Post-release non-target monitoring of *Mogulones cruciger*, a biological control agent released to control *Cynoglossum officinale* in Canada

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**Summary**

Non-target effects of approved biological control agents have raised questions about the safety of biological control of weeds and resulted in an increased emphasis on monitoring and reporting of non-target effects as part of post-release assessments. This is particularly important in the case of the root-mining weevil *Mogulones cruciger* (Herbst), which was approved in Canada to control houndstongue, *Cynoglossum officinale* L., but not in the United States because of concerns over its environmental safety. To address these concerns and the potential for non-target effects, we monitored co-occurring confamilial Boraginaceae species at six *M. cruciger* release sites in Alberta and British Columbia over two years. All four co-occurring species were attacked by the weevil to varying degrees although attack was inconsistent between years and sites, and non-targets were mostly attacked to a lesser degree than houndstongue. There was a positive relationship between the probability of non-target attack and houndstongue attack rate by *M. cruciger* indicating potential spillovers and early evidence suggests non-target attack may be transitory. The comparison between the pre- and post-release evaluations and preliminary plant volatile, electroantennogram, and host-choice behaviour data suggest that chemical ecology may provide an important tool in understanding an insect’s host-choice selection in pre-release host-specificity assessments.

**Keywords**: houndstongue, non-target effects, host-choice behaviour, chemical ecology

**Introduction**

Examples of non-target effects have created widespread interest and concern through both the scientific and public communities about the environmental safety of biological weed control agents (Simberloff and Stiling, 1996; Louda et al., 1997, 2003 a, b; Strong, 1997; Thomas and Willis, 1998; Pemberton, 2000). The predictability of an agent’s host range is often at the center of the debate. Conventional host-specificity tests are used to determine an insect species’ physiological and ecological host range, which is then used to predict an insect’s realized host range once released in the invaded range (Schaffner, 2001; van Klinken and Edwards, 2002). Studies demonstrate that the physiological host range, which can be reliably determined experimentally (e.g. Papaj and Rausher, 1983; Szentesi and Jermy, 1990; van Klinken, 2000; van Klinken and Heard, 2000), appears to be an effective criterion for identifying species at risk of attack by introduced agents, since there is no example of an insect agent attacking a plant outside its physiological host range (Fowler et al., 2000; Pemberton, 2000; van Klinken and Edwards, 2002). A species’ realized host range, however, is almost certainly narrower than its physiological host range, which is evident from the narrowing host range that is frequently observed under increasingly natural testing conditions (i.e. multiple-choice tests and open-field tests). However, adequate pre-release screening protocols to
predict this have not been developed (Van Driesche et al., 2000; Schaffner, 2001; Hopper, 2001; and references therein). Realized host ranges will be limited due to phenological, ecological, and behavioural constraints. While phenological and ecological factors likely differ among continents and habitats, behavioural factors are inherent to the agent and could be examined as part of improved pre-release assessment protocols.

The host selection process of phytophagous insects typically progresses from host finding (dependent upon volatile and visual cues), through host examination (dependent in addition on gustatory and tactile cues), to host acceptance (oviposition or sustained feeding) (Dethier, 1982; Miller and Strickler, 1984; Bernays and Chapman, 1994). Each stage is dependent upon completion of the previous stage. Thus, in nature, host examination and acceptance, as evaluated in no-choice bioassays, cannot occur unless the insect arrives at the potential non-target host during the host-finding stage. Therefore, knowledge of the chemical basis for host-finding behaviour might improve our ability to assess whether a non-target plant species that can support the development of a biocontrol agent would be at risk of attack in the field (Thomas and Willis, 1998; Heard, 2000; Schaffner, 2001).

The Mogulones cruciger Herbst, houndstongue (Cynoglossum officinale L.), system provides a prime example of the importance of such testing. Mogulones cruciger is a root-feeding weevil that has been released in Canada to control the noxious rangeland weed, houndstongue. Release in the United States, however, has been denied because of concerns about its environmental safety. Previous host-specificity testing has demonstrated that M. cruciger's physiological host range is fairly broad, but it has always shown a strong preference for houndstongue (Jordan et al., 1993; De Clerck-Floate et al., 1996; De Clerck-Floate and Schwarzlaender, 2002; Andreas, 2004). A recent study of six field sites in Canada found that M. cruciger was utilizing four confamilial species growing sympatrically with houndstongue (Andreas, 2004). The attacked Boraginaceae species are Cryptantha spiculifera (Eastw.) Payson, Hackelia floribunda (Lehm.) I.M. Johnston, Lappula squarrosa (Retz.) Dumort and Lithospermum ruderale Doug. ex Lehm.. The first three species are within the known physiological host range of M. cruciger (De Clerck-Floate et al., 1996; De Clerck-Floate and Schwarzlaender, 2002; Andreas, 2004) while the latter species has not been sufficiently tested because its cultivation has not been successful. Because M. cruciger is known to prefer houndstongue (Jordan et al., 1993; De Clerck-Floate et al., 1996; De Clerck-Floate and Schwarzlaender, 2002; Andreas, 2004), we hypothesize that these non-target attacks result from ‘spillover’, ‘sensitization/central excitation’ effects or both. In spillover, high population densities on the target result in some insects colonizing non-targets due to random dispersal especially after the target re-source begins to be depleted (Strong, 1997). In sensitization/central excitation, acceptance thresholds for non-targets are lowered after contact with the true host or in the presence of ambient volatile organic compounds emitted by the true host (Marohasy, 1996, 1998; Withers and Barton Browne, 1998). Regardless of the specific mechanism, these recorded non-target attacks indicate a need to determine the risk that M. cruciger poses to native species that are within the physiological host range and within the potential range of the weevil’s dispersal from target host populations. Therefore, we explored the early stages of host-selection behaviour and its underlying phytochemical basis to determine the plant species likely to be colonized by M. cruciger.

