization.

Organisms	**Specialization level**	**Plant fed on**	**References**
Phaedon cochleariae	Oligophagous	Many Brassicaceae species.	Reifenrath & Müller, 2009
Phyllotreta atra	Oligophagous	A limited number of Brassicaceae.	Bullock, 1992
Phyllotreta cruciferae	Oligophagous	Brassicaceae, and a few other plant species in the families Capparidaceae, Tropaeolaceae, and Limnanthaceae.	Feeny et al., 1970
Phyllotreta striolata	Oligophagous	Brassicaceae, and a few other plant species in the families Capparidaceae, Tropaeolaceae, and Limnanthaceae.	Feeny et al., 1970
Phyllotreta undulata	Oligophagous	Many Brassicaceae species.	Freude et al., 1966
Ceutorhynchus pallidactylus	Oligophagous	Many Brassicaceae species.	Diekmann, 1972
Delia floralis	Oligophagous	A limited number of Brassicaceae, particularly crops and turnip rape.	CABI, 2015
Delia radicum	Oligophagous	A limited number of Brassicaceae, particularly crops, and mainly in the tribes within Brassicaceae lineage 2.	Al-Shehbaz, 2012; Finch & Ackley, 1977; Kiefer et al., 2014
Aleyrodes proletella	Polyphagous	Numerous families: Apiaceae, Asteraceae, Balsminaceae, Brassicaceae, Campanulaceae, Euphorbiaceae, Fabaceae, Fagaceae, Papveraceae, Rununculaceae, and Scrophulariaceae.	Mound & Halsey, 1978
Bemisia argentifolii	Polyphagous	More than 500 species of plants from 63 plant families.	Byrne & Bellows, 1991; Greathead, 1986; Mound & Halsey, 1978;
Bemisia tabaci	Polyphagous	More than 500 species of plants from 63 plant families.	Byrne & Bellows 1991; Greathead, 1986; Mound & Halsey, 1978;

Table 2.3 continued. Level of specialization.

Brevicoryne brassicae	Oligophagous	Brassica or other closely related plant species.	Cole, 1997; Gabryś et al., 1997; Nault & Styer, 1972
Lipaphis erysimi	Oligophagous	Host range is restricted primarily to Brassicaceae species.	Nault & Styer, 1972
Myzus persicae	Polyphagous	Brassicaceae as well as Solanaceae, Malvaceae, Asteraceae, Chenopodiaceae, Amaranthaceae, Poaceae, Cucurbitaceae, and Apiaceae.	Cole, 1997
Mamestra brassicae	Polyphagous	Feeds on 70 plant species in 22 different plant families.	Popova, 1993
Trichoplusia ni	Polyphagous	Brassicaceae as well as Chenopodiaceae, Leguminosae, Asparagaceae, Papaveraceae, Umbelliferae, and Scrophulariaceae.	Robinson et al., 2010
Pieris brassicae	Oligophagous	Many plant families that contain glucosinolates.	Feltwell, 1982
Pieris rapae	Oligophagous	Many Brassicaceae and a few species in the Capparidaceae, Tropaeolum, and Resedaceae	Scott, 1986
Plutella xylostella	Oligophagous	Nearly all Brassicaceae plants including crops and several wild crucifers.	Talekar & Shelton, 1993

Table 2.4. Feeding guilds and physical and chemical cues.

Order: Family: Species	**Feeding guild**	**Instances where physical aspects had:**		**Instances where chemical aspects had:**	
		Effect	No effect	Effect	No effect
Coleoptera: Chrysomelidae *Phaedon cochleariae*	Chewing	2	0	1	0
Coleoptera: Chrysomelidae *Phyllotreta atra*	Chewing	1	0		
Coleoptera: Chrysomelidae *Phyllotreta cruciferae*	Chewing	5	2		
Coleoptera: Chrysomelidae *Phyllotreta striolata*	Chewing	1	1		
Coleoptera: Chrysomelidae *Phyllotreta undulata*	Chewing	1	0		
Coleoptera: Curculionidae *Ceutorhynchus pallidactylus*	Boring	1	0		
Diptera: Anthomyiidae *Delia floralis*	Boring			5	0
Diptera: Anthomyiidae *Delia radicum*	Boring	2	0	13	1
Hemiptera: Aleyrodidae *Aleyrodes proletella*	Sap sucking	2	0		
Hemiptera: Aleyrodidae *Bemisia argentifolii*	Sap sucking	3	0		
Hemiptera: Aleyrodidae *Bemisia tabaci*	Sap sucking	1	0		
Hemiptera: Aphididae *Brevicoryne brassicae*	Sap sucking	7	1		
Hemiptera: Aphididae *Lipaphis erysimi*	Sap sucking	1	1		
Hemiptera: Aphididae *Myzus persicae*	Sap sucking	1	1		
Lepidoptera: Noctuidae *Mamestra brassicae*	Chewing	2	0		
Lepidoptera: Noctuidae *Trichoplusia ni*	Chewing	3	0		
Lepidoptera: Pieridae *Pieris brassicae*	Chewing	1	0	1	0
Lepidoptera: Pieridae *Pieris rapae*	Chewing	8	1		
Lepidoptera: Plutellidae *Plutella xylostella*	Chewing	18	2	3	0

Table 2.5 Differences in insect behavior based on life stages.

Order: Family	**Insect**	**Differences**	**References**
Lepidoptera: Noctuidae	*Mamestra brassicae*	In the laboratory, moths preferred to lay eggs on the plant variety on which they had been reared as larvae.	Way & Murdie, 1965
Lepidoptera: Plutellidae	*Plutella xylostella*	Adults preferred to oviposit on glossy plants, but larvae (especially first instars) preferred waxy plants.	Dickson & Eckenrode, 1980 Dickson et al., 1984 Eigenbrode & Pillai, 1998 Eigenbrode & Shelton, 1990 Eigenbrode & Shelton, 1992 Eigenbrode et al., 1991a Eigenbrode et al., 1991b Eigenbrode et al., 1990 Justus et al., 2000 Lin et al., 1983 Lin et al., 1984 Ramachandran et al., 1998 Stoner, 1990 Ulmer et al., 2002

Table 2.6. Differences in insect behavior based on plant growing location.

Order: Family	**Insect**	**Difference**	**References**
Hemiptera: Aleyrodidae	*Bemisia argentifolii*	*B. argentifolii* preferentially selected waxy over glossy collard phenotypes in the field. *B. argentifolii* survives, develops, and reproduces as well on glossy collard phenotypes as it does on waxy phenotypes in no-choice greenhouse experiments.	Jackson et al., 2000
Hemiptera: Aphididae	*Brevicoryne brassicae*	In the field, aphid populations were greatly reduced on glossy lines. Glossy lines grown in the greenhouse were as susceptible to aphid populations as the waxy line.	Cole & Riggall, 1992 Lamb et al., 1993
Lepidoptera: Plutellidae	*Plutella xylostella*	Glossy lines showed to have resistance to *P. xylostella* under field conditions, while in greenhouse experiments there was no observed resistance.	Dickson & Eckenrode, 1980 Dickson et al., 1984 Eigenbrode et al., 1990 Lin et al., 1983 Lin et al., 1984 Ulmer et al., 2002

Table 2.7 Chemical aspects affecting host selection behavior.

Order: Family: Species	**Chemical aspects**	**References**
Coleoptera: Chrysomelidae *Phaedon cochleariae*	Mechanically removing the lower and upper leaf surface waxes of *B. napus* and *N. officinale* with gum Arabic, no GS were detectable. Leaf surface extracts of *N. officinale* applied onto *P. sativum* leaf discs did not evoke feeding.	Reifenrath et al., 2005
Diptera: Anthomyiidae *Delia floralis*	CIF and GS, particularly glucobrassicin, stimulated oviposition. D_2 and D_3- sensilla reacted to glucosinolate fractions. C_5- sensilla reacted to CIF fractions.	Baur et al., 1996a, Gouinguene & Städler, 2006
Diptera: Anthomyiidae *Delia radicum*	Glucosinolates, particularly, glucobrassicin and gluconasturtiin stimulated oviposition. Flies preferred to lay eggs on both the plants and the extracts from the S_n and S_+ treatments. D_3 and D_4- sensilla reacted to glucosinolate fractions (especially to gluconasturtiin, glucobrasssicin, and glucobrassicanapin). C_5-sensilla reacted to CIF.	Baur et al., 1996a, b, Gouinguene & Städler, 2006, Hopkins et al., 1997, Hurter et al., 1999, Marazzi & Städler, 2004, Städler & Reifenrath, 2009
Lepidoptera: Pieridae *Pieris brassicae*	Glucobrassicin stimulated oviposition. Fewer eggs were laid on leaves of jasmonic acid-treated plants compared to control plants. When offered a choice between the purified glucosinolate fraction isolated from leaf surface extracts of jasmonic acid treated plants and that from control plants, they did not discriminate.	Bruinsma et al., 2007, van Loon et al., 1992
Lepidoptera: Pieridae *Pieris rapae*	Fewer eggs were laid on leaves of jasmonic acid-treated plants compared to controls. When offered a choice between the glucosinolate fraction isolated from leaf surface extracts of jasmonic acid treated plants and that from control plants, they did not discriminate.	Bruinsma et al., 2007
Lepidoptera: Plutellidae *Plutella xylostella*	Glucobarbarin and gluconasturtin stimulated oviposition. Females laid more eggs on plant models treated with S_n extracts than on S_0 plants. Sulphur treated plants yielded higher EAG amplitudes than the S_0 extracts. The total glucosinolate content sharply increased from S_0 to S_n plants, whereas it was slightly lower in S_n versus S_+ plants. Glucosinolates were present on the leaf surface of Barbarea spp., but not in *B. napus* or *N. officinale.*	Badenes-Pérez et al., 2011, Marazzi et al., 2004b

Table 2.8. Methodologies for testing chemical cues by species in alphabetical order**.**

Insect species	**Oviposition**	**Electrophysiology**	**Feeding behavior/ movement**	**References**
Delia floralis	(5) • plants • steam leaf surface extracts • methanolic and dichloromethane leaf surface extracts	(2) • methanolic leaf surface extracts using tarsi		Alborn et al., 1985 Baur et al., 1996a Gouinguene & Städler, 2006 Hopkins et al., 1992; 1997
Delia radicum	(11) • plants • mechanically damaged plants • methanolic leaf surface extracts • pure GS • plants grown under one of three sulphur regimes • methanolic leaf surface extracts of plants grown under one of three sulphur regimes	(11) • methanolic leaf surface extracts using tarsi • dichloromethane leaf surface extracts using tarsi • methanolic leaf surface extracts of plants grown under one of three sulphur regimes using tarsi • chloroform, methanol, and aqua bidest leaf surface extracts using tarsi		Baur et al., 1996a, b; Gouinguene & Städler, 2006 Griffiths et al., 2001 Hopkins et al., 1997 Hurter et al., 1999 Isidoro et al., 1994 Marazzi & Städler, 2004 Marazzi et al., 2004a Roessingh & Städler, 1990 Roessingh et al., 1992, 1997 Städler et al., 2002 Städler & Reifenrath, 2009
Phaedon cochleariae			(1) • leaf surface extracts (chloroform, methanol, and water) applied to *P. sativum*	Reifenrath et al., 2005

Table 2.8 continued. Methodologies for testing chemical cues by species in alphabetical order**.**

Insect species	**Oviposition**	**Electrophysiology**	**Feeding behavior/movement**	**References**
Pieris brassicae	(2) • methanolic leaf surface extracts • pure GS methanolic leaf surface extracts from plants sprayed with 1 of 3 different jasmonic acid solution concentrations			Bruinsma et al., 2007 van Loon et al., 1992
Pieris rapae	(1) • methanolic leaf surface extracts from plants sprayed with 1 of 3 different jasmonic acid solution concentrations			Bruinsma et al., 2007
Plutella xylostella	(2) • plants • plants grown under one of three sulphur regimes • methanolic leaf surface extracts of plants grown under one of three sulphur regimes • pure GS identified from gum Arabic leaf surface extracts	(1) • methanolic leaf surface extracts of plants grown under one of three sulphur regimes using antennae		Badenes-Pérez et al., 2011 Marazzi et al., 2004b

Table 2.9. Methodologies for testing physical cues by species in alphabetical order**.**

Insect species	**Infestation levels**	**Oviposition**	**Movement/ overall behavior**	**Feeding behavior**	**Adhesion**	**Reference**
Aleyrodes proletella	(2) • plants					Thompson, 1963 Way & Murdie, 1965
Bemisia argentifolii	(2) • plants	(1) • plants • plants with leaf surface waxes mechanically removed				Blua et al., 1995 Farnham & Elsey, 1995 Jackson et al., 2000
Bemisia tabaci		(1) • plants • plants with leaf surface waxes mechanically removed				Blua et al., 1995
Brevicoryne brassicae	(7) • plants • plants treated with EPTC		(1) • plants • plants with leaf surface waxes mechanically removed			Åhman, 1990 Cole & Riggall, 1992 Eigenbrode et al., 2000 Stoner, 1990 Stoner, 1992a Stoner, 1992b Thompson, 1963 Way & Murdie, 1965
Ceutorhynchus pallidactylus	(1) • plants					Way & Murdie, 1965

Table 2.9 continued. Methodologies for testing physical cues by species in alphabetical order.

Insect species	**Infestation levels**	**Oviposition**	**Movement/ overall behavior**	**Feeding behavior**	**Adhesion**	**Reference**
Delia radicum		(2) • plants • paraffin coated plant models				Roessingh & Städler, 1990 Way & Murdie, 1965
Lipaphis erysimi	(1) • plants		(1) • plants • plants with leaf surface waxes mechanically removed			Åhman, 1990 Lamb et al., 1993
Mamestra brassicae	(1) • plants	(1) • plants				Picoaga et al., 2003 Way & Murdie, 1965
Myzus persicae	(2) • plants					Stoner, 1990 Way & Murdie, 1965
Phaedon cochleariae				(1) • plants • plants with leaf surface waxes mechanically removed	(1) • plants	Reifenrath et al., 2005 Stork, 1980
Phyllotreta atra	(1) • plants					Way & Murdie, 1965

Table 2.9 continued. Methodologies for testing physical cues by species in alphabetical order.

Insect species	**Infestation levels**	**Oviposition**	**Movement/overall behavior**	**Feeding behavior**	**References**
Phyllotreta cruciferae	(5) • plants • plants treated with EPTC			(2) • plants • plants with leaf edges removed • plants with surface waxes mechanically removed • plants using antennectomized beetles	Bodnaryk, 1992a Bodnaryk, 1992b Eigenbrode et al., 2000 Stoner, 1990 Stoner, 1992a Stoner, 1992b Way & Murdie, 1965
Phyllotreta striolata	(2) • plants				Stoner, 1992a Stoner, 1992b
Phyllotreta undulata	(1) • plants				Way & Murdie, 1965
Pieris brassicae	(1) • plants				Picoaga et al., 2003
Pieris rapae	(7) • plants • plant treated with EPTC		(1) • plants	(1) • plants	Dickson & Eckenrode, 1980 Dickson et al., 1984 Eigenbrode et al., 1993 Picoaga et al., 2003 Stoner, 1990 Stoner, 1992a Stoner, 1992b Stoner, 1997 Way & Murdie, 1965

Table 2.9 continued. Methodologies for testing physical cues by species in alphabetical order.

Insect species	**Infestation levels**	**Oviposition**	**Movement/overall behavior**	**Feeding behavior**	**References**
Plutella xylostella	(7) • plants • plant treated with EPTC	(8) • plants • plants with leaf surface waxes mechanically removed • plants with leaf surface waxes chemically removed • plant treated with EPTC • plants treated with surfactant Latron CS-7 • plants kept under light and dark conditions	(6) • plants • plants with leaf surface waxes mechanically removed • plants with leaf surface waxes chemically removed • plant treated with EPTC • surface wax components of glossy leaf extracts mixed with waxy leaf extracts deposited as a film on glass	(5) • plants	Badenes-Pérez et al., 2004 Dickson & Eckenrode, 1980 Dickson et al., 1984 Eigenbrode & Pillai, 1998 Eigenbrode & Shelton, 1990 Eigenbrode & Shelton, 1992 Eigenbrode et al., 1990 Eigenbrode et al., 2000 Eigenbrode et al., 1991a Eigenbrode et al., 1991b Eigenbrode et al., 1993 Justus et al., 2000 Lin et al., 1983 Lin et al., 1984 Ramachandran et al., 1998 Riggin-Bucci et al., 1998 Stoner, 1990 Stoner, 1992a Uematsu & Sakanoshita, 1989 Ulmer et al., 2002
Trichoplusia ni	(3) • plants • plant treated with EPTC				Dickson & Eckenrode, 1980 Dickson et al., 1984 Eigenbrode et al., 1993

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Chapter 3

EXAMINING PRE-FEEDING BEHAVIOR AS PART OF HOST-SPECIFICITY TESTING OF WEED BIOLOGICAL CONTROL AGENTS

Abstract

Comprehensive host-specificity testing has been conducted for *Ceutorhynchus cardariae*, a classical biological control candidate for the Eurasian *Lepidium draba,* a perennial mustard that is invasive in western North America. Under natural field conditions, *C. cardariae* is a host-specific specialist. Under laboratory, no-choice conditions, its fundamental host range includes two distantly related confamilial species in the genus *Streptanthus: S. anceps* and *S. flavescens*. Pre-release host-specificity testing of weed biological control agents can assess which plants are attacked but not *why* they are attacked. Detailed examination of post-alightment behavior on host and non-host plants can help determine what plant cues influence host choice. To investigate this, we quantified the pre-feeding behavior of female *C. cardariae* on leaves of its host plant *L. draba,* and on leaves of a non-host plant, *Ocimum basilicum* (basil). We grouped behavioral activities into chemosensory activities (antennal waving, antennal contact, and tarsal drumming) and non-chemosensory activities (walking/running, grooming, resting, and time not on the plant). We distinguished two distinct phases of pre-feeding behavior: acclimation and pre-consumption. When on the leaf surface of *L. draba*, all 12 weevils engaged in all four acclimation behaviors: resting, walking, walking with antennal waving, and grooming. When on the leaf surface of basil, all 12 weevils engaged in three acclimation behaviors: walking, walking with antennal waving, and grooming. Only nine weevils on basil engaged in resting. During the pre-consumption

phase, weevils engaged in one of two behaviors: antennal contact and/or tarsal drumming. Ten of the 12 weevils on *L. draba* engaged in a combined total of 30 pre-consumption behavioral events, 17 of which resulted in feeding. These feeding events were always preceded by weevils dragging their antennae along or just above the leaf surface. When offered basil leaves, nine of the 12 weevils engaged in a combined total of 13 pre-consumption behavioral events, none of which led to feeding. When comparing behavioral models between the two plant species, weevils exhibited more resting and antennal contact events on *L. draba* than on basil, but spent more time grooming and less time engaging in antennal contact on basil than on *L. draba*. These data suggest that *C. cardariae* assesses and responds differently to cues present on or immediately above the leaf surface of its host plant and a non-host. A better knowledge of these compounds should improve the understanding of whether a plant species would be vulnerable to attack by the weevil.

Introduction

Classical biological control of weeds relies on the use of sufficiently host-specific specialist herbivore organisms to avoid post-release non-target attack (Heard, 1999; Louda et al., 2003; Sheppard et al., 2005; Suckling & Sforza, 2014). Conventionally, the risk is assessed pre-release through host-specificity testing of candidates using no-choice or choice tests, under differing conditions such as confined plants or under open field conditions. These tests can assess feeding, oviposition and larval development of a candidate species on a set of confamilial plant species ranging from very closely to distantly related to the targeted invasive plant species (Briese, 2005; Schaffner, 2001; Sheppard et al., 2005). Host-specificity tests determine a biological control agent candidate’s fundamental and ecological host range.

The fundamental host range has been defined as the range of plant species that are capable of supporting a biological control agent’s full life cycle (Van Klinken, 1999) and is typically assessed through no-choice tests. As no-choice tests are generally performed by confining the insect in a cage with one plant, they are only able to act out a subset of their full sequence of host selection behaviors. The ecological host range is typically a subset of the fundamental host range. It is assessed in series of field tests in which the biological control agent candidates can choose between plant species and express their host choice behaviors. It therefore comprises plant species that have passed through species-specific behavioral and ecological filters (Nechols et al., 1992; Schaffner, 2001; Schaffner et al., 2018; Van Klinken, 1999; Wapshere, 1989). Open field tests are an important component of the assessment of the ecological host range because they allow the insects to also display its full sequence of host selection behavior during the pre-alightment phase, which is primarily triggered by volatile and visual plant cues (Briese, 1999; Clement & Cristofaro, 1995; Schaffner, 2001; Schaffner et a., 2018; Van Klinken, 1999; Wapshere, 1989). Yet, there are often discrepancies between the no-choice and open field host range testing results (Schaffner et al., 2018). The fundamental host range, as determined by no-choice tests, is often broader than what is observed in the open field tests, which are more indicative of the ecological host range (Schaffner, 2001). These differences can lead to uncertainty as to whether certain plant species may or may not be attacked after the release of the agent (McFadyen et al., 2002).

Test plant species are traditionally selected according to the centrifugal phylogenetic method, which assumes that more closely related plant species to the target weed are also more likely to support development of a biological control candidate compared to more distantly related plant species (Wapshere, 1974; 1989). While the centrifugal phylogenetic approach has been the standard practice for assessing a biological control agent’s likelihood of non-target attack (Harris & Zwölfer, 1968; Wapshere, 1974; 1989), it does not explain well why discrepancies may exist between no-choice and open field tests, or the physiological or behavioral processes involved in a biological control candidate’s choice for one plant species over another (Louda et al., 2003; Marohasy, 1998; Rapo, 2012; Smith & Beck, 2013; 2015).

The host selection behavior of an insect herbivore can be divided into three main phases: finding, examining, and accepting (Bernays & Chapman, 1994; Miller & Strickler, 1984). Finding occurs before the insect has contact with the plant, and examining and acceptance occur after contact. Acceptance or rejection of a potential host plant during the finding and examining phases depends on the insect’s response to visual, volatile, contact, and gustatory plant cues (Bernays & Chapman, 1994; Dethier, 1982; Heard, 1999; Miller & Strickler, 1984; Wheeler & Schaffner, 2013). These stimuli dictate behavioral and physiological patterns of feeding and oviposition (Beck, 1965; De Wilde & Schoonhoven, 1969; Jermy, 1966; Marohasy, 1998; Miller & Strickler, 1984; Thorsteinson, 1960). Pre-release risk assessment could be improved by observing and describing the host selection behavior of an agent both pre- and post-alightment to determine which cue or cue modality may be particularly important in its host plant choice (Hinz et al., 2014; Knolhoff & Heckel, 2014; Wheeler & Schaffner, 2013). If a specific cue or cue modality is shared between the target plant and non-target plant species, this could indicate that the non-target species is vulnerable to attack in the field.

The Eurasian clonal perennial mustard *Lepidium draba* (L.) Desv. (Brassicaceae), which is invasive in western North America, and one of its biological control candidates, the stem gall-forming weevil *Ceutorhynchus cardariae* Korotyaev (Coleoptera: Curculionidae), offer an interesting system to study the host selection behavior in a biological weed control system. Host-specificity testing data has demonstrated a high level of specialization of *C. cardariae,* however, under no-choice conditions the weevil is able to readily develop on two distantly related plant species in the tribe Thelypodieae, *Streptanthus anceps* (Payson) Hoover and *Streptanthus flavescens* Hook, than would have been expected based only the centrifugal phylogenetic method (Weyl et al., 2018).

Little is known about the different behavioral phases involved in the host selection and acceptance of *C. cardariae*, but adults likely employ olfactory, visual, gustatory and contact cues to identify their host plant. Other Brassicaceae feeding beetles, e.g. *Ceutorhynchus assimilis* (Paykull), *Phaedon cochleariae* (F.) and *Phyllotreta cruciferae* (Goeze), are known to use olfactory (Bartlet et al., 1993; Blight et al., 1995), visual (Kühnle & Müller, 2011; Smart & Blight, 1997), and surface wax cues (Bodnaryk, 1992a; Bodnaryk, 1992b; Reifenrath et al., 2005) during host selection.

Preliminary tests investigating the role of volatiles in the pre-alightment host selection behavior of *C. cardariae* were performed as a means to demonstrate the causes of its host selection and specificity (Rendon et al., unpublished data). These tests involved offering *C. cardariae* the choice between the scent of *L. draba* plants and the scent of air in one of three experimental setups: 1) various y-tube setups using airflow, 2) various static systems utilizing simple chambered cylinders, and 3) a four-arm olfactometer. In all olfactometers, the weevils did not demonstrate a preference for *L. draba* volatiles over air. For example, a total of 38 weevils were observed individually in the four-arm olfactometer. Two inlet arms were connected via tubing to potted plants whose foliage was hermetically sealed within Teflon bags. The other two inlet arms were connected to Teflon bags that contained only air. Four push pumps were used to deliver air to each inlet arm and another pump was used to pull air from the basal outlet. At the end of testing, approximately 45% of the weevils were recorded in the *L. draba* quadrant, approximately 43% in the air quadrant, and approximaetly12% were not responsive and remained on or returned to the center of the arena (Rendon et al., unpublished data).

Because all initial pre-alightment volatile tests did not reveal substantial information about *C. cardariae's* host selection behavior, an alternative approach of the characterization the weevil's post-alightment behavior was taken. Examination of the post-alightment behavior on host and non-host plants can be used a basis for understanding factors that are influencing host plant choice of candidate biological control agents, in the same way it has been used for insect herbivores. For example, Henderson et al., (2004) examined the post-alightment behavior of the crucifer flea beetle, *Phyllotreta cruciferae* (Goeze) and gained insight into underlying mechanisms influencing the beetle's host selection behavior. The pre-feeding behaviors of adults were recorded and described on seedlings of a host plant, and compared to behaviors on seedlings of two non-host plants. Both antennal and tarsal chemoreceptors were shown to be important in determination of host plant quality. Differences in time spent on plant tissue and the frequency and duration of pre-feeding behaviors provide insight into possible mechanisms of resistance to flea beetles in the non-host plants. Specifically, non-host plants may contain deterrent phytochemicals that appear to be volatile in nature on one non-host plant, and non-volatile on the other non-host plant. This observation could be utilized to direct further study to identify the exact phytochemical compounds involved.

The objective of this study was to characterize the sequence of behaviors that precede *C. cardariae* adult feeding after alightment on *L. draba* and on an unrelated non-target plant species, basil, *Ocimum basilicum* L. (Lamiaceae). We sought to identify specific behavioral patterns occurring after alightment and compare these between its field host *L. draba* and the non-target basil. We hypothesized that the specific behaviors, their frequency, and duration would differ between the two plant species and that these behavioral differences would indicate the types of plant cues that mediate host plant selection of *C. cardariae*.

Materials and methods

Study system and materials

Lepidium draba is a clonal perennial mustard native to Eurasia that can invade open, disturbed, and degraded habitats, such as croplands, pastures, rangelands, and riparian areas, and form dense monospecific stands in North America (Scurfield, 1962). It is a declared noxious weed in 15 US states and three Canadian provinces (USDA-NRCS, 2018). *Ceutorhynchus cardariae* is a petiole and stem galling weevil native to Europe and Asia (Hinz & Diaconu, 2015; Korotyaev 1992). In its native range *C. cardariae* has only been found on *L. draba* and on rare occasions on the closely related *Lepidium campestre* (L.) (Francis & Warwick, 2008; Hinz & Diaconu, 2015; Korotyaev, 1992). Females lay eggs in early spring into stems, leaf stalks, and midribs of *L. draba* rosettes and bolting plants, which is thought to cause the formation of galls (Hinz & Diaconu, 2015). Larvae of *C. cardariae* hatch and then feed on the parenchymatic tissue of developing galls, which can stunt or even kill shoots (Hinz & Diaconu, 2015). The host-specificity of *C. cardariae* has been studied at CABI Switzerland since 2003. Testing methodologies included no-choice, multiple-choice, and open field cage tests. A total of 156 test plant species, over half of which are native to North America, and ten federally listed threatened or endangered (T&E) species, were tested for non-target attack including feeding, gall development, and adult emergence (Weyl et al., 2018). Results of the host-specificity testing indicate that *C. cardariae* has a fundamental host range that partially includes species in the tribes Lepidieae, Cardamineae, and Thelypodieae. In contrast, the ecological host range of *C. cardariae* is narrower and includes only few species (Weyl et al., 2018). When using the relative performance threshold based on results of laboratory tests to predict non-target attack post-release for New Zealand (Paynter et al., 2015), four native non-target species were above the predicted relative threshold for non-target attack (Weyl et al., 2018). These included two species in the tribe Lepidieae: *Lepidium oblongum* Small and *Lepidium paysonii* Rollins, and two in the tribe Thelypodieae: *Streptanthus anceps* (Payson) Hoover and *Streptanthus flavescens* Hook. (Weyl et al., 2018). The Brassicaceae family is separated into three distinct lineages[1], (Beilstein et al., 2006), with the Cardamineae and the Lepidieae tribe belonging to lineage I, and the Thelypodieae belonging to lineage II (Kiefer et al., 2014). Thus, attack on the two *Streptanthus* species does not fit the assumption underlying the centrifugal phylogenetic method, i.e. that species closely related to the target weed are more likely to be attacked than more distantly related species (Weyl et al., 2018) and results in *C. cardariae* exhibiting a ‘phylogenetically disjunct’ host range.

Approximately 400 female adult *C. cardariae* were transferred from a rearing colony at CABI Switzerland to the Northwestern Biocontrol Insectary and Quarantine (NWBIQ) at

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[1] Lineages were determined using the chloroplast gene *ndhF*. Using parsimony, likelihood, and Bayesian methods, Beilstein et al., (2006) reconstructed the phylogeny of the gene. The genera were then able to be grouped into monophyletic groups called lineages.

Washington State University, Pullman, WA in winter of 2015. Weevils were separated into groups of 10 and were placed in transparent plastic cylinders (15 cm height, 11 cm in diameter) covered with a mesh lid. Cylinders were kept in an environmental chamber (Percival Scientific Model E-30BC, Boone, IA) set to spring conditions: L10: D14, at 12 °C during light and 2.5 °C during darkness, and 75 % relative humidity. These environmental conditions correspond with the time at which females lay eggs. Weevils were fed foliage of *L. draba* as needed. All weevils were maintained under these environmental conditions until experimentation.

Lepidium draba and *Ocimum basilicum* (basil) plants (Marseilles basil; Dwarf sweet basil; The Cooks Garden, Warminster, PA) were propagated from root cuttings and seeds grown in an environmentally controlled greenhouse at the University of Idaho's Parker Research Farm, Moscow, ID. Plants were maintained at the Parker Research Farm since 2007. Plants were potted in 3-liter black plastic pots (15 cm in diameter and 17 cm tall) and filled with a soil mixture consisting of Sunshine Professional Growing Mix #1 (SunGrow Horticulture, Agawam, MA), top soil with added trace elements (FRIT Industries, Inc., Ozark, AL), chelated iron 10% (Grow More Inc., Gardena, CA), fertilizers (Bonide Triple Super Phosphate, Bonide Products Inc., Oriskany, NY); and Osmocote Flower and Vegetable Fertilizer, (The Scotts Company LLC., Marysville, OH). Plants were kept at 24 °C day, 13 °C night, and a 15-hour photoperiod. Leaves from plants were clipped as needed and used immediately in behavioral bioassays. Basil was chosen for its ease of propagation, absence of glucosinolates, similar leaf architecture and relative length of petiole and leaf blade compared to *L. draba*, and because of its phylogenetic distance to *L. draba* since the two species are located in distinct clades within the Eudicots (Byng et al., 2016).

Behavioral bioassays

The experiments performed in Henderson et al., (2004) provided the basis for the experiments performed here. The experimental arena (Fig. 3.1) consisted of a leaf of *L. draba* or basil placed inside a florist's water pick which was then placed in a larger square plastic dish (5 cm high and 20 cm^2 with rounded edges) filled with water to create a moat surrounding the water pick. This set-up resulted in *C. cardariae* adults being able to walk on the leaf or on the arena floor. Leaves used were approximately 9 cm long (approximately 4 cm petiole and 5 cm leaf blade length) and 5 cm^2 in area. Water picks were 5.5 cm high and 3 cm in diameter. Only females were used in experiments. Weevils were starved for 24 hours prior to their use in behavioral assays. Twelve *C. cardariae* were placed on leaves of either *L. draba* or basil and recorded individually for 30 minutes. To initiate a bioassay, a single weevil was placed in a supine position at a random location on the arena floor. Recording began when weevils became active.

Weevil behavior was viewed using a microscope camera (USB Digital Microscope, 20x-200x, Leuchtturm Albenverlag GmbH & Co. KG und Torquato AG, Geesthacht, Germany) at 20x magnification. The microscope was handheld to allow for tracking of the weevils' intricate movements in 3D space. The microscope camera displayed real time video onto a laptop computer screen. This real time video was simultaneously recorded and saved using a Contour HD wearable camcorder 1080P (Contour Inc., Seattle, WA). Video files saved onto the camcorder were later viewed and analyzed.

All recorded behaviors were analyzed using the behavioral software Noldus Observer XT 11 (Noldus Information Technology BV, Wageningen, The Netherlands). Observations for *C. cardariae* were categorized by behavior when the weevils were on the plant. When weevils were off the plant on the arena, behaviors were not quantified as we wished to quantify only those behaviors post-alightment. Behavioral activities were classified as chemosensory (antennal waving, antennal contact, and tarsal drumming), or non-chemosensory (walking/running, grooming, resting, and off the leaf on the arena floor) (Table 3.1). Behavioral activities were also categorized into two pre-feeding behavioral phases: acclimation (antennal waving, walking/running, grooming, resting) and pre-consumption (antennal contact, and tarsal drumming). Antennal contact was defined as a motion in which weevils would tilt their antennae down towards the leaf surface and drag the antennae along or just above the leaf surface. Tarsal drumming was defined as rapidly moving tarsi up and down the leaf surface. Resting was included in non-chemosensory activities since the weevils were not actively waving their antennae or drumming their tarsi during resting. Feeding was recorded and included in the overall qualitative behavioral descriptions, but not included in the statistical analysis as we wished to characterize only the pre-feeding behaviors. The total time and frequency of each behavior was determined. The recorded behaviors were used to develop an ethogram of the post-alightment host selection behavior of the weevil and compared between the host plant *L. draba* and the non-target host plant basil.

Statistical analysis

Between species, data were analyzed as follows: frequency data were standardized to the number of behavioral events per minute. A one-way analysis of variance (ANOVA) using a generalized linear mixed model assuming a Poisson distribution was used to compare the frequency per minute of each behavior between species. Duration data were standardized to percentage of total time engaged in each behavior during the 30-minute observation period. A one-way ANOVA using a generalized linear mixed model assuming a beta distribution was used to determine differences among species. Means separation was determined at $\alpha = .05$. All statistical analyses were conducted using the statistical software package SAS (Version 9.4; SAS Institute Inc., 2013).

Results

Based on the recorded behavior of *C. cardariae*, ethograms of the post-alightment pre-feeding behavior on *L. draba* and basil were developed as flowcharts (Fig. 3.2). At the beginning of the bioassays all weevils (n=24) turned over on their own after they were placed on the arena floor in supine position, began to walk, and soon after approached and climbed onto the leaf petiole surface (Fig. 3.2). Once weevils moved from the arena floor onto the leaf petiole all 12 weevils on *L. draba* engaged in all four acclimation behaviors: resting, walking, walking with antennal waving (Fig. 3.3a), and grooming. Grooming behavior (Fig. 3.3b) included weevils rubbing legs or antennae with the tarsi, or using a tarsus to press the distal tips of the antenna onto the leaf surface. When weevils were walking but not waving antennae, the antennae were observed to be held relatively motionless as compared to when they were actively being waved. Once weevils moved from the arena floor onto the leaf petiole on basil all 12 weevils engaged in three acclimation behaviors, walking, walking with antennal waving, and grooming, while only nine weevils engaged in resting.

During the pre-consumption phase, weevils engaged in one of two behaviors: antennal contact (Fig. 3.3d) and/or tarsal drumming (Fig. 3.3c). Ten of the 12 weevils (83%) on *L. draba* engaged in at least one pre-consumption behavior. The ten weevils engaged in a combined total of 30 pre-consumption behavioral events (27 antennal contact events, two tarsal drumming events, and one antennal contact with tarsal drumming event) on *L. draba* (Fig 3.2a). Thirteen out of the 30 pre-consumption behaviors led to weevils re-entering the acclimation phase. Ten out of these 13 pre-consumption events were followed by antennal waving and walking, and three by grooming. Seventeen out of the 30 pre-consumption events resulted in feeding. Each of the 17 feeding events was always preceded by the weevil engaging in antennal contact. Seven of the feeding events were followed by antennal waving and walking, three by resting, one by grooming, and five weevils were feeding when the 30-minute recording time was concluded.

When offered basil leaves, nine of the 12 weevils (75%) engaged in at least one pre-consumption behavior (Fig. 3.2b). A total of 13 pre-consumption behavioral events (10 antennal contact and three antennal contact with tarsal drumming) occurred on basil (Fig. 3.2b). None of the weevils on basil initiated feeding. After engaging in pre-consumption behaviors weevils would return to the acclimation phase. Seven out the 13 pre-feeding behaviors were then followed by antennal waving and walking behaviors, two by resting, and four by grooming.

Between the two plant species, frequencies of resting (R) and antennal contact (AC) differed (Fig. 3.4a). Weevils rested more frequently (*P*=0.008) and made more frequent antennal contact (*P*=0.042) on *L. draba* compared to basil.

The duration of grooming (G) and antennal contact (AC) behaviors differed between plant species (Fig. 3.4b). Weevils spent more time grooming (*P*=0.012) on basil than on *L. draba*, and more time engaging in antennal contact (*P*=0.019) on *L. draba* than on basil.

Discussion

While there is a large body of literature on the host selection behavior of herbivorous insect species (Bernays & Chapman, 1994; Knolhoff & Heckel, 2014; and references therein), there are few accounts on the host selection behavior of biological weed control candidate species. For example, olfactory cues (Andreas et al., 2008; Kafle, 2017) and visual cues (Reeves et al., 2009) have been investigated on the host selection behavior of biological control agents in only a few studies, and only two studies have examined olfactory and visual cues combined in the context of biological weed control (Müller & Nentwig, 2011; Park et al., 2018). To our knowledge this is the first study to specifically examine the post-alightment behaviors of a potential biological control agent. As hypothesized, some of the weevils' specific behaviors, their frequency, and duration differed between the two plant species and these behavioral differences may indicate which plant cues are of particularl importance in *C. cardariae* host selection. Fewer antennal contact events and less overall duration of antennal contact events on basil compared to *L. draba* may indicate that the weevils on basil did not find the precise chemosensory cues that were required to indicate an acceptable host plant, or that plant cues on basil were sufficient to identify the plant as a non-host. Subsequently, the weevils were not stimulated to continue to assess the leaf surface via antennal contact to the same degree as on *L. draba*. These results could indicate that 1) basil lacks stimulatory compounds, or that 2) basil has deterrent compounds. Compounds on the leaf surface or volatiles near the leaf surface of *L. draba* may stimulate the weevils to engage in more antennal contact events with longer duration and trigger subsequent feeding. Frequent antennal contact events with the leaf surface and a longer amount of time spent for antennal contacts may indicate the appropriate behavioral cascade to identify *L. draba* as the field host.

There are numerous examples in the literature documenting how chemical cues on the leaf surface influence the host selection behavior of other Brassicaceae feeding herbivores (Adati & Matsuda, 1993; Badenes-Pérez et al., 2011; Eigenbrode, 1996; Eigenbrode & Espelie, 1995; Reifenrath et al., 2005; Städler and Reifenrath, 2009; Städler et al., 2002). These chemical cues are often glucosinolates which are unique sulfur- and nitrogen-containing secondary metabolites that are found in all plants in this family and are considered to be their main chemical defensive compounds (Fahey et al., 2001). A total of 17 glucosinolates have been found in *L. draba* by analyzing plant material that was freeze dried and pulverized (Müller & Martens, 2005; Puliafico, 2008). Several of these glucosinolates have been found of the leaf surface of *Brassica oleracea* (L.) and have been documented to influence other Brassicaceae feeding herbivores including: glucobrassicin (Gouinguene & Städler, 2006; Roessingh et al., 1992; Städler & Reifenrath, 2009), glucobrassicanapin (Roessingh et al., 1992), and 2-Propenyl (Allyl, Sinigrin) (Barker et al., 2006). It is not known if these compounds are also present on the leaf surface of *L. draba* and consequently detected by *C. cardariae* as it assesses the leaf surface. Other compounds have also been found to be present on the leaf surface of some Brassicaceae plants: the thia-triaza-fluorene compounds, commonly named cabbage identification factors (CIF). These compounds can act as powerful oviposition stimulant for cabbage fly *Delia radicum* (L.) (Baur et al., 1996; Gouinguene & Städler, 2006; Hurter et al., 1999; Roessingh et al., 1997) and turnip root fly *Delia floralis* Fallén (Gouinguene & Städler, 2006). However, it is not known whether CIF compounds are present on *L. draba* leaves and whether they affect post-alightment host selection by *C. cardariae.*

Fewer resting events and longer grooming duration on basil may indicate displacement behavior - a behavior that is performed when an insect is under stress (in this case being confined to a non-host plant) (Zhukovskaya et al., 2013). If weevils did not encounter necessary host plant cues, or were deterred by cues present on basil, it follows that they would be influenced to not arrest on the plant, would engage in less resting events on basil, and engage more in other activities such as grooming. Another reason that insects engage in grooming is to remove foreign particles from their sensory organs to keep these organs functioning properly (Böröczky et al., 2013; Zhukovskaya et al., 2013). For example, *Phaedon cochleariae* (F.) beetles were observed to adhere better to glossy leaves compared to waxy leaves and the setae on their tarsi (and by extension, tarsal chemosensory cells) were hindered by the vertical rods and dendritic plates that were accumulated from the waxy leaves (Stork, 1980). Antennal grooming has been shown to remove foreign particles from the antennae of the American cockroach, *Periplaneta Americana* (L.), which aided in maintaining their olfactory acuity (Böröczky et al., 2013). When antennal grooming was prevented, ungroomed antennae accumulated three to four times more cuticular hydrocarbons than groomed antennae and were significantly less responsive than groomed antennae to the sex pheromones (Böröczky et al., 2013). The same study detected similar effects in carpenter ants and houseflies, suggesting grooming as a means to keep sensory organs functioning properly is widespread. The differences in wax load between *L. draba* and basil, and whether weevils accumulated more debris on their tarsi on basil is not known.

The feeding events on *L. draba* were always preceded by antennal contact events. This may further indicate that important chemosensory information is being assessed on the leaf surface via antennae just prior to feeding, and that this stimulates the weevil to begin feeding. Chemosensory information assessed on the leaf surface of basil never led to a feeding event. On both *L. draba* and basil, weevils most often engaged in antennal waving and walking after returning from the pre-consumption phase and were back into the acclimation phase. Why they chose to engage in this behavior first is unclear. As they were starved for 24 hours prior to testing, it may be that they were stimulated to continue to look for new feeding sites, even when on a non-host. Likewise, after feeding on *L. draba*, weevils again engaged most often in antennal waving and walking after a feeding event. After feeding on *L. draba* they never entered the pre-consumption phase directly, but would enter back into the acclimation phase. This may indicate that weevils preferred to search for new feeding sties as opposed to re-assessing the surface on which they had just fed.

One specific difference between the solely volatile tests performed in previous studies (Rendon et al., unpublished data), in which *C. cardariae* did not react, and the tests performed in this study, is that weevils in this study had access to plant contact cues. These different test conditions, and their subsequent results, may suggest that the weevil does not react to volatiles when they are not in contact with the leaf surface. Physical contact (contact cues) with the plant may be necessary for host recognition, or in combination with volatile stimuli once on the plant.

The pre-feeding antennae behavior - dragging them along or just above the leaf surface always before initiating a feeding event - indicates that chemical cues are being assessed while in physical contact with the plant. Host acceptance and rejection by *C.*

cardariae appears to be mediated by olfactory cues accessible when the insect is in contact with the leaf surface. Identifying compounds present on or near the leaf surface that are electrophysiologically active would be the next step in determining the factors underlying the host plant selection of *C. cardariae*.

Table 3.1: Description of behaviors observed for *C. cardariae.*

Behavior	Label	Description	Fig. No.
Non-chemosensory			
In arena	A	On the arena floor and not on the leaf surface.	
Walking	W	Walking or running on the leaf surface.	
Grooming	G	Tarsi and/or legs would rub each other, the body, or the antennae.	3.3b
Resting	R	No movement, except occasional and slight antennal waving.	
Chemosensory			
Antennal waving	AW	Antennae moving up and down rapidly.	3.3a
Tarsal drumming	T	Tarsi moved up and down rapidly over the leaf surface.	3.3c
Antennal contact	AC	The tips of the antenna were bent down toward to the leaf surface and dragged along or just above it.	3.3d
Feeding	F	Feeding on the leaf surface.	3.3e

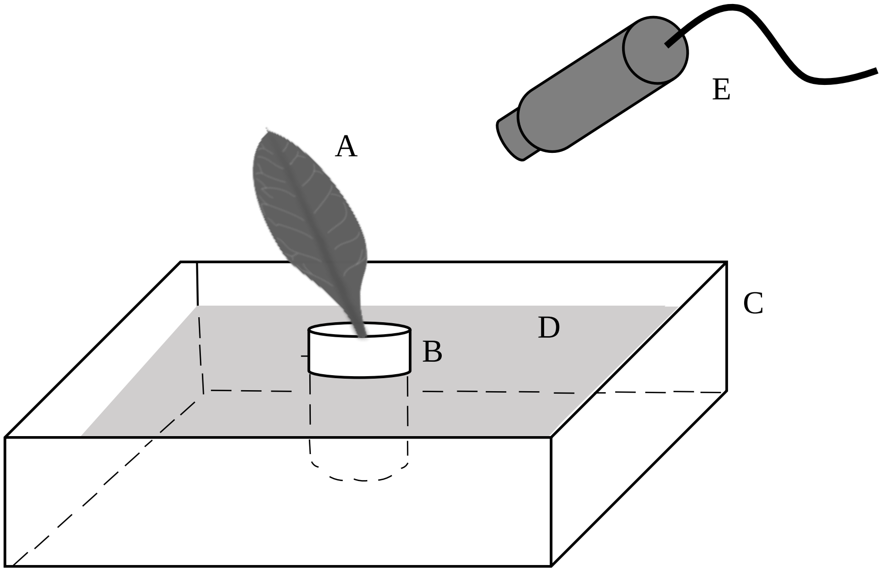

Figure 3.1: Experimental arena (not drawn to scale). A: leaf approximately 9 cm length (4 cm petiole and 5 cm leaf rib) and approximately 5 cm^2 leaf area, B: water picks 5.5 cm tall and 3 cm in diameter, C: plastic water dish with 5 cm tall side wall and 20 cm by 20 cm side length, D: dish was filled with water to avoid weevil movement out of experimental arena, E: hand-held microscope camera.

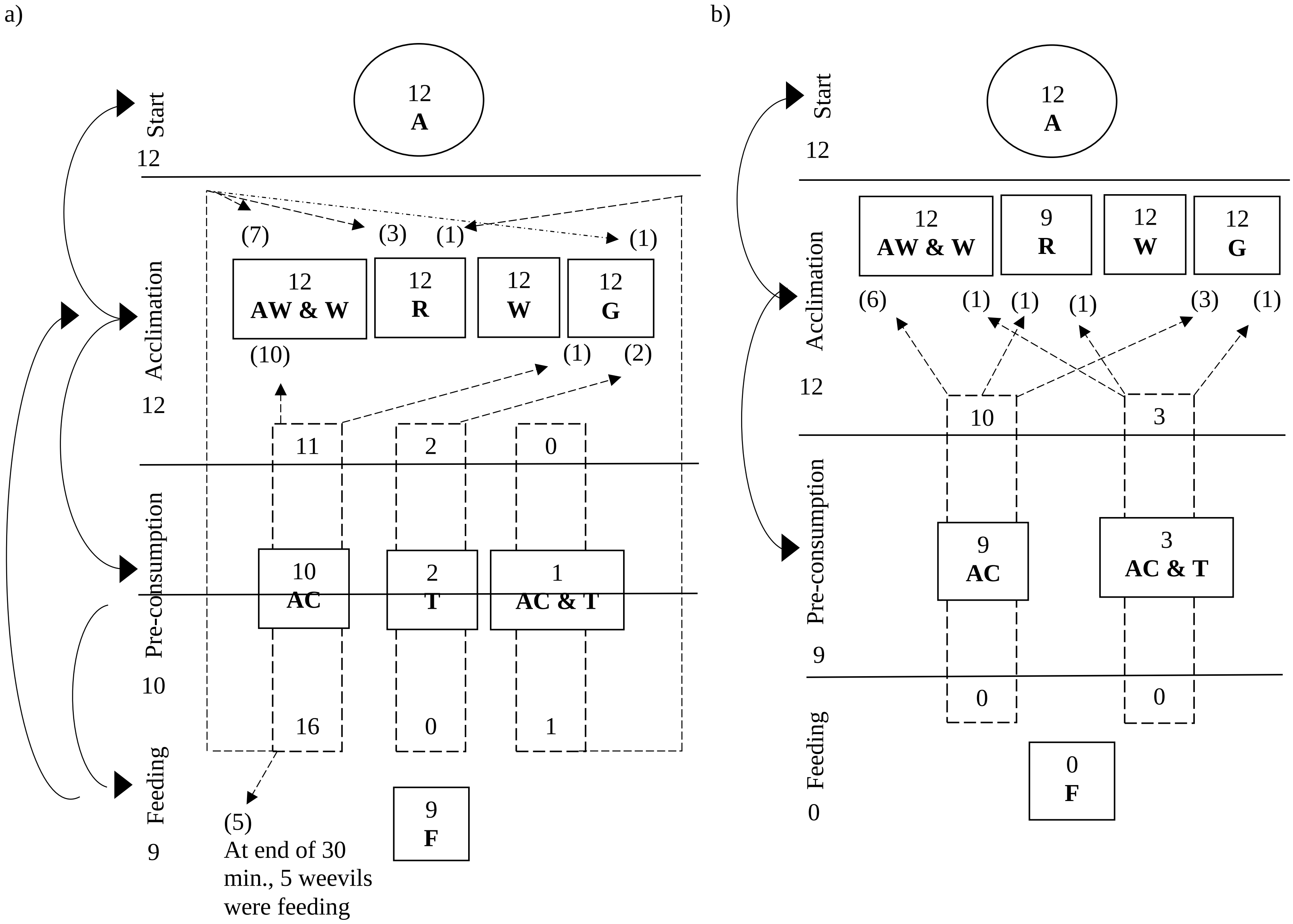
a)
Start
12
12
A
(7)
(3)
(1)
(1)
12
AW & W
12
R
12
W
12
G
Acclimation
12
(10)
(1)
(2)
11
2
0
Pre-consumption
10
AC
2
T
1
AC & T
10
16
0
1
Feeding
9
(5)
At end of 30 min., 5 weevils were feeding
9
F
b)
Start
12
12
A
12
AW & W
9
R
12
W
12
G
Acclimation
(6)
(1)
(1)
(1)
(3)
(1)
12
10
3
Pre-consumption
9
AC
3
AC & T
9
0
0
Feeding
0
0
F

Figure 3.2: Ethogram of observed categorized pre-feeding behaviors of *Ceutorhynchus cardariae* in an experimental arena when offered a) *Lepidium draba* (n=12) and b) basil (n=12). Starting label: A- arena. Behavioral labels: W- walking or running on leaf, R-resting, G-grooming, AW- antennal waving, AC- antennal contact, T- tarsal drumming, F- feeding. Each graph contains four stages (start, acclimation, pre-consumption, and feeding) as indicated on the left side of each graph. The number underneath each stage label indicates how many weevils entered that stage at least once. The lines with arrows on the left side of the graph depict at what stage the weevils were observed to move to and from. The start of the bioassay is denoted by a circle, and the number 12 indicates that 12 weevils started on the arena floor. In the acclimation, pre-consumption, and feeding stages, the boxes with solid lines indicate the behavior that was observed and the number of weevils that engaged in that behavior at least once. Boxes with dashed lines contain two units of information. First, the numbers on the top indicate the number of times the behavior in the box with solid lines was performed and was followed by an acclimation behavior. Second, the numbers on the bottom indicate the number of times the behavior in the box with solid lines was performed and was followed by feeding. The dashed arrows that lead to a number in parentheses indicate what specific behavior was performed when switching between the stages. These flowcharts were modeled after Henderson et al., (2004)

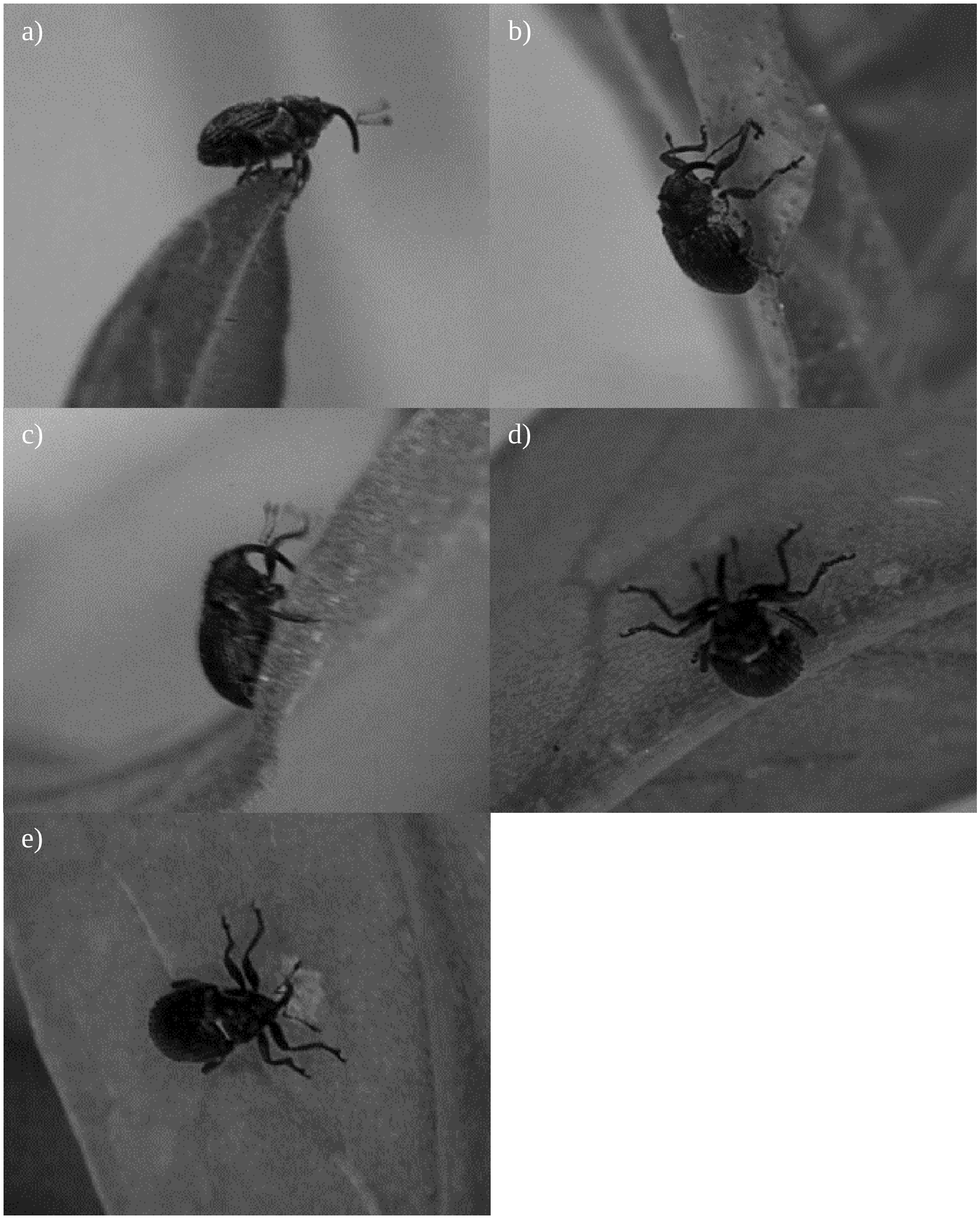

Figure 3.3: Images depicting *C. cardariae* behaviors during pre-feeding on the leaf surface of *L. draba*. a) Antennal waving (AW), b) grooming (G) (photo shows specific type of grooming with antennal contact with leaf surface), c) tarsal drumming (T), d) antennal contact with leaf surface (AC), and e) feeding (F).

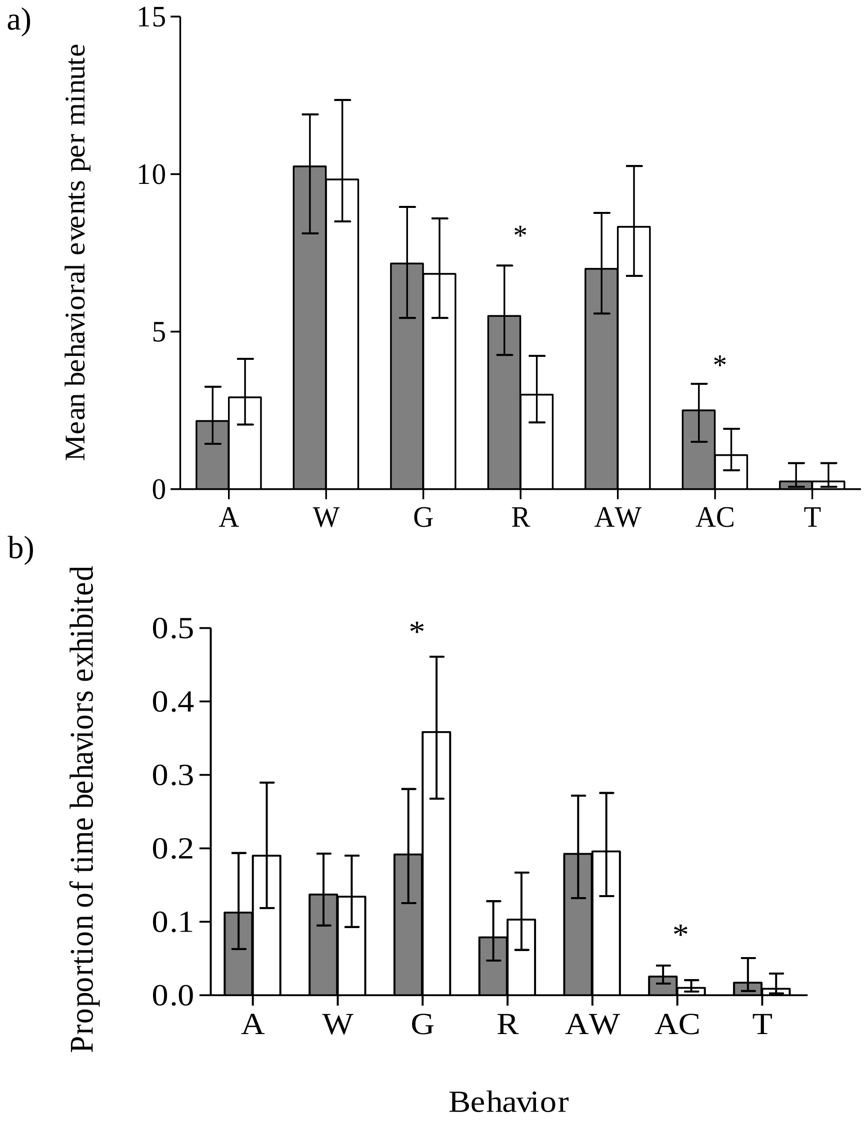

Figure 3.4: a) Mean behavioral events per minute by *C. cardariae* on *L. draba* (*n*= 12), and basil (n=12) and b) Mean percentage of time spent engaged in individual behaviors by *C. cardariae* on *L. draba* (n=12), and basil (n=12). *L. draba* shown in grey, basil in white. Behavior labels: A- on arena floor; W- walking or running on leaf surface; G- grooming; R- rest; AW- antennal waving; AC- antennal contact with the leaf surface; T- tarsal drumming. Error bars are 95% confidence intervals. Bars superscripted by asterisk denote significant differences among behavioral category between species.

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Chapter 4

ROLE OF VISUAL, VOLATILE, AND CONTACT CUES IN THE HOST SELECTION BEHAVIOR OF *CEUTORHYNCHUS CARDARIAE*, A BIOLOGICAL CONTROL CANDIDATE FOR *LEPIDIUM DRABA*

Abstract

The gall-forming weevil *Ceutorhynchus cardariae* is a classical biological control candidate for the Eurasian *Lepidium draba,* a perennial mustard that is invasive in western North America. Previous investigations indicated that the fundamental host range of *C. cardariae* includes a relatively wide range of plant species within the family Brassicaceae, while in open-field tests, the weevil exhibited a very restricted host range. In a series of experimentswe therefore investigated the importance of visual, volatile and contact cues in the host finding behavior of *C. cardariae*. For these experiments we developed a walking arena, which allowed for the manipulation of visual, volatile, and contact cues using whole plants, plant cuttings (one petiole with one leaf attached), or paper models. Cues were examined individually and in combination. The time weevils spent on plants or plant models, respectively, was analyzed. Visual cues, particularly whole plants or cuttings of *L. draba*, but also green paper plant models, showed a consistently strong attraction for *C. cardariae.* While volatile and contact cues showed some attraction for *C. cardariae*, when offered individually, these cues were insufficient to allow for field host recognition. Our data suggest that *C. cardariae* mainly relies on visual cues to identify a potential host plant prior to contact, while after contact volatile and contact cues are important for final host recognition. Results also indicate that volatile and contact cues are equally important for final host choice.

Introduction

Classical biological control of weeds relies on the use of sufficiently host-specific specialist herbivore organisms to avoid post-release non-target attack (Heard, 1999; Louda et al., 2003; Sheppard et al., 2005; Suckling & Sforza, 2014). Conventionally, the risk is assessed pre-release through host-specificity testing of candidates using no-choice or choice tests, under differing conditions such as confined plants or under open field conditions. These tests can assess feeding, oviposition and larval development of a candidate species on a set of plant species ranging from very closely to distantly related to the targeted invasive plant species (Briese, 2005; Schaffner, 2001; Sheppard et al., 2005). Host-specificity tests determine a biological control candidate's fundamental and ecological host range. The fundamental host range has been defined as the range of plant species that are capable of supporting a biological control agent's full life cycle (Van Klinken, 1999) and is typically assessed through no-choice tests. No-choice tests are generally performed by confining the insect in a cage with one plant, restricting the full sequence of behaviors in play during host selection. The ecological host range, which is typically narrower than the fundamental host range, is assessed in field tests in which the candidate biological control agent can choose among plant species (Schaffner, 2001). In these tests, the pre-alightment phase of host selection, mediated by volatile and visual plant cues, becomes important (Briese, 1999; Clement & Cristofaro, 1995; Schaffner, 2001; Schaffner et al., 2018; Van Klinken, 1999; Wapshere, 1989). Overreliance on assessments of the fundamental host range rather than the ecological host range can lead to uncertainty about whether non-target plant species are at risk of attack after the release of the agent (McFadyen et al., 2002).

The host selection behavior of an insect herbivore can be divided into three main phases: finding, examining, and accepting (Bernays & Chapman, 1994; Miller & Strickler, 1984). Finding takes place prior to contact with the plant, and examining and acceptance take place following contact. Acceptance or rejection of a potential host plant during the finding and examining phases depends on the insects’ response to visual, volatile, contact, and gustatory plant cues (Bernays & Chapman, 1994; Dethier, 1982; Heard, 1999; Miller & Strickler, 1984; Wheeler & Schaffner, 2013). These stimuli dictate behavioral and physiological patterns of feeding and oviposition (De Wilde & Schoonhoven, 1969; Jermy, 1966; Marohasy, 1998; Miller & Strickler, 1984; Thorsteinson, 1960). Examining host selection behavior in detail may lead to a better understanding of how and why certain plant species are attacked. Pre-release risk assessment could thus be improved by observing and describing the host selection behavior of an agent both pre- and post-alightment to determine which cues or cue modalities may be particularly important in its host plant choice (Hinz et al., 2014; Knolhoff & Heckel, 2014; Wheeler & Schaffner, 2013).

While there is a large body of literature on the host selection behavior of herbivorous insect species (Bernays & Chapman, 1994; Knolhoff & Heckel, 2014; and references therein), there are few accounts on the host selection behavior of biological weed control candidate species. For example, the influence of olfactory (Andreas et al., 2008; Kafle, 2017) and visual cues (Reeves et al., 2009) on the host selection behavior of biological control agents have been investigated in only a few studies, and only two studies have examined olfactory *and* visual cues combined in the context of biological weed control (Müller & Nentwig, 2011; Park et al., 2018).

The Eurasian clonal perennial mustard *Lepidium draba* (L.) Desv. (Brassicaceae), which is invasive in western North America, and one of its biological control candidates, the stem gall-forming weevil *Ceutorhynchus cardariae* Korotyaev (Coleoptera: Curculionidae), offer an ideal system to study the host selection behavior in a biological weed control system. Host range testing including no-choice, choice, and open field tests, has demonstrated that *C. cardariae* is highly host-specific under natural test settings, but under no-choice conditions the weevil readily attacks and develops on two distantly related plant species in the tribe Thelypodieae, *Streptanthus anceps* (Payson) Hoover and *Streptanthus flavescens* Hook (Weyl et al., 2018). This pattern does not fit the assumption underlying the centrifugal phylogenetic method, i.e. that species closely related to the target weed are more likely to be attacked than more distantly related species (Weyl et al., 2018). *C. cardariae* exhibits a 'phylogenetically disjunct' host range.

Volatile, visual, and contact cues have been found to mediate the host selection and acceptance behavior of other Brassicaceae feeding beetles, e.g. *Ceutorhynchus assimilis* (Paykull), *Phaedon cochleariae* (F.), and *Phyllotreta cruciferae* (Goeze)) with regard to volatile cues (Bartlet et al., 1993; Blight et al., 1995), visual cues (Kühnle & Müller, 2011; Smart & Blight, 1997), and contact cues (Bodnaryk, 1992a; Bodnaryk, 1992b; Reifenrath et al., 2005). Since *C. cardariae* demonstrates a high level of specificity under field conditions [it has only been reported from *L. draba* and occasionally from the closely related *L. campestre* in its native range (Hinz & Diaconu, 2015)], it is assumed that specific visual, volatile organic compounds, and/or contact cues are conferring that specificity during the host selection process (Bernays & Chapman, 1994). In behavioral studies of the post-alightment pre-feeding behavior of *C. cardariae* on *L. draba* and a non-target plant (basil), results suggested that important chemical information of the host plant is assessed on the leaf surface or in volatilized form right above the surface by either contact chemoreceptors on the weevils' antennae, tarsi, or both (see Chapter 3).

Here we report on both pre- and post-alightment tests of host selection by *C. cardariae*. Our objective was to determine of the relative contributions of visual, volatile, and contact cues of *L. draba* both individually and in combination that mediated the host selection behavior of *C. cardariae.* We hypothesized that female weevils use a specific combination of visual, volatile, and/or contact cues to select *L. draba* as host. To test this hypothesis, we constructed a bioassay arena in which we could manipulate visual, volatile and contact cues of *L. draba* or plant models and record the behavior of walking *C. cardariae* in response to these cues.

Materials and methods

Study system and materials

Lepidium draba is a clonal perennial mustard native to Eurasia that can invade open, disturbed, and degraded habitats, such as croplands, pastures, rangelands, and riparian areas, and form dense monospecific stands in North America (Scurfield, 1962). It is a declared noxious weed in 15 US states and three Canadian provinces (USDA-NRCS, 2018). *Ceutorhynchus cardariae* is a petiole and stem galling weevil native to Europe and Asia (Hinz & Diaconu, 2015; Korotyaev, 1992). Females lay their eggs in early spring into stems, leaf stalks, and midribs of *L. draba* rosettes and bolting plants, which causes the formation of galls (Hinz & Diaconu, 2015). Larvae of *C. cardariae* hatch and then feed on the parenchymatic tissue of developing galls, which can stunt or even kill shoots (Hinz & Diaconu, 2015

For these experiments, approximately 400 female adult *C. cardariae* were transferred from a rearing colony at CABI in Switzerland to the Northwestern Biocontrol Insectary and Quarantine (NWBIQ) at Washington State University, Pullman, WA, in winter of 2015. Weevils were separated into groups of 10 and were placed in transparent plastic cylinders (15 cm height, 11 cm in diameter) covered with a mesh lid. Cylinders were kept in an environmental chamber (Percival Scientific Model E-30BC, Boone, IA) set to "spring" conditions: L10: D14, at 12 °C during light and 2.5 °C during darkness, and 75 % relative humidity. These environmental conditions correspond with the time of year, in their native range, during which females lay eggs. Weevils were fed foliage of *L. draba* as needed. All weevils were maintained under these environmental conditions until experimentation.

Specific populations of *L. draba* plants were maintained at the Parker Research Farm since 2007. Populations had been identified as closely related genetic populations based on amplified fragment length polymorphism (AFLP) analysis (J.F. Gaskin, unpublished data; Puliafico, 2008). Oregon population E was utilized in this study (Puliafico, 2008). From these plants, root cuttings were collected, propagated, and maintained in an environmentally controlled greenhouse at the University of Idaho's Parker Research Farm, Moscow, ID. Plants were potted in 3-liter black plastic pots (15 cm in diameter and 17 cm tall) and filled with a soil mixture consisting of Sunshine professional Growing mix #1 (SunGrow Horticulture, Agawam, MA), top soil with added trace elements (FRIT Industries, Inc., Ozark, AL), chelated iron 10 % (Grow More Inc., Gardena, CA), and fertilizers (Bonide Triple Super Phosphate, Bonide Products Inc., Oriskany, NY; Osmocote Flower and

Vegetable Fertilizer, (The Scotts Company LLC., Marysville, OH). Plants were kept at 24 °C day, 13 °C night, and a 15-hour photoperiod.

Bioassays

Bioassays were conducted in a walking arena (Fig. 4.1) similar to that used by Heisswolf et al., (2007). This type of arena allows for the manipulation of visual, volatile, and contact cues using plants, plant cuttings (petioles with leaves attached), or paper models. Cues could be examined individually or in combination. The walking arena consisted of a circular plastic lid (Cambro, Huntington Beach, CA) with an indentation around the edge. The indentation was filled with water to create a moat to prevent *C. cardariae* from escaping the arena. The outer edge of the lid was 31 cm in diameter, and the moat was 2.5 cm in width, thus creating an inner arena diameter of 28.5 cm. Four holes, evenly spaced around the inner edge, were drilled into the plastic lid. Plant material or plant models were placed inside a water pick, which was then pushed through the holes until flush with the plastic lid surface. A thin layer of moistened sand was spread on the bottom of the arena, and a moistened filter paper disk (Whatman Grade 1 Filter Paper, GE Health Care Life Sciences, Piscataway, NJ) was placed on top of the sand. Plants and plant models were changed after each replicate consisting of three weevils used in a bioassay. To avoid any directional bias, the arena was rotated 90 degrees after each replicate. The arena was surrounded by a square white cardboard box with a side length of 61.5 cm and an open top. Illumination was from overhead fluorescent lighting and two Jansjö LED lamps (Inter Ikea System B.V., Delft, The Netherlands) placed directly above the center of the arena.

Plant cuttings and plant models used were approximately 8 cm tall. Generally, two options were offered to weevils in an alternating pattern (e.g. a cutting of *L. draba*, a white filter paper stick, a cutting of *L. draba*, and a white filter paper stick) around the arena, unless otherwise noted (Fig. 4.1). Unless stated otherwise, all plant material of *L. draba* was offered as cut petioles with attached leaves. All white paper models were made from Whatman Grade 1 Filter Paper (GE Health Care Life Sciences, Piscataway, NJ). All colored paper models (red, yellow, and blue) were made from construction paper (Pacon Creative Products, Pacon Corporation Appleton, WI).

For each bioassay, 12 adult female *C. cardariae* were starved for 24 hours before recording their behavior in the walking arena. Unless otherwise noted, these 12 weevils were grouped into four replicates of three weevils. While both adult males and females need to locate their host plant, we focused on females since they lay eggs and can initiate gall formation. Three weevils were placed together in the center of the arena in supine positions and timing began for each weevil after it turned over and started walking. Each weevil's movement through 60 minutes was recorded using a Contour HD Camcorder 1080P (Contour Inc., Seattle, WA). The length of time spent on a plant, plant model or on the arena floor was recorded. Twenty-four hours prior to testing, the three weevils were marked distinctively so they could be distinguished on video recordings. One received a small dab of low volatile organic compound white paint (Valspar Perfect Sample Pure White #57351 0 g/L VOC, Minneapolis, MN) applied to the end of its abdomen, another had a large dab of white paint applied to the end of its abdomen, and the third was unmarked. Weevils were generally only used once in the walking arena. However, in a few cases weevils were used twice, with these tests performed at least one week apart from each other. If any weevil dropped into the water moat, all weevils were removed from the arena, and a new set of three weevils was used.

In order to establish baseline data for weevil responses and to test the functionality of the arena, five initial bioassays (pre- and post-alightment) were performed. These included offering *C. cardariae*: a) cuttings of *L. draba* placed in one of the four arena openings, while three openings remained empty and were closed off (as controls), b) cuttings of *L. draba* and white paper sticks; for this bioassay 12 weevils were recorded in the arena one at a time (rather than four sets of three weevils at a time in the arena). This bioassay was only conducted to test whether using three weevils simultaneously in the arena would lead to differing results compared to using only one weevil in the arena at a time, c) petioles with attached leaves (of two intact potted plants) of *L. draba* that were pushed through two of the openings in the arena with the other two openings containing white paper sticks. This bioassay was conducted to test whether *C. cardariae's* responses to cuttings of *L. draba* would be comparable to responses to petioles with leaves attached to intact plants, d) two cuttings of *L. draba* and two white paper sticks, and e) two cuttings of *L. draba* and two green paper plant models resembling *L. draba (*Fig. 4.1).

In order to examine the effect of color and shape on *C. cardariae* choice (pre-alightment), the weevils were offered: a) one green paper plant model and three empty openings as controls, b-g) green paper sticks, green paper plant models, white paper sticks, and white paper plant models in all six possible paired combinations, h) green paper plant models and green paper grass models (Fig. 4.1), i-k) green paper sticks were compared to red paper sticks, yellow paper sticks, or blue paper sticks, and l-n) green paper plant models were compared to red, yellow, or blue paper plant models. In order to examine the role of visual cues alone, *C. cardariae* were offered choices between: o) cuttings of *L. draba* that were covered with a glass cylinder (3.5 in tall, 1.5 in diameter), preventing volatile and contact cues, with empty glass cylinders as the alternative, and p) cuttings of *L. draba* that were covered with a glass cylinder and glass cylinders containing a green paper plant model.

In order to examine the role of volatile cues only (pre-alightment), *C. cardariae* were offered cuttings of *L. draba* that had been wrapped in perforated filter paper tubes, preventing contact and visual cues from being available to weevils, and empty perforated filter paper tubes. The perforation of the filter papers was performed with a needle (20 holes; hole diameter: 0.5 mm).

In order to study the effects of combined volatile and visual cues (pre-alightment), *C. cardariae* were offered: a) *L. draba* placed inside a fine mesh cage. Cages were constructed from a plastic vial (approximately 7 cm height, 4 cm in diameter), from which ¾ of its plastic vertical surface area was removed and replaced with a fine cloth mesh (approximately 4.5 cm x 6.5 cm). This enabled weevils to see and smell, but not to touch the plants, along with empty mesh cages as alternative choices, and b) either *L. draba* or green paper plant models placed within mesh cages as described above.

In order to examine the effects of combined visual and contact cues, and to exclude volatile cues (pre- and post-alightment), *C. cardariae* had their antennae removed with a scalpel and a pair of dissecting scissors. Weevils were allowed to recover for three days and then offered either a) *L. draba* and white paper sticks, or b) *L. draba* and green paper plant models. These bioassays were compared to bioassays which offered the same treatments, but used weevils with intact antennae.

In order to examine the role of contact cues (post-alightment) in the form of surface waxes in the host selection of *C. cardariae*, a series of bioassays using hexane and methanol extracts was conducted. Hexane was chosen to extract any non-polar bioactive chemicals that may have been present on the leaf surface of *L. draba*. Methanol was chosen to extract any polar bioactive chemicals that may have been present on the leaf surface of *L. draba*. These extracts were then applied to neutral models - green paper sticks and green paper plants. In this way we could examine the effects of surface waxes separate from volatile visual cues of *L. draba*. To prevent damage to the leaf surface and prevent penetration of the inner leaf tissue, quick immersions into hexane (30 seconds), methanol (10 seconds), and a 1:1 mixture of hexane and methanol (20 seconds) were adapted from De Vos et al., (2008), Eigenbrode et al., (1991), Reifenrath et al., (2005) and Städler & Reifenrath, (2009). These short rinsing times, as well as not using solvents such as chloroform and dichloromethane (which can penetrate the leaf tissue) were utilized as we were interested in cues present on and not within the leaf surface.

To detect potential repellency of hexane or methanol on *C. cardariae* host selection, weevils were offered green paper sticks and green paper sticks that had been dipped twice for five seconds into either a) hexane (EMD Millipore Corporation, Billerica, MA), b) methanol (EMD Millipore Corporation, Billerica, MA), or c) a 1:1 mixture of hexane and methanol. For subsequent bioassays, 50 leaves of *L. draba* were dipped into 500 ml of either d) hexane for 30 seconds, e) methanol for 10 seconds or, f and g) a 1:1 mixture of hexane and methanol for 20 seconds. The duration of time leaves of *L. draba* were dipped into these mixtures (d, e, f, and g) is different from the time used in the previous bioassays (a, b, and c). This is because more than five seconds is required to adequately dissolve the leaf surface waxes from *L. draba* into the mixture (Eigenbrode, personal communication). Five hundred ml of these mixtures were then poured separately over four green paper sheets (22 cm x 28 cm) and allowed to evaporate until completely dry. From this, green paper sticks and green paper plants were made and offered to weevils alongside identical green paper sticks, or green paper plants that had only been treated with hexane, methanol, or a 1:1 mixture of hexane and methanol for a duration of five seconds. As each leaf surface area was approximately 12 cm^2, 600 cm^2 of leaf surface area was extracted and applied to 616 cm^2 of paper.

Another examination of the role of leaf surface waxes (post-alightment), was conducted by removing the waxes from one half of a leaf of *L. draba* and compare *C. cardariae* feeding preferences. Two non-destructive methods to remove leaf surface waxes without damaging the underlying leaf tissue were employed: gum Arabic and cellulose acetate. These two methods have been used previously in Reifenrath et al., (2005) and Müller and Hilker, (2001), respectively. The respective leaf surface (left half of adaxial side and right side of abaxial side) was coated with a 50% (w/w) aqueous solution of gum Arabic (Frontier Co-op, Norway, IA) using a small paint brush. After three hours, a dry and stable polymer film had formed. The epicuticular waxes could then be carefully removed along with the dried polymer film using forceps, leaving the leaves physically intact and without damaging epidermal and mesophyll tissue. The half-side treated leaves were offered in behavioral assays. A total of 24 leaves were offered to 24 weevils. Water picks holding the half-side treated leaves were placed inside plastic cylinders that had mesh on top. One female weevil was placed inside each cylinder. After 24 hours, leaves were photographed and percent of consumed area was determined using Image6 software (Media Cybernetics, Inc., Rockville, MD). Cellulose acetate (Sigma-Aldrich Co. LLC, St. Louis, MO) was also used for the mechanical removal of surface waxes. Methods were almost identical to those using gum Arabic except 1) cellulose acetate was dissolved in acetone (Sigma-Aldrich Co. LLC, St. Louis, MO) (10% w/w), 2) this solution was allowed to dry and evaporate on the leaf surface for only 15 minutes, and 3) a total of 33 leaves were offered to 33 weevils.

Statistical analysis

All statistical analyses were conducted using the statistical software package SAS (Version 9.4; SAS Institute Inc., 2013). Data from the bioassays conducted in the walking arena were analyzed using a logistic regression with a general logit transformation to determine the probability of weevils being on one of the two treatments or on the arena floor. It should be noted that if weevils move randomly throughout the arena, it is expected that they should be found more often on the arena floor, since it is larger than the plant cuttings and plant models offered. Weevil time spent on the arena floor can be viewed one of two ways, 1) weevils are 'non-responders' and are not attracted to any of the offered cues, or 2) weevils are actively searching for their host plant. Emphasis in the subsequent analysis and discussion is given to which of the two treatments weevils preferred in each bioassay. For bioassays in which *L. draba* leaves had their surface waxes removed, feeding amounts on the treated and untreated leaves were compared to one another using a Proc Glimmix procedure to determine if the amount of feeding differed between the two treatments. This procedure fits statistical models to data with correlations or non-constant variability and where the response is not necessarily normally distributed.

Results

Bioassays establishing a baseline (pre- and post-alightment)

Ceutorhynchus cardariae strongly preferred *L. draba* over three empty control openings ($P<0.05$), which confirmed that females exhibited normal host selection behavior in the bioassay arena (Fig. 4.2a). The behavior of individual weevils, as opposed to groups of three weevils, did not differ and both strongly preferred *L. draba* over white paper sticks ($P<0.05$) (Fig. 4.2b vs. 4.2d). There was also no difference in the choice of *C. cardariae* whether offered un-excised or excised leaves of *L. draba* (Fig. 4.2c vs. 4.2d). *Ceutorhynchus cardariae* strongly preferred *L. draba* over green paper plants ($P<0.05$) (Fig. 4.2e).

Visual bioassays (pre-alightment)

Females of *C. cardariae* consistently preferred green paper plants and (even) green paper sticks to white paper plants and sticks (Figs. 4.3a-h). When offered white paper plants and sticks only, weevils had the highest probability of being on the arena floor (Fig. 4.3e). However, when offered a choice of different colors, weevils preferred red and yellow paper plants more than green paper plants (Fig. 4.3l+m), while blue paper plants or sticks were not attractive (Fig. 4.3k+n). In general, paper plants appear to be more attractive than paper sticks (except for Fig. 4.3c). *Ceutorhynchus cardariae* highly preferred cuttings of *L. draba* or a green paper plant under glass (visual cues only) to an empty control (Fig. 4.3o+p).

Volatile bioassay (pre-alightment)

Ceutorhynchus cardariae preferred filter paper tubes that contained *L. draba* over empty filter paper tubes ($P<0.05$) (Fig. 4.4). However, weevils had the same probability of being on the arena floor or filter paper tubes containing *L. draba*.

Volatile and visual bioassays (pre-alightment)

Ceutorhynchus cardariae preferred mesh cylinders (which allowed access to volatile and visual cues but not contact cues) that contained *L. draba* over empty mesh cylinders ($P<0.05$) (Fig. 4.5a). *Ceutorhynchus cardariae* equally preferred mesh cylinders that contained *L. draba* and mesh cylinders that contained green paper plant models ($P<0.05$) (Fig. 4.5b).

Visual and contact bioassays (pre- and post-alightment)

Antennectomized *C. cardariae* strongly preferred *L. draba* over white paper sticks ($P<0.05$) (Fig. 4.6a). Antennectomized *C. cardariae* equally preferred *L. draba* and green paper plant models ($P<0.05$) (Fig. 4.7b).

Surface wax bioassays (post-alightment)

Ceutorhynchus cardariae was not repelled or attracted by hexane or a 1:1 mixture of hexane and methanol into which green paper sticks had been dipped ($P<0.05$) (Fig 4.7a, c). *Ceutorhynchus cardariae* slightly preferred green paper sticks from which hexane and methanol that had been evaporated over *L. draba* evaporated hexane and methanol extracts applied onto green paper sticks ($P<0.05$) (Fig. 4.7d, e). This pattern did not hold when applying a mixture of hexane and methanol (Fig. 4.7f). However, when the *L. draba* extract using hexane and methanol was applied to a green paper model, it was more attractive than the pure hexane/methanol mixture applied to a plant model (Fig. 4.7g).

Ceutorhynchus cardariae fed equally on the intact leaf surface and the leaf surface that had been removed using gum Arabic (0.600) (*P*=0.055). *Ceutorhynchus cardariae* fed more on the leaf surface from which wax had been removed using cellulose acetate, than on the intact leaf surface (0.682) (*P*<0.001).

Discussion

Taken as a whole, the visual bioassays suggest that visual cues play an important role in the host finding of *C. cardariae;* in particular, the color green and the shape of leaves are attractive to the weevil. Weevils also showed a clear preference for the color yellow (Fig 4.3j, m). Several herbivorous insects are attracted to the color yellow and it has been hypothesized that yellow may constitute a super-normal foliage type stimulus for many foliage-seeking insects (Prokopy & Owens, 1983). A super-normal stimulus is a paradoxical effect whereby an animal shows a greater responsiveness to a stimulus that differs substantially from the "natural" stimulus (Staddon, 1975; Tinbergen, 1948). Other *Ceutorhynchus* weevils including *C. picitarsis* Gyllenhal (Büchi, 1986), *C. assimilis* and *C. pallidactylus* (Láska et al., 1986; Smart et al., 1997) have all demonstrated a preference for the color yellow; in the field, greater numbers of these weevils were caught in yellow colored traps over other colored traps. However, these studies did not provide explanations for these preferences. Interestingly, weevils preferred red paper plants over green ones (Fig. 4.3l). The reason for this is not known. The ancestral set of visual receptors for class Insecta is believed to be UV, blue, and green, with red receptors evolving the most recently (Briscoe & Chittka, 2001). In their review on the evolution of color vision in insects, Briscoe & Chittka, (2001) report several insect species that are attracted to the color red. But for several of these species, the adaptive significance of such red preference is not well understood, and they were not able to identify a common selective pressure underling their evolution. For example, certain island subspecies of *Bombus terrestris* (L.), *sassaricus* and *canariensis*, preferred the color red in behavioral bioassays, but their natural host ranges include few red colored flowers, and these seem to not be utilized by bumble bees and were pollinated instead by beetles (Briscoe & Chittka, 2001; Dafni et al., 1990). Briscoe & Chittka, (2001) suggest that this preference may have evolve because it was not selected against and that chance evolutionary events play a more important role in sensory ecology than has previously been recognized.

The importance of visual and/or volatile cues is also supported by results of an open-field test with *C. cardariae,* in which plots of test and *L. draba* plants were exposed in increasing distances from a central weevil release point. Test plants were only attacked when exposed in close proximity (0-2.5 m) to the central release point, while *L. draba* was still attacked when exposed at a distance of up to 10 m. This suggests that visual and/or volatile cues allow long-distance host recognition.

Volatile cues alone were not sufficient to elicit a clear host choice of *C. cardariae* (see Fig. 4.4 and Fig. 4.7a-g), and only appeared attractive in combination with the ‘correct’ visual cues (Fig. 4.7f vs. 4.7g). It has been proposed that specialist insect herbivores may rely more on visual and less on olfactory cues, while generalist insect herbivores may be visual generalists and rely more on olfactory plant cues (Prokopy & Owens, 1978). This could be because the formation of an innate search image would be less efficient and less adaptive for generalists (who utilize a greater variety of plant species) compared to specialists (who utilized very few plant species) (Prokopy & Owens, 1978). Additionally, it has been proposed that monophagous herbivores with limited dispersal ability that inhabit persistent habitats, that use host plants that dominate plant communities, and that are predictable in time and space, may commonly evolve vision as the main or even exclusive host plant location mechanism (Reeves, 2011; Stenberg & Ericson, 2007). This could also be the case for *C. cardariae* as a specialized herbivore on a clonal plant that is dominant, at least within patches. Also, *C. cardariae* likely has limited dispersal capabilities as 1) adults have never been observed to fly (Hinz unpublished data), 2) in flight experiments weevils could not be induced to fly (Rendon unpublished data), and 3) the larvae are stem miners, as opposed to free-moving larvae with the potential for up to two generations to occur in the same host plant patch.

Ceutorhynchus cardariae could support the theory of Finch & Collier, (2000) who proposed that pest insects of cruciferous crops search for hosts against a diverse background and use visual cues to land indiscriminately on green objects. Once landed on a potential host plant, additional plant cues are assessed and the plant is either accepted or rejected (Finch & Collier, 2000). While *C. cardariae* does not attack Brassicaceae crops, it is a Brassicaceae specialist. Our results suggest that visual cues are of primary importance for the initial host selection of *C. cardariae* before contact with a potential host plant is made, and that the weevils are attracted to the basic shape and color of a green plant. However, when given a choice, *C. cardariae* strongly preferred *L. draba* compared to a simple green plant paper model, suggesting that additional volatiles or contact cues are utilized by the weevil to discriminate its host.

According to our results, volatile and contact cues appear to be of similar importance in the host recognition of *C. cardariae* (Fig. 4.5b and 4.6b). When weevils were offered foliar volatile cues of *L. draba,* they preferred these over empty controls but they spent equal time on the arena floor, suggesting that foliar volatile cues are of less importance (Fig. 4.4). Even when visual and foliar volatile cues were offered to *C. cardariae,* the sensory information was insufficient for the recognition of *L. draba* as host over a plant model (Fig. 4.5b). Consequently, this indicates that contact cues may be of greater importance for host discrimination in *C. cardariae*. Another specialist herbivore, the tortoise beetle *Cassida canaliculata* (Laich.), which is monophagous on meadow sage *Salvia pratensis* (L.), showed weak attraction to its host plant when only foliar volatiles and visual plant cues were offered (Heisswolf et al., 2007). However, when contact cues were added, a strong preference for its host plant over non-target plants and controls was observed (Heisswolf et al., 2007).

When antennectomized weevils were offered visual *and* contact cues of *L. draba,* results suggest that weevils are attracted to a green plant-like object, but without volatiles cues they cannot distinguish between its host plant and a green plant model (see Fig 4.6b). These results are again in accordance with the hypothesis that volatile cues are the necessary chemosensory step for acceptance or rejection of a potential host plant (Finch & Collier, 2000).

Results suggest that contact cues present on the leaf surface of *L. draba* also play a role in the host finding of *C. cardariae*. Weevils were more attracted to green paper plant models that had *L. draba* surface wax extracts applied to them (when using a 1:1 mixture of hexane and methanol), over green paper plant models that did not have *L. draba* surface wax extracts applied (Fig. 4.7g). When using gum Arabic to remove leaf surface waxes, weevils fed equally on treated and untreated leaf surfaces, but when using cellulous acetate to remove leaf surface waxes, weevils preferred to feed on the treated leaf surface. It is not clear why gum Arabic and cellulose acetate results differed from one another. There are no examples in the literature of these two methods being employed and compared using the same study system. Müller and Hilker, (2001) used cellulose acetate to remove the surface waxes of *Tanacetum vulgare* (L.), the host plant of *Cassida stigmatica* Suff. When only adaxial leaf surfaces were offered, adult females did not discriminate between intact and stripped leaflets for oviposition. When only abaxial leaf surfaces (which are generally used as oviposition sites) were offered, adult females preferred to lay eggs on intact leaflets. Reifenrath et al., (2005) used gum Arabic to remove the epicuticular surface waxes of *Brassica napus* (L.) and *Nasturtium officinale* W.T. Aiton, which are hosts for *Phaedon cochleariae* (F.). Adults preferred both the adaxial and abaxial leaf surfaces of host plants that had been treated with gum Arabic. Unlike *C. stigmatica*, beetles were not restricted to only one side of the leaf surface. These two examples demonstrate that removing surface waxes can cause differing results between species (one prefers untreated, and another treated), as well as differing results when offering only the lower or upper leaf surface.

Summary

Our data suggest that *C. cardariae* mainly relies on visual cues for pre-alightment host selection, and that volatile and contact cues are necessary for final host recognition. It also appears that volatile and contact cues are equally important and that females need to be able to perceive both in order to make a final host choice. Previous experiments reported that weevils always drag their antennae along or just above the leaf surface prior to the onset of feeding, indicating that close-range volatiles are being assessed while the weevil is in contact with the plant (see Chapter 3). This study provides evidence of the importance of volatile cues, but further indicates that the weevils must also see and/or be in physical contact with the plant for host recognition.

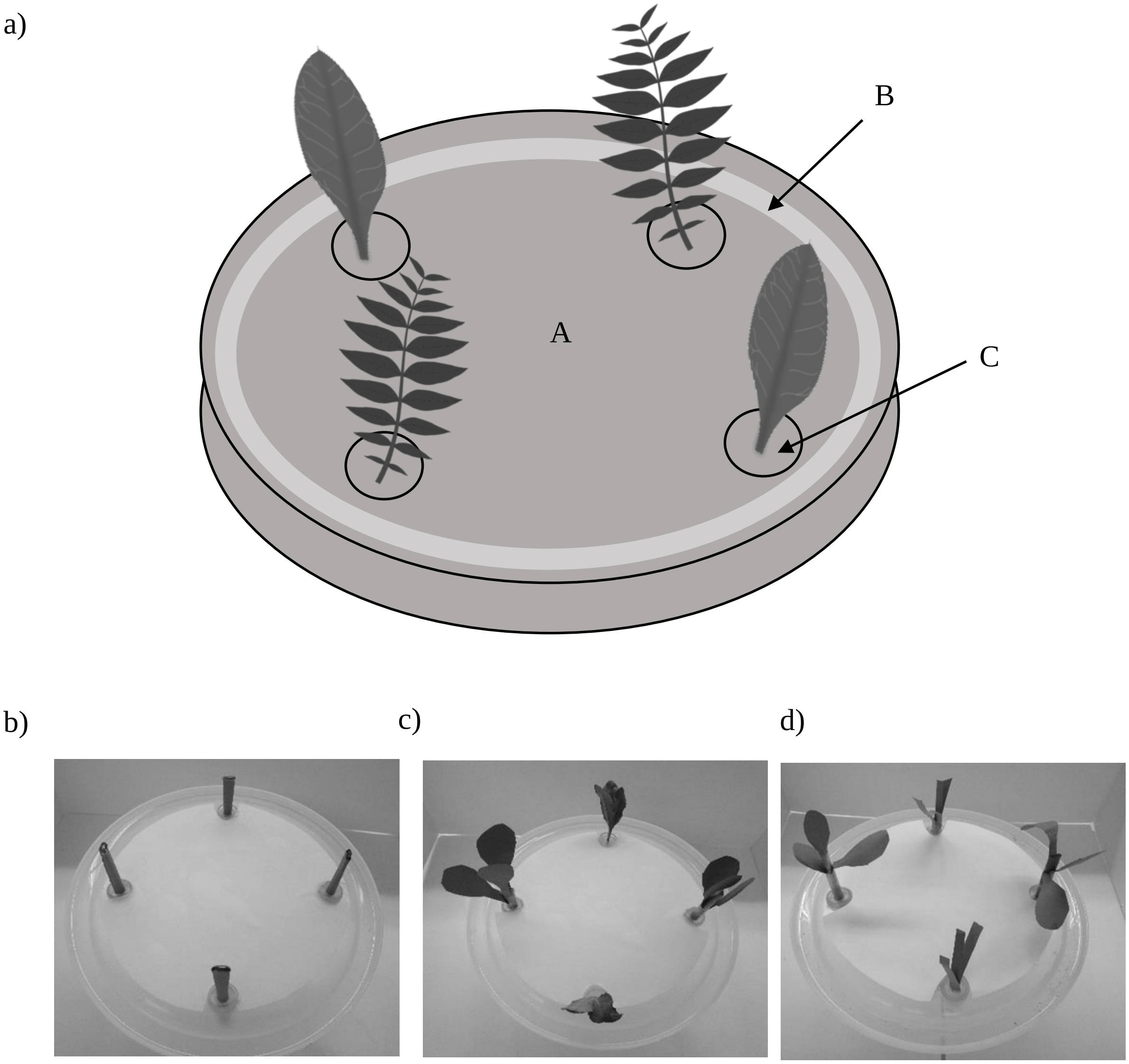


Figure 4.1: a) Walking arena schematic containing two different plant species. A: plastic walking arena, B: water moat, C: top of water pick, b) paper sticks, c) *L. draba* petioles with attached leaves and plant models, d) grass plant models and plant models.

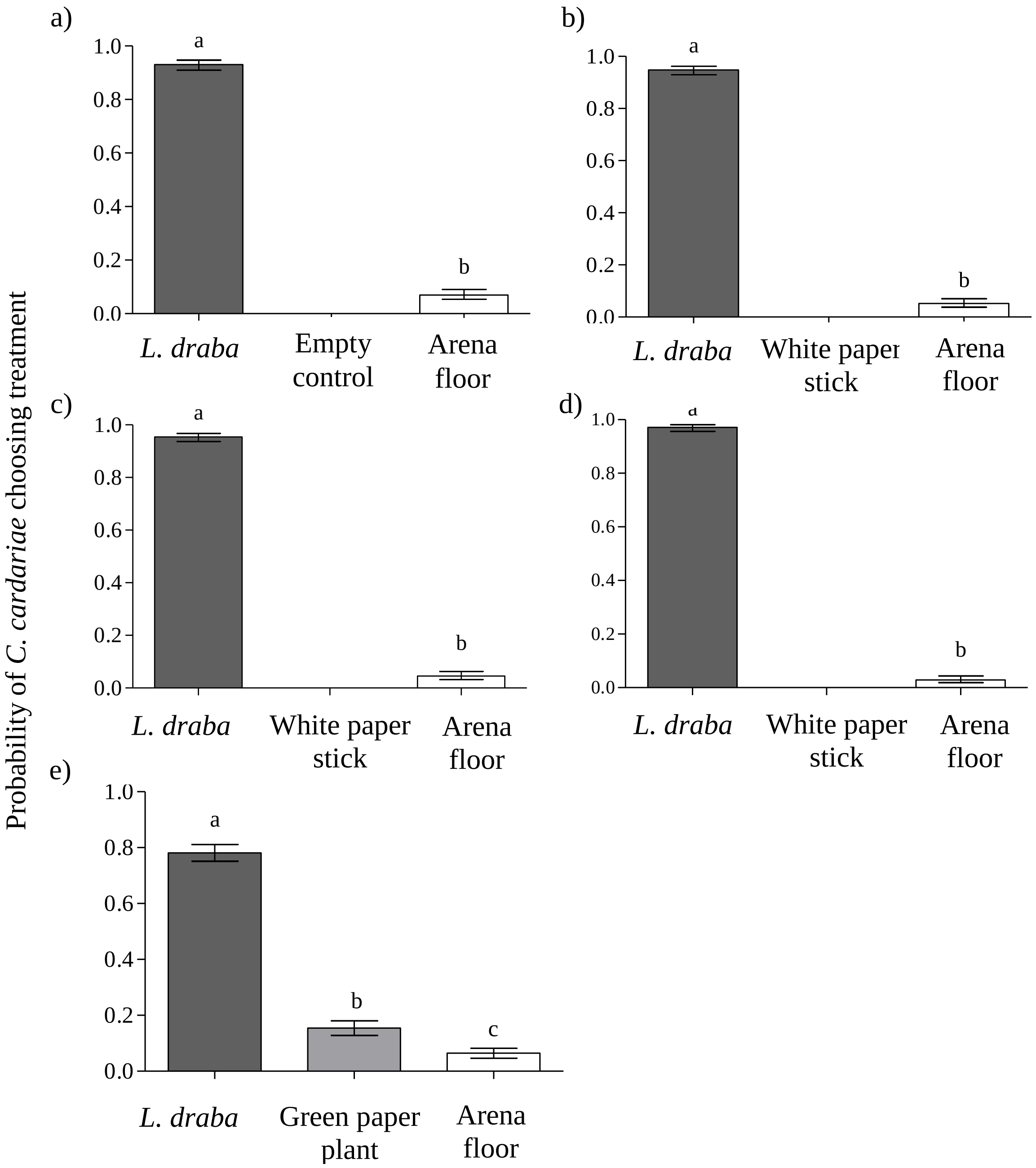
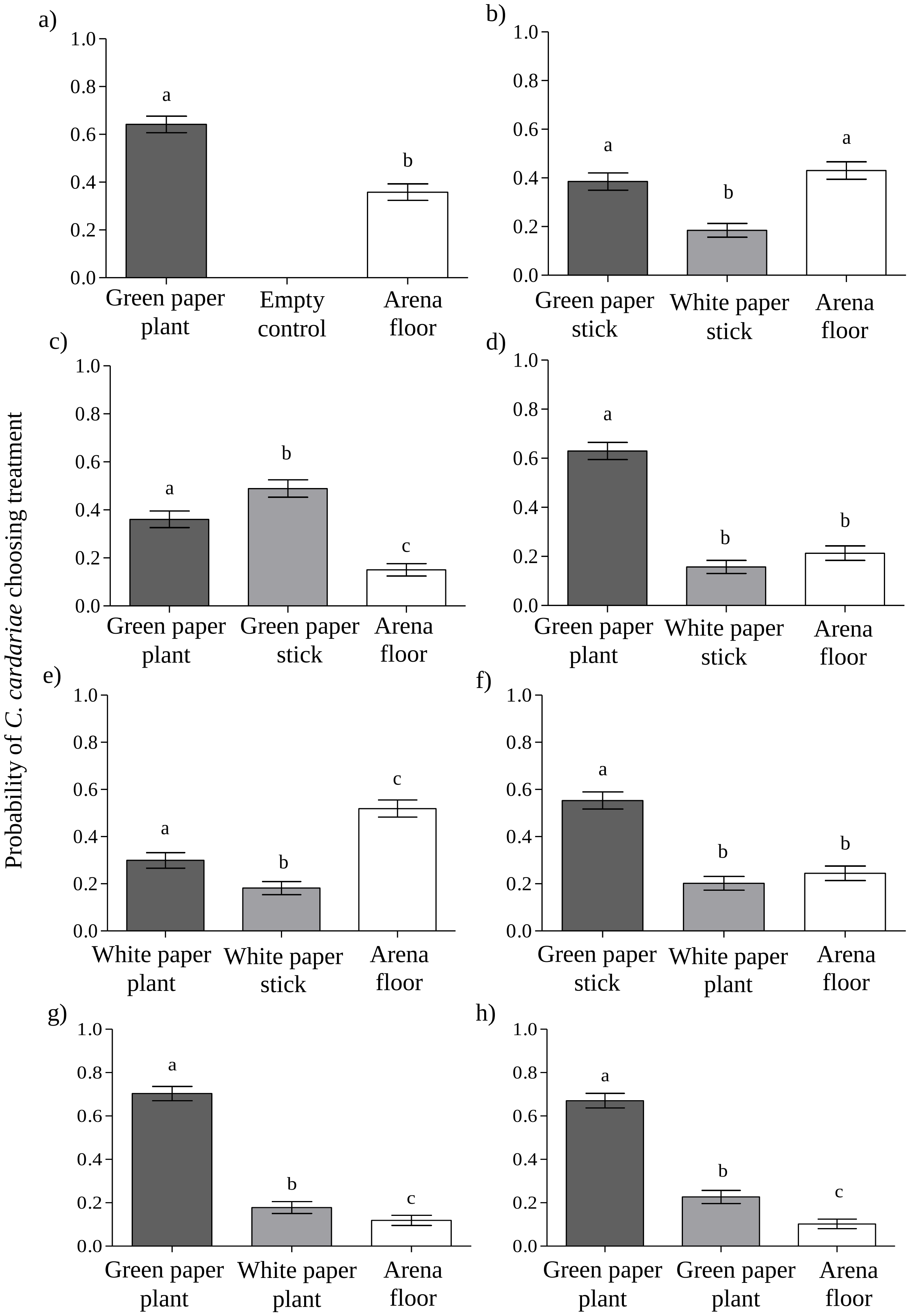


Figure 4.2: Responses of *C. cardariae* in bioassays testing different controls and simplified models: a) *Lepidium draba* versus three empty openings, b) *Lepidium draba* versus white paper sticks (12 individual weevils), c) *Lepidium draba* versus white paper sticks (un-excised leaves), d) *Lepidium draba* versus white paper sticks, and e) *Lepidium draba* versus green paper plant models. Mean (± CI) for each treatment are depicted. Differing letters on top of bars denote significant differences between those treatments (P<0.05).


Probability of *C. cardariae* choosing treatment
a)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
Green paper plant
Empty control
Arena floor
b)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
a
Green paper stick
White paper stick
Arena floor
c)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
Green paper plant
Green paper stick
Arena floor
d)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
Green paper plant
White paper stick
Arena floor
e)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
White paper plant
White paper stick
Arena floor
f)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
Green paper stick
White paper plant
Arena floor
g)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
Green paper plant
White paper plant
Arena floor
h)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
Green paper plant
Green paper plant
Arena floor

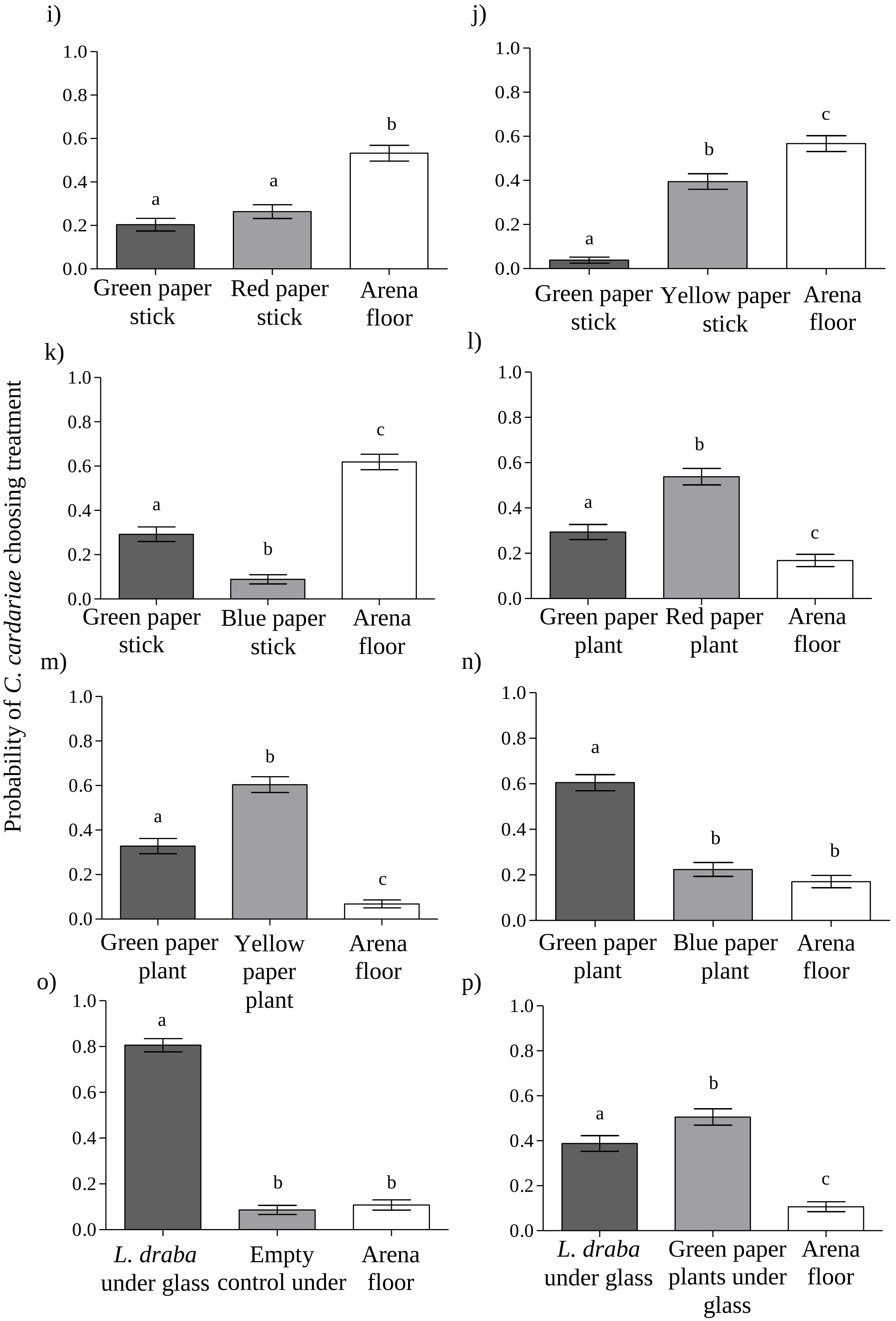

Probability of *C. cardariae* choosing treatment
i)
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
Green paper stick
Red paper stick
Arena floor
j)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
Green paper stick
Yellow paper stick
Arena floor
k)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
Green paper stick
Blue paper stick
Arena floor
l)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
Green paper plant
Red paper plant
Arena floor
m)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
Green paper plant
Yellow paper plant
Arena floor
n)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
Green paper plant
Blue paper plant
Arena floor
o)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
L. draba under glass
Empty control under
Arena floor
p)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
L. draba under glass
Green paper plants under glass
Arena floor

Figure 4.3: Responses of *C. cardariae* in bioassays testing visual cues of plant models and *Lepidium draba*. a) Green paper plants versus three empty controls, b) Green paper sticks versus white paper sticks, c) Green paper plants versus green paper sticks, d) Green paper plants versus white paper sticks, e) White paper plants versus white paper sticks, f) Green paper sticks versus white paper plants, g) Green paper plants versus white paper plants, h) Green paper plants versus green paper grass, i) Green paper sticks versus red paper sticks, j) Green paper sticks versus yellow paper sticks, k) Green paper sticks versus blue paper sticks, l) Green paper plants versus red paper plants, m) Green paper plants versus yellow paper plants, n) Green paper plants versus blue paper sticks, o) Cuttings of *Lepidium draba* covered by glass cylinders versus empty glass cylinders, and p) Cuttings of *Lepidium draba* covered by glass cylinders versus green paper plants covered by glass cylinders. Mean (± CI) for each treatment are depicted. Differing letters on top of bars denote significant differences between those treatments based on (summarize statistical procedure used) ($P<0.05$).

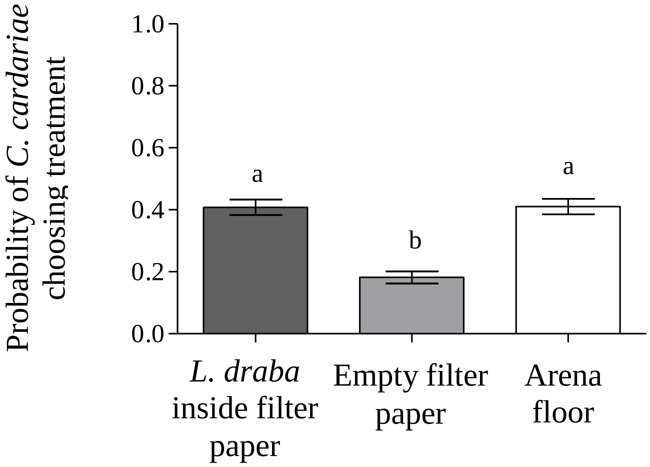


Figure 4.4: Responses of *C. cardariae* in bioassays testing the volatile cues of *Lepidium draba.* Cuttings of *Lepidium draba* wrapped in perforated filter paper tubes (preventing contact and visual cues being accessible to the weevils) versus empty perforated filter paper tubes. Mean (± CI) for each treatment are depicted. Differing letters on top of bars denote significant differences between those treatments (P<0.05).

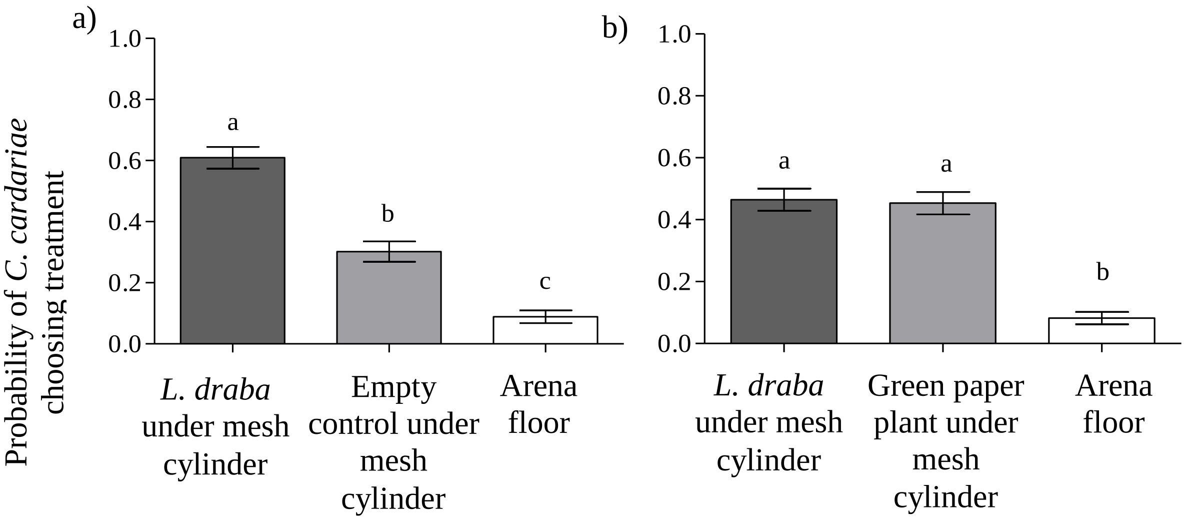


Figure 4.5: Responses of *C. cardariae* in bioassays testing the volatile and visual cues of *Lepidium draba.* a) *Lepidium draba* covered by mesh cylinders (allowing weevils to see and smell but not touch the treatments) versus empty mesh cylinders, and b) cuttings of *Lepidium draba* covered by mesh cylinders versus green paper plants covered by mesh cylinders. Mean (± CI) for each treatment are depicted. Differing letters on top of bars denote significant differences between those treatments ($P<0.05$).

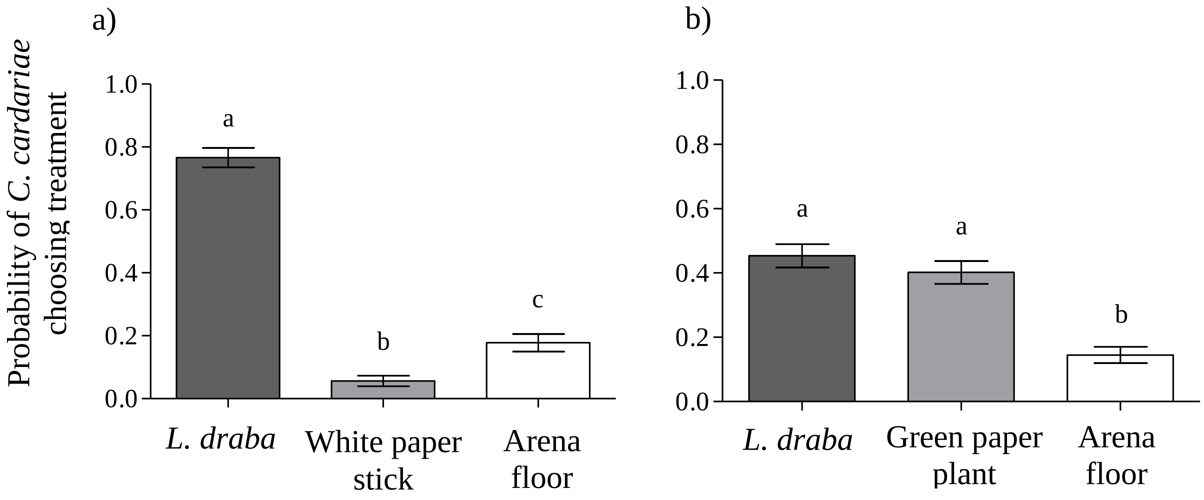


Figure 4.6: Responses of antennectomized *C. cardariae* in bioassays testing visual and contact cues of *Lepidium draba*. a) *Lepidium draba* versus white paper sticks, and b) *Lepidium draba* versus green paper plants. Mean (± CI) for each treatment are depicted. Differing letters on top of bars denote significant differences between those treatments ($P<0.05$).

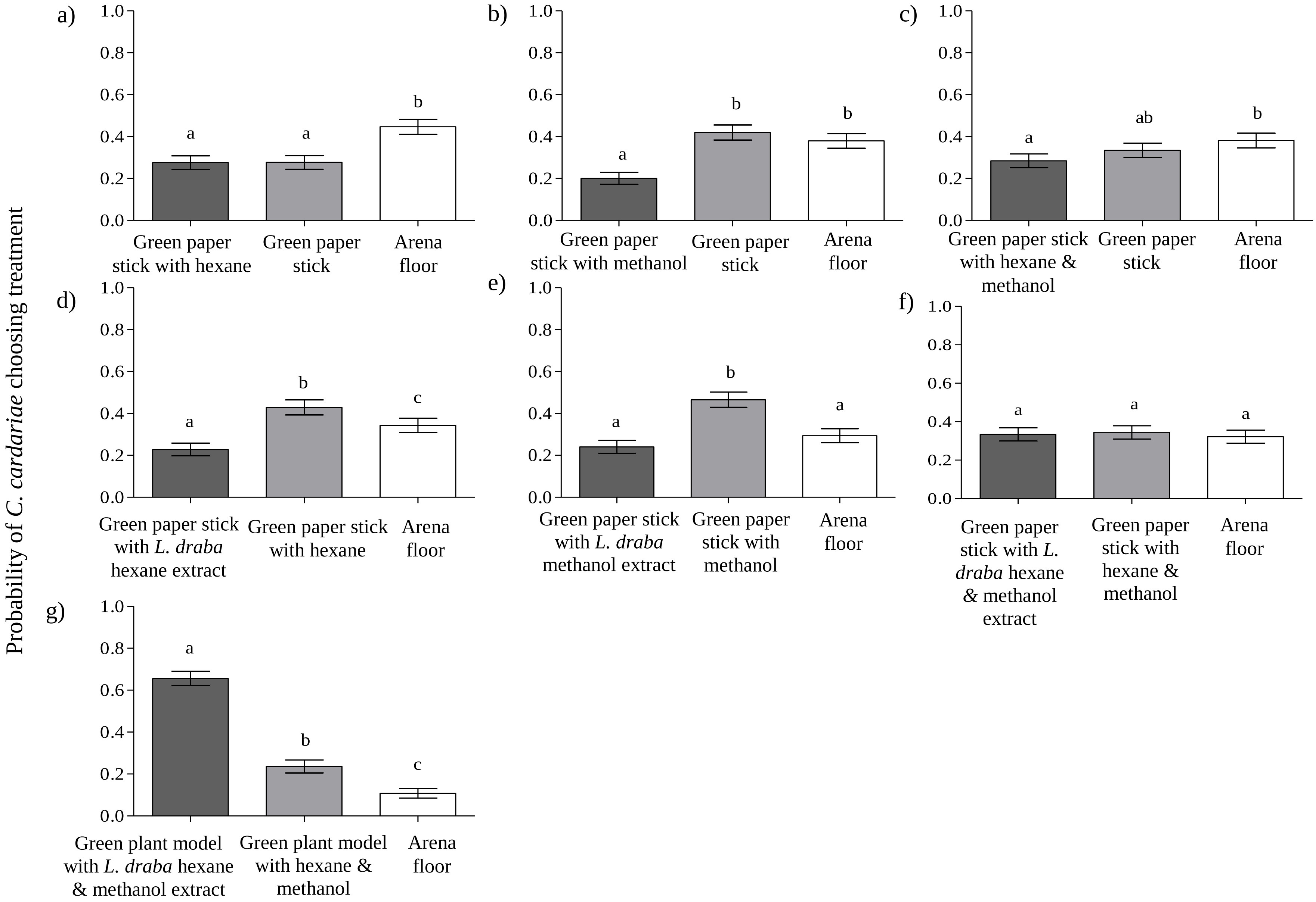

Probability of *C. cardariae* choosing treatment
a)
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
Green paper stick with hexane
Green paper stick
Arena floor
b)
a
b
b
Green paper stick with methanol
Green paper stick
Arena floor
c)
a
ab
b
Green paper stick with hexane & methanol
Green paper stick
Arena floor
d)
a
b
c
Green paper stick with *L. draba* hexane extract
Green paper stick with hexane
Arena floor
e)
a
b
a
Green paper stick with *L. draba* methanol extract
Green paper stick with methanol
Arena floor
f)
a
a
a
Green paper stick with *L. draba* hexane & methanol extract
Green paper stick with hexane & methanol
Arena floor
g)
a
b
c
Green plant model with *L. draba* hexane & methanol extract
Green plant model with hexane & methanol
Arena floor

Figure 4.7: Responses of *C. cardariae* in bioassays testing surface wax cues of *Lepidium draba.* a) Green paper sticks that had been dipped into hexane versus green paper sticks, b) Green paper sticks that had been dipped into methanol versus green paper sticks, c) Green paper sticks that had been dipped into 1:1 hexane and methanol versus green paper sticks, d) *Lepidium draba* hexane extract applied to green paper sticks versus green paper sticks dipped into hexane, e) *Lepidium draba* methanol extract applied to green paper sticks versus green paper sticks dipped into methanol, f) *Lepidium draba* 1:1 hexane and methanol extract applied to green paper sticks versus green paper sticks dipped into 1:1 hexane and methanol, and g) *Lepidium draba* 1:1 hexane and methanol extract applied to green paper plants versus green paper plants dipped into 1:1 hexane and methanol. Mean (± CI) for each treatment are depicted. Differing letters on top of bars denote significant differences between those treatments ($P<0.05$).

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Chapter 5

IDENTIFICATION OF FOLIAR VOLATILE ORGANIC COMPOUNDS IN THE HEADSPACE OF *LEPIDIUM DRABA* AND 13 CONFAMILIAL NATIVE NORTH AMERICAN PLANT SPECIES

Abstract

Previous host-specificity testing with the weevil *Ceutorhynchus cardariae,* a classical biological control candidate for the invasive plant *Lepidium draba,* indicated that it is a host-specific specialist under field conditions, but that its fundamental host range includes relatively distantly related confamilial species in the endemic North American genus *Streptanthus*. Pre-release host-specificity testing of weed biological control agents can demonstrate well which plants may be at risk of attack but not *why* they will be attacked. Previous work has indicated that unidentified volatile organic compounds (VOCs) from *L. draba* play a role in the weevils' host selection. Identifying the volatiles that may be influencing the host selection of *C. cardariae,* is a first step towards a better understanding of whether a plant species that could support the development of the weevil would actually be attacked post-release. Here we analysed the similarity of the VOCs from four different *L. draba* populations (closely related genetic populations) and 13 native North American confamilial species and assessed whether the similarity of the VOCs from the test plant species was related to their level of attack observed in host-specificity testing. VOCs were collected from greenhouse propagated plants using a portable volatile collection system. A total of 46 VOCs were identified across all plant species using GC-MS but the volatile blends could not be differentiated among plant species using principal component analyses (PCA). PCA among volatile blends of the four populations of *L. draba, S. anceps,* and *S. flavescens*

only, showed slight separation among the populations and species. Follow-up experiments using gas chromatography-flame ionization detector-electroantennogram detection (GC-FID/EAD) to measure antennal responses in female *C. cardariae* when exposed to volatile blends of *L. draba* did not yield any consistent responses.

Introduction

Classical biological control of weeds relies on the use of sufficiently host-specific specialist herbivore organisms to avoid post-release non-target attack (Heard, 1999; Louda et al., 2003; Sheppard et al., 2005; Suckling & Sforza, 2014). Conventionally, the risk is assessed pre-release through host-specificity testing of candidates using no-choice or choice tests, under differing conditions such as confined plants or under open field conditions. These tests can assess feeding, oviposition and larval development of a candidate species on a set of confamilial plant species ranging from very closely to distantly related to the targeted invasive plant species (Briese, 2005; Schaffner, 2001; Sheppard et al., 2005). Host-specificity tests determine a biological control agent candidate’s fundamental and ecological host range. The fundamental host range has been defined as the range of plant species that are capable of supporting a biological control agent’s full life cycle (Van Klinken, 1999) and is typically assessed through no-choice tests. As no-choice tests are generally performed by confining the insect in a cage with one plant, they are only able to act out a subset of their full sequence of host selection behaviors. The ecological host range is typically a subset of the fundamental host range. It is assessed in series of field tests in which the biological control agent candidates can choose between plant species and express their host choice behaviors. It therefore comprises plant species that have passed through species-specific behavioral and ecological filters (Nechols et al., 1992; Schaffner, 2001; Schaffner et al., 2018; Van Klinken, 1999; Wapshere, 1989). Open field tests are an important component of the assessment of the ecological host range because it allows the insects to also display the full sequence of host selection behavior during the pre-alightment phase, which is primarily triggered by volatile and visual plant cues (Briese, 1999; Clement & Cristofaro, 1995; Schaffner, 2001; Schaffner et al., 2018; Van Klinken, 1999; Wapshere, 1989). Yet, there are often discrepancies between the no-choice and open field host range testing results (Schaffner et al., 2018). The fundamental host range, as determined by no-choice tests, is often broader than what is observed in the open field tests, which are more indicative of the ecological host range (Schaffner, 2001). These differences can lead to uncertainty as to whether certain plant species may or may not be attacked after the release of the agent (McFadyen et al., 2002).

The host selection behavior of an insect herbivore can be divided into three phases: finding, examining, and accepting (Bernays & Chapman, 1994; Miller & Strickler, 1984). Finding takes place prior to contact with the plant, and examining and acceptance take place following contact. Acceptance or rejection of a potential host plant during the finding and examining phases depends on the insects’ response to visual, volatile, contact, and gustatory plant cues (Bernays & Chapman, 1994; Dethier, 1982; Heard, 1999; Miller & Strickler, 1984; Wheeler & Schaffner, 2013). These stimuli mediate behavioral and physiological patterns of feeding and oviposition (Beck, 1965; Jermy, 1966; Marohasy, 1998; Miller & Strickler, 1984; Thorsteinson, 1960). As acceptance or rejection of a potential host plant during the finding and examining phases depends on the insects’ response to these different cues (Bernays & Chapman, 1994), examining this behavior in detail may lead to a better understanding of how and why certain plant species are attacked.

Test plant species are traditionally selected according to the centrifugal phylogenetic method, which assumes that more closely related plant species to the target weed are also more likely to support development of a biological control candidate compared to more distantly related plant species (Wapshere, 1974; 1989). While the centrifugal phylogenetic approach has been the standard practice for assessing a biological control agent's likelihood of non-target attack (Harris & Zwölfer, 1968; Wapshere, 1974; 1989), it does not assess the physiological or behavioral processes involved in the host choice of a biological control candidate (Louda et al., 2003; Marohasy, 1998; Rapo, 2012; Smith & Beck, 2013; 2015). If specialist herbivores are relying on specific secondary plant metabolites for their host selection behavior, then chemical similarity may be a better predictor of host utilization than strictly phylogenetic relationships (Becerra & Venable, 1999; Wheeler & Schaffner, 2013). If a specific cue or cue modality is shared between the target plant and related species, this could indicate that the non-target species is vulnerable to attack in the field.

The Eurasian clonal perennial mustard *Lepidium draba* (L.) Desv. (Brassicaceae: Lepidieae) which is invasive in western North America, and one of its biological control candidates, the stem gall-forming weevil *Ceutorhynchus cardariae* Korotyaev (Coleoptera: Curculionidae), offer an interesting system to study the potential host selection cues in a biological weed control system. Host-specificity testing data has demonstrated a high level of specialization of *C. cardariae*, but two distantly related plant species in the tribe Thelypodieae, *Streptanthus anceps* (Payson) Hoover and *Streptanthus flavescens* Hook, were also attacked to a certain extent under open-field conditions (Weyl et al., 2018). Thus, *C. cardariae* exhibits a 'phylogenetically disjunct' fundamental host range.

In behavioral studies of the pre- and post-alightment behavior of *C. cardariae* where weevils were offered an array of plant models in a walking arena, it was found that female *C. cardariae* utilize volatiles from *L. draba* in combination with visual and contact cues during host selection (see Chapter 4). Volatiles have been a determining factor in many herbivorous insects' host plant preferences (Bernays & Chapman, 1994; Bruce & Pickett, 2011; Bruce et al., 2005) and several weevil species in the *Ceutorhynchini* tribe are attracted to olfactory cues (Bartlet et al., 1993; Blight et al., 1995; Free & Williams, 1978; Kafle, 2017; Park et al., 2018; Tansey et al., 2010).

A comparison of the volatile profiles of the plant species tested in the host-specificity tests may reveal volatiles that are shared between *L. draba, S. anceps,* and *S. flavescens* but not the other non-target species, helping to explain *C. cardariae's* host selection behavior. Here we report volatile profiles for four populations of *L. draba* and 13 confamilial native North American plant species (previously used in host-specificity testing) that were selected based on *C. cardariae* attack levels, as well as our attempt to identify biologically active volatiles for *C. cardariae*, i.e., triggering a physiological response of the weevil's antenna. Our specific objectives were to (1) compare and contrast similarities or dissimilarities of volatile profiles between plant species with emphasis on *S. anceps* and *S. flavescens,* and to (2) determine whether there are specific volatiles associated with the host selection behavior of *C. cardariae.* We hypothesized that the volatile profile of *L. draba* may be more similar to that of *S. anceps* and *S. flavescens* when compared to the other 11 plant species. We further hypothesized that specific biologically active volatiles are present in *L. draba.*

Materials and methods

Study system and materials

Lepidium draba is a clonal perennial mustard native to Eurasia that can invade open, disturbed, and degraded habitats, such as croplands, pastures, rangelands, and riparian areas, and form dense monospecific stands in North America (Scurfield, 1962). It is a declared noxious weed in 15 US states and three Canadian provinces (USDA-NRCS, 2018). *Ceutorhynchus cardariae* is a petiole and stem galling weevil native to Europe and Asia (Korotyaev, 1992; Hinz & Diaconu, 2015). In its native range *C. cardariae* has only been found on *L. draba* and on rare occasions on the closely related *Lepidium campestre* (L.) (Francis & Warwick, 2008; Hinz & Diaconu, 2015; Korotyaev, 1992). Females lay eggs in early spring into stems, leaf stalks, and midribs of L. draba rosettes and bolting plants, which is thought to cause the formation of galls (Hinz & Diaconu, 2015). Larvae of *C. cardariae* hatch and then feed on the parenchymatic tissue of developing galls, which can stunt or even kill shoots (Hinz & Diaconu, 2015). The host-specificity of *C. cardariae* has been studied at CABI Switzerland since 2003. Testing methodologies included no-choice and multiple-choice tests under confined potted plant and open field test conditions. A total of 156 test plant species, over half of which are native to North America, were tested for non-target attack including feeding, gall development, and adult emergence (Weyl et al., 2018). Results of the host-specificity testing indicate that *C. cardariae* has a fundamental host range that partially includes species in the tribes Lepidieae, Cardamineae, and Thelypodieae. In contrast, the ecological host range of *C. cardariae* is narrower and includes only few species (Weyl et al., 2018). When using the relative performance threshold based on results of laboratory tests to predict non-target attack post-release for New Zealand (Paynter et al., 2015), four native non-target species were above the predicted relative threshold for non-target attack (Weyl et al., 2018). These included two species in the tribe Lepidieae: *Lepidium oblonglum* Small *and Lepidium paysonii* Rollins, and two in the tribe Thelypodieae: *Streptanthus anceps* (Payson) Hoover and *Streptanthus flavescens* Hook. (Weyl et al., 2018). The Brassicaceae family is separated into three distinct lineages[1], (Beilstein et al., 2006), with the Cardamineae and the Lepidieae tribe belonging to lineage I, and the Thelypodieae belonging to lineage II (Kiefer et al., 2014). Thus, attack on the two *Streptanthus* species does not fit the assumption underlying the centrifugal phylogenetic method, i.e. that species closely related to the target weed are more likely to be attacked than more distantly related species (Weyl et al., 2018) and results in *C. cardariae* exhibiting a 'phylogenetically disjunct' host range.

Approximately 400 female adult *C. cardariae* were transferred from a rearing colony at CABI Switzerland to the Northwestern Biocontrol Insectary and Quarantine (NWBIQ) at Washington State University, Pullman, WA in winter of 2015. Weevils were separated into groups of 10 and were placed in transparent plastic cylinders (15 cm height, 11 cm in diameter) covered with a mesh lid. Cylinders were kept in an environmental chamber (Percival Scientific Model E-30BC, Boone, IA) set to spring conditions: L10: D14, at 12 °C during light and 2.5 °C during darkness, and 75 % relative humidity. These environmental conditions correspond with the conditions in the native range at the time at which females lay eggs. Weevils were fed foliage of *L. draba* as needed. All weevils were maintained under these environmental conditions until experimentation.

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[1] Lineages were determined using the chloroplast gene *ndhF*. Using parsimony, likelihood, and Bayesian methods, Beilstein et al., (2006) reconstructed the phylogeny of the gene. The genera were then able to be grouped into monophyletic groups called lineages.

Specific populations of *L. draba* plants were maintained at the Parker Research Farm since 2007. Populations had been identified as closely related genetic populations based on amplified fragment length polymorphism (AFLP) analysis (J.F. Gaskin, unpublished data; Puliafico, 2008). Four populations were utilized here: *L. draba* population A that was first collected from Nevada, population D from Montana, Population E from Oregon, and Population F from Washington (Puliafico, 2008). From these plants, root cuttings were collected, propagated, and maintained in an environmentally controlled greenhouse at the University of Idaho's Parker Research Farm, Moscow, ID. Plants were potted in 3-liter black plastic pots (15 cm in diameter and 17 cm tall) and filled with a soil mixture consisting of Sunshine Professional Growing Mix #1 (SunGrow Horticulture, Agawam, MA), top soil with added trace elements (FRIT Industries, Inc., Ozark, AL), chelated iron 10% (Grow More Inc., Gardena, CA), and fertilizers (Bonide Triple Super Phosphate, Bonide Products Inc., Oriskany, NY; Osmocote Flower and Vegetable Fertilizer, (The Scotts Company LLC., Marysville, OH). Plants were kept at 24 °C day, 13 °C night, and a 15-hour photoperiod.

Test plants included 13 native North American Brassicaceae species from the Lepidieae and Thelypodieae tribes. These plant species were selected in such a way so three different attack levels that were observed in *C. cardariae* host-specificity testing would be represented within each tribe (Table 5.1). Seeds from *Lepidium crenatum, Lepidium latipes, Lepidium oblongum, Lepidium papilliferum, Stanleya pinnata, Stanleya viridiflora, Streptanthus anceps, Streptanthus crassicaulis, Streptanthus farnsworthianus, Streptanthus flavescens, Streptanthus glandulosus, Streptanthus inflatus,* and *Streptanthus niger* were provided by CABI Switzerland, the Rancho Santa Ana Botanical Gardens in Santa Ana, CA, the Chicago Botanical Gardens in Glencoe, IL, the USDA National Plant Germplasm System in Beltsville, MD, and from Seedhunt in Freedom, CA (Table 5.2).

Foliar volatile collection, identification, and analysis

To collect foliar volatiles, polyvinyl acetate bags (Reynolds Consumer Products LLC., Richmond, VA) were cut into 15 cm x 15 cm bags and sealed on three sides with a vacuum sealer. The bags were sterilized in a drying oven at 180 °C for three hours. Forty mg Porapak Q volatile collection traps (Southern Scientific Inc. Micanopy, FL) were rinsed with 30 ml of dichloromethane and then dried overnight under a vacuum hood. Volatiles of the four populations of *L. draba* and the 13 native North American Brassicaceae species were collected at the University of Idaho's Parker Research Farm, Moscow, ID using a portable volatile collection system (PVCS) (Park et al., 2018) (Fig. 5.1a-b). Leaf stems of each plant species were covered with the polyvinyl bag and the bottom open end was sealed with a cotton ball and a cable tie. A Rena 400 pump (RENA, Chalfont, PA, Rena® Air 400, Mars Fishcare North America, Inc., Chalfont, PA) was used for headspace volatile collection. Two holes were cut into opposite sides of the top of the bag. The inlet port of the pump was connected to an activated charcoal filter (Orbo™, Sulpelco, Sigma-Aldrich Co. LLC, St. Louis, MO) to remove contaminants and secured to one of the top holes. The outlet port of the pump was connected to the 40 mg Porapak Q volatile collection trap and secured to the other top hole of the bag. Both ports were set to the same air pressure using flowmeters (470 ml per min^{-1}, Clack Solutions, Hudson, MA). When collecting volatiles, a full spectrum florescent light bulb illuminated the plants. For each plant species, volatiles from three plants along with an empty control were collected for six hours. 200 µl of dichloromethane (Sigma-

Aldrich Co. LLC, St. Louis, MO) were then used to elute each Porapak Q trap into a 2 ml clear GC vial (National C5000-180, Thermo Fisher Scientific Inc., Rockwood, TN). The eluted volatile samples were transported in a portable cooler, and kept in a freezer at -20 °C until further use.

To identify the headspace volatiles of the four populations of *L. draba* and the 13 native North American Brassicaceae species, an Agilent 7890 gas chromatograph (Agilent Technologies Inc., Santa Clara, CA) was coupled with a Hewlett-Packard 5973 Mass Selective Detector (Agilent Technologies Inc., Santa Clara, CA). The injector port was heated to 250 °C and 1 μL of volatile extract was injected in splitless mode onto a fused silica HP-5MS capillary column (30 m×0.25 mm×0.25 μm, Agilent Technologies Inc., Santa Clara, CA). The carrier gas was helium. The initial oven temperature was set at 40 °C, held for 1 minute, and then increased to 200 °C at a rate of 5 °C per minute. It was then further increased to 300 °C at a rate of 10 °C per minute and held at that temperature for 2 minutes. Total run time was 45 minutes. Subsequent volatile compounds were tentatively identified by comparing their fragmentation pattern in the NIST library database (National Institute of Standards and Technology, Gaithersburg, MD) and standardized retention indices. The relative amount of identified volatiles was determined based on peak area normalization of total ion concentration (TIC). Only compounds with a relative peak area of 0.1 % or more of the total peak area in at least 2 of the 3 samples were selected. The volatiles that were detected in both plant and control samples were regarded as contaminants and discarded. Volatiles from each plant species were collected and averaged across three individual plants, except for *L. draba* population D, *S. viridiflora*, *S. anceps*, and *S. niger*, from which there were only two viable collections made. The third individual of *L. draba* population D, *S.*

viridiflora, and *S. anceps,* only contained 3 volatiles and the third individual of *S. niger* contained only one volatile; none of these were shared with the other two individuals of the same plant species.

Two principal component analyses (PCA) were performed using the statistical software package SAS (Version 9.4; SAS Institute Inc., 2013) to differentiate volatile profiles of tested plant species based on the volatiles identified by the GC-MS. One PCA was performed using all plant species, and another only analyzing the four populations of *L. draba* and the two *Streptanthus* species *S. anceps* and *S. flavescens*.

GC-FID/EAD

In order to detect electro-physiologically active volatiles, the headspace volatiles of the four populations of *L. draba* and 20 female *C. cardariae* were subjected to coupled gas chromatography-flame ionization detection and electroantennogram detection (GC-FID/EAD). For antennal preparation, female weevils were decapitated and one antennal tip was cut using a scalpel blade and microdissection kit. The basal portion of the head was placed over a reference electrode and the antenna was connected to the recording electrode. The base of the head and antennal tip were partly submerged in Spectra® 360 electrode gel (Parker Laboratories Inc, Fairfield, NJ) (Fig 5.2). 1 µL of volatile extract was injected in the splitless mode of an Agilent 6890N GC equipped with a capillary column (30 m×0.25 mm×0.25 cm, Agilent Technologies Inc., Santa Clara, CA) with a column splitter. The temperature of injector was 250 °C and helium was used as the carrier gas. The initial oven temperature was set to 40 °C and held for 1 minute. It was then increased to 200 °C at a rate of 5 °C per minute, and then further increased to 300 °C at a rate of 10 °C per minute and held at that temperature for 2 minutes. Total run time was 45 minutes. The effluent from the column was split into two parts and transferred to FID and to the EAD in a ratio of 1:1. The column of EAD outlet was introduced into a 5 mm diameter glass tube with a constant stream of purified and humidified air generated with a stimulus controller (CS-05; Syntech, Hilversum, The Netherlands). Mounted antennae were placed 0.5 cm away from the end of glass tube. The signals from the antennae were detected through the EAG probe (Syntech, Hilversum, The Netherlands) and processed with a data acquisition controller (IDAC-232, Syntech, Hilversum, The Netherlands). The GC-EAD recordings were analyzed using GC-EAD2000 software (Syntech, Hilversum, The Netherlands). Antennal responses to volatiles were reviewed and compounds that elicited a response in at least 5 of the 20 recordings were considered electrophysiologically active.

Results

A total of 46 volatiles were identified in the headspace of *L. draba* and the 13 confamilial Brassicaceae species (Table 5.3). These included 13 monoterpenes, three alcohols, three sesquiterpenes, one isothiocyanate, one nitrile, seven aldehydes, nine esters, four hydrocarbons, two phenols, one furan, one ketone, and one imine. *Stanleya viridiflora* had the most volatiles with 18, and *Streptanthus niger* had the fewest volatiles with 1 (Table 5.3). The species in the genus *Lepidium,* as a whole (excluding all *L. draba* populations), shared 15 volatiles with the *L. draba* populations, the genus *Stanleya* shared 11 volatiles with the *L. draba* populations, and the genus *Streptanthus* shared six volatiles with the *L. draba* populations (Table 5.3). All volatiles found in individuals of *L. draba* populations were also found in at least one confamilial plant species, except for 2- phenethyl acetate, which was unique to *L. draba* population E. Four volatiles were shared between at least one *L. draba* population and either *S. anceps* or *S. flavescens* (Table 5.3).

The principal component analysis using volatile profiles of all plant species used in this study did not differentiate plant species, as only 33.58% of the variation was explained (PC1= 19.46, PC2= 14.12) (Fig. 5.3a-b). The principal component analysis using the four populations of *L. draba*, *S. anceps,* and *S. flavescens* showed moderate separation of plant species based on volatile profiles with 59.02% of the variation explained (PC1= 36.80, PC2= 22.22) (Fig. 5.4). *Lepidium draba* population D was separated from the three other *L. draba* populations and the two *Streptanthus* species (Fig. 5.4). Of the 20 volatiles identified in *L. draba* headspace samples, none elicited consistent EAD responses in female *C. cardariae* and other potential antennal responses were inconsistent among plant replicates, and thus could not be categorized as biologically active.

Discussion

Brassicaceae volatiles can act as attractants or repellents for many insect species (Heil, 2014; Mitchell, 1994; Moyes & Raybould, 2001; Tansey et al., 2010; Visser, 1986). Working from the premise that volatiles are also an important influence on *C. cardariae's* host selection behavior, we expected to find a higher degree of separation between *L. draba* populations and of the confamilial plant species in the PCA, and perhaps clustering of plant species based on the observed *C. cardariae* attack level. We also expected that the volatile profiles of *L. draba* would be more similar to those of *S. anceps* and *S. flavescens* than the other non-target species. However, results of the two PCA's do not indicate these expectations. Additionally, in the second PCA, *L. draba* population D was surprisingly separated from the other three *L. draba* populations. As they are the same species, it would be expected that all populations of *L. draba* are very similar to one another. This separation appears to be due to the fact that volatiles could only be measured for two plant individuals of *L. draba* population D instead of three.

While many of the specific volatiles found in the headspace of the plant species used for this study have been found to act as attractants and/or repellents for other insect species (Gruber et al., 2009; Reddy & Guerrero, 2000; Rojas, 1999; Smart & Blight, 1997; Smid et al., 2002), here we were not able to observe consistent electrophysiological responses in the antennae of *C. cardariae* to *L. draba* foliar volatiles. Based on the two PCA results and without identifying any bioactive volatiles, one would not necessarily expect to detect differences among the blends using multivariate methods. If there are specific bioactive compounds, or a blend of bioactive compounds shared between *L. draba* and non-target species, including *S. anceps* and *S. flavescens*, it may be that additional visual or contact cues are more important for *C. cardariae's* host selection behavior. Weevils may integrate information from several cue modalities during their response to VOCs (Andreas et al., 2008; Park et al., 2018). This scenario is likely for *C. cardariae* based on series of bioassays conducted in a walking arena, which indicated that *C. cardariae* may only respond to volatile cues when in contact with its host plant (see Chapter 4).

Two other host specific weevils in the *Ceutorhynchini* tribe - *Mogulones borraginis* (F) and *Mogulones crucifer* (Pallas), along with their host plant, *Cynoglossum officinale* (L) - offer interesting systems to contrast the *C. cardariae- L. draba* system presented here. Both *Mogulones* weevils respond to bioactive volatiles found in their host plant (ten and six respectively), and based on these bioactive volatiles, the host plant and confamilial plant species (four and eight respectively) showed a fair amount of separation under PCA (69.50% and 67.60% respectively) (Kafle, 2017; Park et al., 2018). However, a PCA was not conducted using all volatiles found within the plant species, only those that were bioactive. It would be informative to see this data set and compare and contrast to the data set here.

There could be several explanations for the lack of the antennal response in *C. cardariae*. First, the antennae of *C. cardariae* may truly not be sensitive to foliar volatiles of its field host. Second, weevil antennae could be sensitive to VOCs but the collected volatile samples used were insufficient to elicit antennal responses for an unknown reason, e.g. the time or length of volatile collection. Emission of plant VOC can vary throughout the photoperiod and maximum emission can occur either during day or night (Dudareva et al., 2004; Helsper et al., 1998; Jakobsen & Olsen, 1994; Kolosova et al., 2001). The lengths of volatile collection may also affect EAD results. We collected VOC for six hours, as this is a commonly used collection time for foliar volatiles studies (Kafle, 2017; Park et al., 2018) and a collection time of five hours has been used in a study on EAD responses of insects to Brassicaceae volatiles (Smid et al., 2002). However, in other studies longer VOC collection times of up to 24 hours were used successfully, likely to increase VOC concentrations in the traps (Fraser et al., 2003; Ngumbi et al., 2009; Proffit et al., 2011).

Other studies have found very low volatile concentrations in the headspace of undamaged plants (Finch, 1978; Rohloff & Bones, 2005; Tollsten & Bergström, 1988). In addition it has also been concluded that low VOC concentrations make it unlikely that herbivorous insects locate Brassicaceae host plants over large distances by olfaction alone (Städler & Reifenrath, 2009). The role of volatile isothiocyanates in long distance host-finding by Brassicaceae specialists varies, whereas glucosinolates seem to have a stimulatory effect when insects are in contact with the plant (Städler & Reifenrath, 2009). Pre-feeding behavioral and arena bioassay data indicate that foliar volatiles are at least partially important for the host location in *C. cardariae* (see Chapter 4). Weevils did exhibit distinctive pre-feeding behaviors on *L. draba:* prior to the onset of feeding, their antennae were dragged along or just above the leaf surface (see Chapter 3). It may be that for *C. cardariae,* the presence of volatiles concentrated directly above the leaf surface act synergistically with non-volatile compounds in the host plant and are assessed simultaneously while in contact with the plant. This may be supported by data that volatiles alone did not seem to be sufficient for host recognition by *C. cardariae,* but may act synergistically with contact cues (see Chapter 4). Another highly specialized insect, the tortoise beetle *Cassida canaliculata* (Laich.), which is strictly monophagous on meadow sage *Salvia pratensis* (L.), has also shown a very weak attraction to its host plant when only offered volatile and visual plant cues (Heisswolf et al., 2007). However, when contact cues were also offered to the beetles, a strong preference for its host plant over non-target plants and controls was observed (Heisswolf et al., 2007).

It may also be that in this study certain biotic or abiotic conditions, such as mycorrhizal fungi, plant competition, or nutrient availability did not replicate the conditions necessary for plants to emit volatiles in a way they would be encountered by *C. cardariae* under natural conditions (Fontana et al., 2009; Gouinguene & Turlings, 2002; Jallow et al., 2008; Kigathi et al., 2013; Sampedro et al., 2010). If this was the case, collecting volatiles in situ may be necessary.

While we could not detect any antennal response to foliar VOC of *L. draba, C. cardariae* may respond to VOC when the weevil is in physical contact with the plant. This would however require electrophysiological studies on responses of live *C. cardariae* weevils while being in physical contact with the leaf surface to investigate the influence of volatiles and contact cues synergistically. This type of study may be experimentally challenging, but may help in understating *C. cardariae’s* host selection behavior.

Table 5.1: The host plant, *L. draba*, and the 13 native North American species for which volatiles were profiled.

Tribe	Species	Level of attack designation and reasoning[1,2] (3- highest, 1-lowest)
Lepidieae	*Lepidium draba* (L)	3: host plant
	Lepidium crenatum (Greene) Rydb.	1: limited feeding
	Lepidium latipes Hook.	2: supported gall formation, but limited adult emergence
	Lepidium oblongum Small	2: supported limited adult development
	Lepidium papilliferum (L.F. Hend.) A. Nelson & J.F. Macbr.	1: hardly any feeding
Thelypodieae	*Stanleya pinnata* (Pursh) Britton	2: supported gall formation
	Stanleya viridiflora Nutt.	1: limited feeding
	Streptanthus anceps (Payson) Hoover	3: supported adult development
	Streptanthus crassicaulis Torr.	1: no feeding at all
	Streptanthus farnsworthianus J.T. Howell	1: limited feeding
	Streptanthus flavescens Hook.	3: supported adult development
	Streptanthus glandulosus ssp. glandulosus Hook.	2: supported limited adult development
	Streptanthus glandulosus ssp. niger Hook.	2: supported gall formation
	Streptanthus inflatus Greene	2: supported limited adult development

[1] Weyl et al., (2018)

[2] Hinz unpublished data.

Table 5.2: North American confamilial plant species and their seed sources.

Plant species	Seed source				
	CABI Switzerland	Rancho Santa Ana Botanical Gardens	Chicago Botanical Gardens	USDA National Plant Germplasm System	Seedhunt
Lepidium crenatum	x			x	
Lepidium latipes	x				
Lepidium oblongum	x		x		
Lepidium papilliferum	x				
Stanleya pinnata	x	x			
Stanleya viridiflora	x				
Streptanthus anceps		x			x
Streptanthus crassicaulis	x	x			
Streptanthus farnsworthianus	x	x			x
Streptanthus flavescens					x
Streptanthus glandulosus					x
Streptanthus niger	x				
Streptanthus inflatus		x			x

Table 5.3: Relative Total Ion Concentration (TIC) peak area percentage of volatile organic compounds collected from the headspace of plant species.[2]

Chemical Class	Volatile Organic Compound	LD-A	LD-D	LD-E	LD-F	LC	LL	LO	LP
Monoterpenes	α-Pyronene		0.40			0.22			
	β-Pinene		0.98		0.20	14.29			
	Limonene		1.23			0.11			
	Linalool			2.69					
	(Z)-β-Ocimene	1.42	1.00	7.05	3.81	19.50			
Alcohols	Hexan-1-ol					0.81			
	(Z)-3-Hexen-1-ol					0.82			
Sesquiterpenes	Caryophyllene			1.08	0.30				
	Farnesene	2.68			0.60	0.89			
	Nerolidol			1.06				0.27	
Isothiocyanates	Benzyl isocyanate						0.27		
Nitriles	2-Phenylacetonitrile					0.43	0.59		
Aldehydes	2-Phenylacetaldehyde						0.45		
	3,5-Ditert-butyl-4-hydroxybenzaldehyde						0.05	0.12	
	Benzaldehyde					0.91	2.74	0.20	
	Decanal						0.25		

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[2] Tentative identification of compounds was based on comparison of their mass-spectra in the NIST library. For most species, values are averaged across three individual plants. For *Lepidium draba* population D, *Stanleya viridiflora*, *Streptanthus anceps*, and *Streptanthus niger*, values are average across only two plants. The four *L. draba* populations are shaded in light grey. (a) Plant species, LD-A: *Lepidium draba* population A, LD-D: *Lepidium draba* population D, LD-E: *Lepidium draba* population E, LD-F: *Lepidium draba* population F, LC: *Lepidium crenatum*, LL: *Lepidium latipes*, LO: *Lepidium oblongum*, LP: *Lepidium papilliferum.* (b) SP: *Stanleya pinnata*, SV: *Stanleya viridiflora*, SA: *Streptanthus anceps*, SC: *Streptanthus crassicaulis*, SFA: *Streptanthus farnsworthianus*, SFL: *Streptanthus flavescens*, SGG: *Streptanthus glandulosus ssp. glandulosus*, SI: *Streptanthus inflatus*, SN: *Streptanthus niger*.

Table 5.3 continued.

Chemical Class	Volatile Organic Compound	LD-A	LD-D	LD-E	LD-F	LC	LL	LO	LP
Aldehydes	Nonanal						0.76		
	(Z)-2-Heptenal						0.31		
Esters	2-Phenethyl acetate			0.69					
	4-Hexen-1-ol acetate	0.59	2.60	0.80	2.47	1.63			
	δ-Valerolactone				0.43			0.26	
	Hexyl acetate	0.48	1.33		0.27	0.09			
	Methyl benzoate			0.44		0.21			
	(E)-3-Hexenyl butyrate					.73			
Hydrocarbons	1,2 (1,3) (1,4)-Xylene			0.46					
	2-Ethenyl-1,1-dimethyl-3-methylidenecyclohexane	3.90	3.90	20.16	22.57	25.74			
	2-Methylnaphthalene		0.74					0.20	1.92
	Naphthalene	0.19	0.86					0.21	0.72
Phenols	2,4-Ditert-butylphenol						1.51		
	2-Ethyl-5-propylphenol								
Furans	Denderalasin		4.06	16.04	3.49	3.79			
Ketones	1-(4-Ethylphenyl) ethanone			4.53					
Imines	Methyl (Z)-N-hydroxy-benzenecarboximidate								0.76
Total number of volatile compounds		6	10	12	10	16	9	6	3
Total number of volatile compounds shared with at least one *L. draba* population		6	6	6	9	10	0	4	2

Table 5.3 continued.

Chemical Class	Volatile Organic Compound	SP	SV	SA	SC	SFA	SFL	SI	SGG	SN
Monoterpenes	α-Pinene		1.95							
	α-Terpineol		0.28							
	β-Pinene				11.9					
	β-Thujene		0.81							
	δ-Terpinene		0.36							
	Eucalyptol		2.35							
	Limonene		20.1							
	Linalool	0.64	0.62							
	Perillen								0.82	
	Sabinene		14.9							
	(Z)-β-Ocimene		9.01							
	(Z)-β-Terpineol		0.26							
Alcohols	Cuminol	0.52								
	(Z)-3-Hexen-1-ol	0.41		0.42					3.02	17.8
Sesquiterpenes	Caryophyllene	3.18								
	Farnesene	0.25	0.51							
Aldehydes	Benzaldehyde		0.09							
	Nonanal								0.36	
	(E)-2-Decenal		0.10							
Esters	4-Hexen-1-ol acetate	6.99			4.90	19.3	13.2	4.14	0.77	
	5-Hexen-1-ol acetate			5.59		1.52				
	δ-Valerolactone			1.35	0.34				0.54	
	Hexyl acetate	0.42			0.23	0.40				
	Methyl salicylate		2.01							
	(E)-3-Hexenyl butyrate	0.26								
	(Z)-2-Penten-1-ol acetate	0.35								

Table 5.3 continued.

Chemical Class	Volatile Organic Compound	SP	SV	SA	SC	SFA	SFL	SI	SGG	SN
Hydrocarbons	1,2 (1,3) (1,4)-Xylene					0.97	0.73			
	2-Ethenyl-1,1-dimethyl-3-methylidenecyclohexane		11.3							
	2-Methylnaphthalene				0.84			0.55		
Phenols	2,4-Ditert-butylphenol		0.26		33.5		7.68		54.1	
	2-Ethyl-5-propylphenol	0.31								
Furans	Denderalasin		2.98							
Ketones	1-(4-Ethylphenyl) ethanone			1.57		0.98	0.35			
Total # of volatile compounds		10	18	4	6	5	4	2	6	1
Total # of volatile compounds shared with at least one *L. draba* population		4	8	2	5	4	3	2	2	0

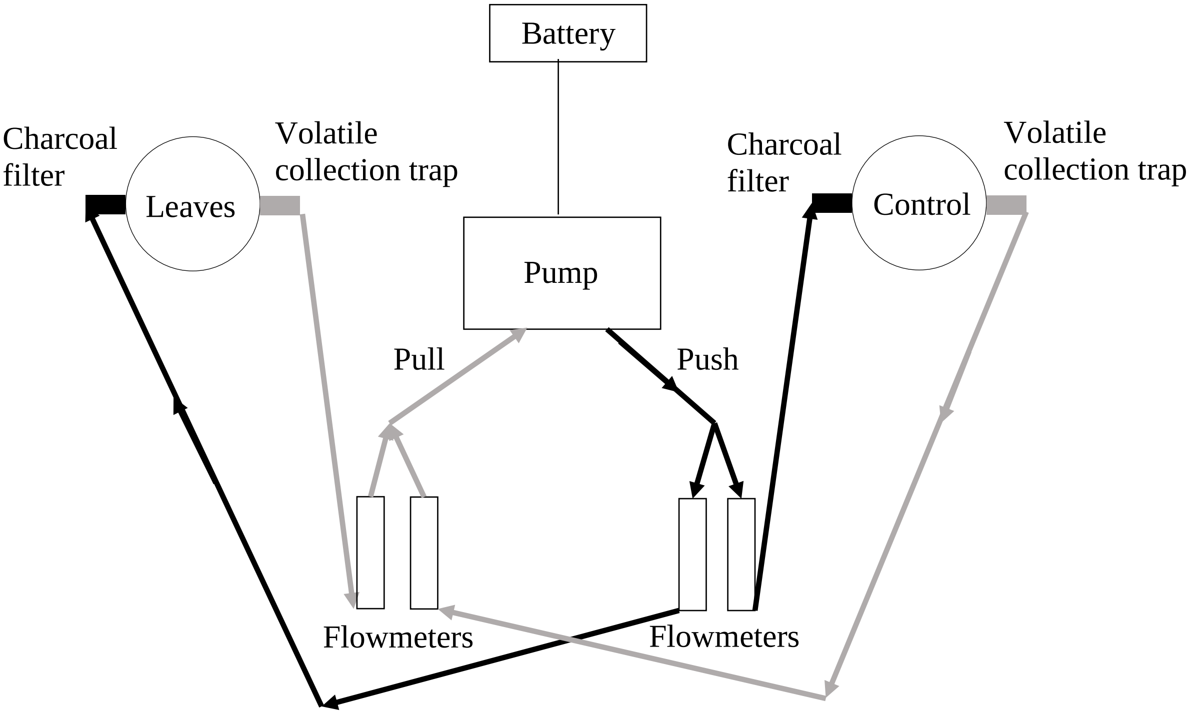


Figure 5.1: Schematic drawing of the portable volatile collection system (PVCS) (Park et al., 2018, modified).

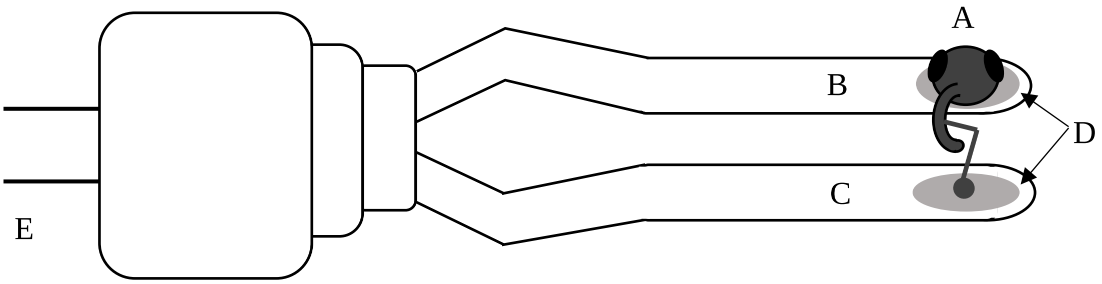
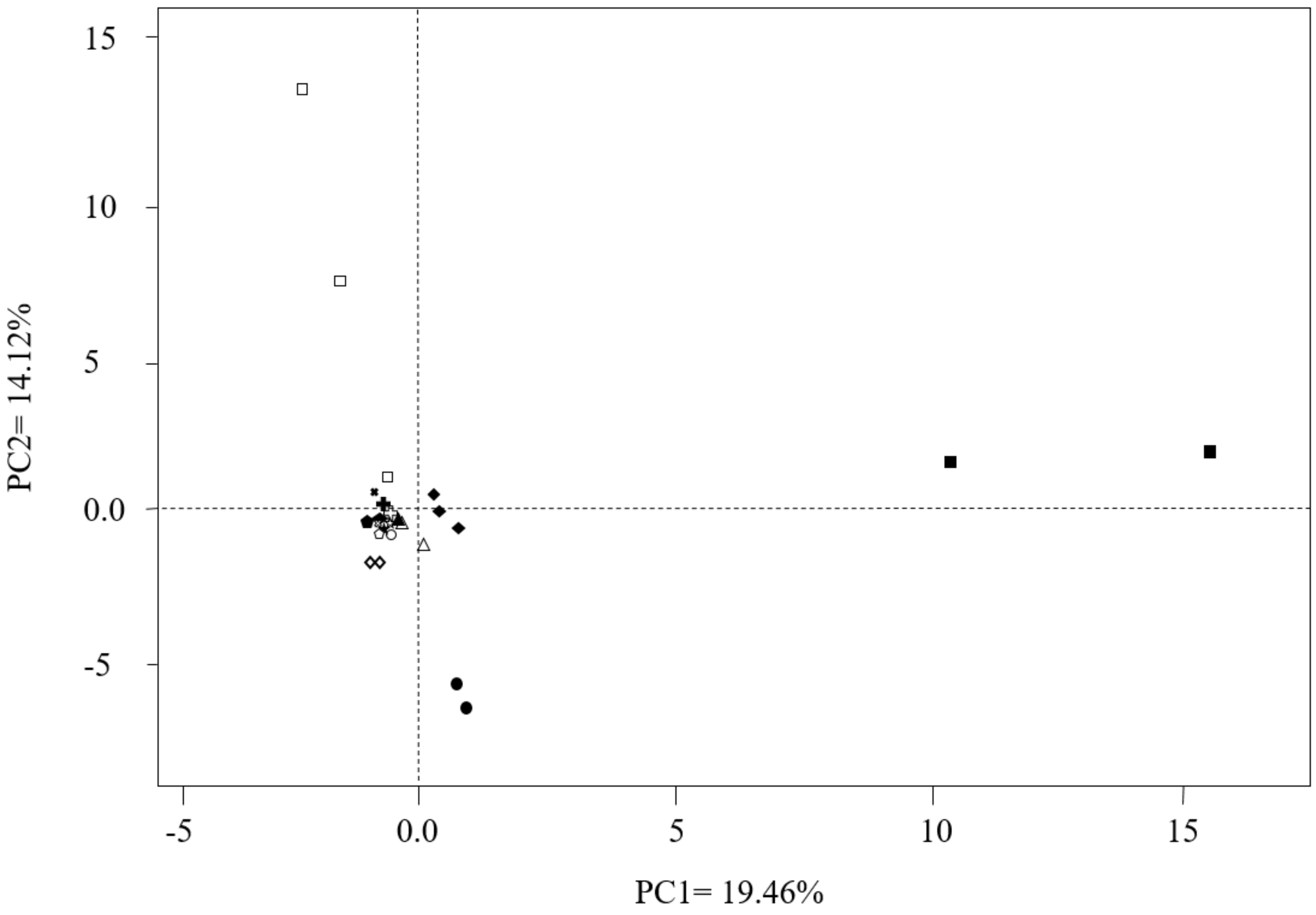


Figure 5.2: Schematic drawing (not to scale) of EAD probe and antennal preparation. The basal portion of the head (A) was placed over a reference electrode (B) and an antenna was connected to the recording electrode (C). The base of the head and antennal tip were partly submerged in the Spectra® 360 electrode gel (D). Any electrical signals were sent from the probe to the data acquisition controller via connecting wires (E).

a)



b)

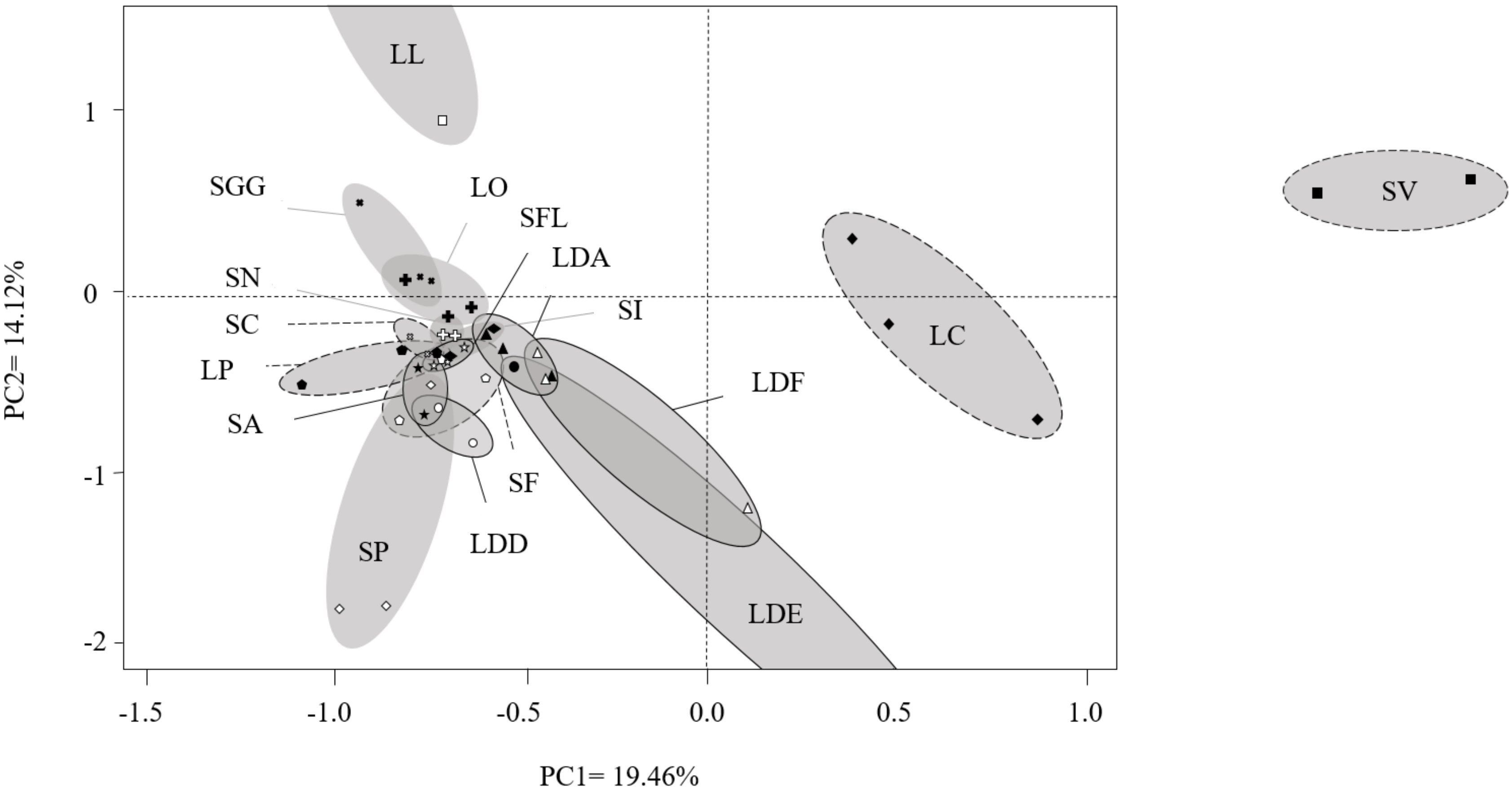


Figure 5.3: Principal component analysis score plot based on volatiles found in four populations of *Lepidium draba* and 13 related plant species depicting a) all data points; points are tightly compacted and overlapping, and b) close-up of plot; ellipses that extend off the graph indicate that plant species within those ellipses lie outside this close-up graph. Plant species- LDA: *Lepidium draba*

population A, LDD: *Lepidium draba* population D, LDE: *Lepidium draba* population E, LDF: *Lepidium draba* population F, LC: *Lepidium crenatum*, LL: *Lepidium latipes*, LO: *Lepidium oblongum*, LP: *Lepidium papilliferum*, SP: *Stanleya pinnata*, SV: *Stanleya viridiflora*, SA: *Streptanthus anceps*, SC: *Streptanthus crassicaulis*, SFA: *Streptanthus farnsworthianus*, SFL: *Streptanthus flavescens*, SGG: *Streptanthus glandulosus ssp. glandulosus*, SI: *Streptanthus inflatus*, SN: *Streptanthus niger*. As host specificity testing data allowed us to designate plant species into one of three attack levels, level 3 plant species are denoted by circles and arrows with solid black outlines, level 2 plants with circles without outlines and with grey arrows, and level 1 plants with circles with dashed outlines and dashed arrows.

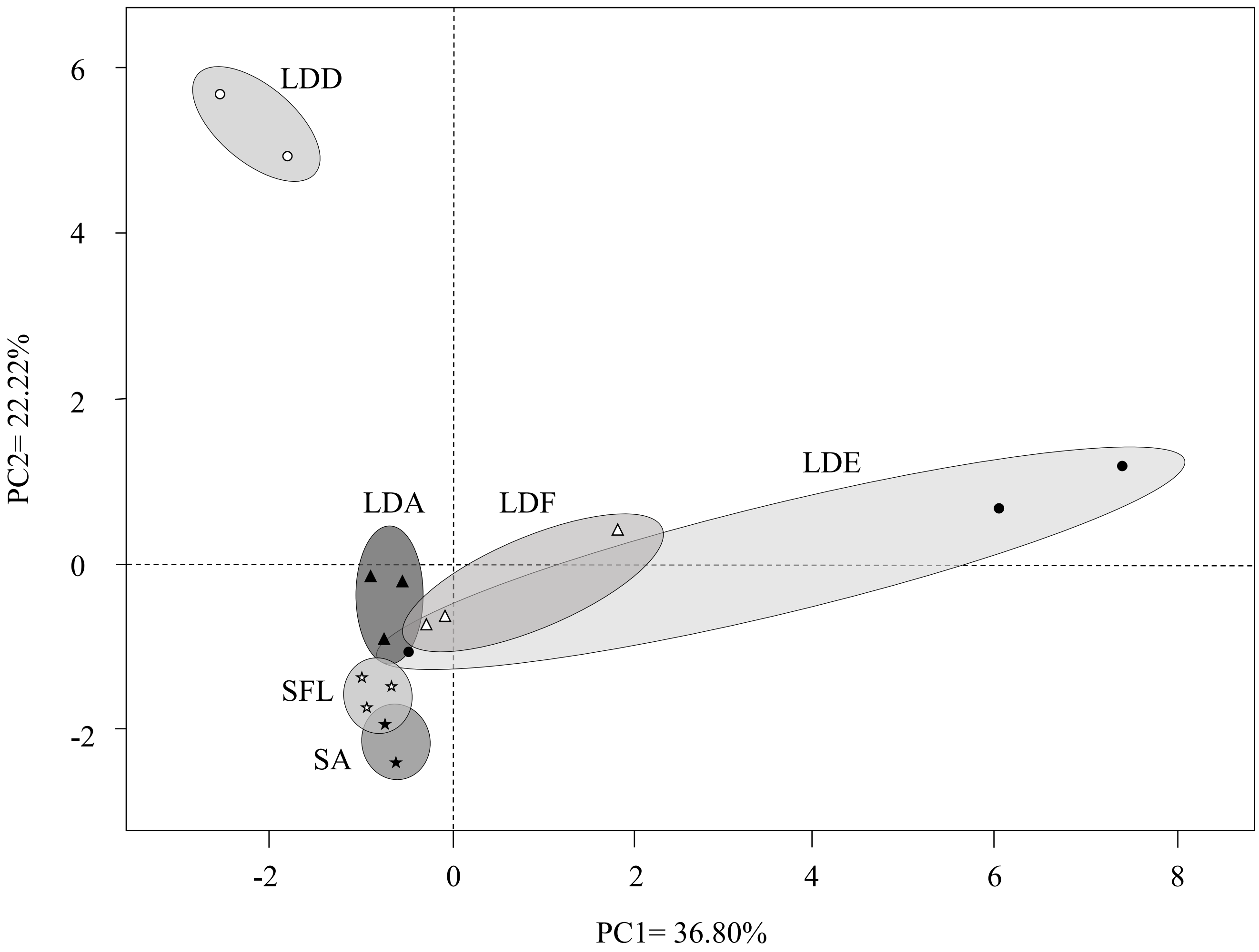


Figure 5.4: Principal component analysis score plot based on volatiles found in *Streptanthus anceps* (SA), *Streptanthus flavescens* (SFL), and four populations of *Lepidium draba* (LDA, LDD, LDE, LDF).

References

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