**Materials and methods**

**Study organisms**

Houndstongue is a Eurasian herbaceous, facultative, short-lived perennial. Plants produce rosettes in the first year and typically reproduce in the second or third year (Wesselingh et al., 1997). After sexual reproduction, barbed nutlets are formed and dispersed via epizoochory (De Clerck-Floate, 1997). In Europe, the plant is found in sand dunes, roadsides, and open woodlands (Tansley and Adamson, 1925; Tutin et al., 1972; Hegi, 1975; Klinkhamer and de Jong, 1988). In North America, this ruderal species colonizes disturbed areas, rangelands, pastures, and forests (Macoun, 1884; Upadhyaya and Cranston, 1991).

Mogulones cruciger is a root-feeding weevil native to central Europe. In spring, after hibernation, adults occur on above-ground plant parts, where they feed on leaves, mate, and oviposito into leaf petioles (Schwarzlaender, 1997). Larvae hatch from eggs 7-10 days after oviposition and begin to mine down into the root crown where they feed primarily in the vascular cylinder. Mogulones cruciger has three instars and pupates in the surrounding soil (Schwarzlaender, 1997). In late summer, adults emerge and begin feeding on houndstongue rosettes between July and October. Oviposition begins in late August until temperatures cool and oviposition sites become unavailable. Adult weevils hibernate in leaf litter (Schwarzlaender, 1997; De Clerk-Floate and Schwarzlaender, 2002). Mogulones cruciger often has overlapping generations. As a consequence, eggs and larvae can be found in houndstongue roots and leaf petioles at most times of the year.

The native North American Boraginaceae species Hackelia venusta (Piper) St. John was chosen as a non-target species in this study because it is listed as endangered by the United States Fish and Wildlife Service (USFWS) and laboratory tests indicated that M. cruciger is capable of partial larval development on this species (Andreas, 2004). Its distribution is limited to one small remaining population of approximately 150 individuals
in a ponderosa pine (*Pinus ponderosa* P. & C. Lawson) and Douglas fir (*Pseudotsuga menziesii* (Mirbel) Franco) clearing in Chelan County, Washington, just south of the Canada/U.S. border (Center for Plant Conservation, 2004). Fire suppression has been an important factor in the reduction of *H. venusta* populations (Center for Plant Conservation, 2004). *Cryptantha spiculifera* and *Hackelia floribunda* were selected for this experiment because they co-occur with houndstongue at field sites in Canada (Kartesz, 1999) and were monitored in a post-release open field study (Andreas, 2004).

**Olfactometer bioassay**

Our methods for an olfactometer bioassay and for electroantennogram (EAG) and GC/EAD have been adapted from those proven effective for a close relative of *M. cruciger*, the cabbage seed weevil, *Ceutorhynchus obstrictus* Marsham (*C. assimilis* Payk.) (Evans and Allen-Williams, 1992; Evans and Bergeron, 1994). The olfactometer was a four-arm configuration (Vet et al., 1983) (Syntech, Hilversum, The Netherlands). We conducted preliminary 3-way choice bioassays in the olfactometer with air streams directed over intact plants of *H. floribunda*, *C. spiculifera*, houndstongue, and a blank (carrying humidified air). Intake air for all treatments was prefiltered through activated charcoal. Airflow was balanced at 300 ml/min through each arm of the olfactometer.

Illumination was with overhead fluorescent fixtures, diffused through a white, translucent plastic pail inverted over the olfactometer. The lens of a video camera was inserted through the top center of the pail to permit continuous recording of weevil behaviour during the bioassay. Twelve female weevils (having previously been exposed to houndstongue but not having encountered other hosts) were tested simultaneously in a single run. The weevils were placed at the center of the olfactometer and their positions and movements recorded on videotape for 30 minutes for later review and recording using a computer program (Noldus Observer, Wageningen, The Netherlands). Relative attractiveness of each source was quantified in terms of the proportion of time spent in the respective quadrants of the olfactometer.

**VOC analysis**

Volatile organic compounds (VOCs) were trapped from the headspace of houndstongue, *H. venusta*, *H. floribunda*, and *C. spiculifera* with a volatile collection apparatus (Analytical Research Systems Inc., Gainesville, FL) following methods modified from Eigenbrode et al. (2002). The headspace VOC profiles were compared for similarity based on the number of shared compounds detected and by calculating similarity coefficients based on occurrence and relative abundance of each compound. We employed two binary coefficients, Jaccard’s and Sørensen’s, and one quantitative coefficient, Bray-Curtis, commonly used for ecological studies (Southwood and Henderson, 2000).

**EAG and GC/EAD/FID**

EAG assay methods were modified from those of Evans and Allen-Williams (1992). Recordings were taken from single antennae on partially excised heads using sharpened stainless steel electrodes coated with an electrolyte gel. To measure antennal response to the total blend of VOCs from each species, samples of headspace volatile that had been standardized based on plant dry weight were applied in solvent to a filter paper strip and delivered to the antenna in a puff of prefiltered air. Antennal response to VOCs of each species was standardized on the basis of a response to a single concentration of linalool. Responses from ten weevils (three males and seven females) were obtained from *H. floribunda*.

Gas chromatography using EAG and flame ionization detectors (GC/EAD/FID) (Bjostad, 1998) can help identify the components of potential host VOCs that are most important for observed behavioural responses. A 1-μl sample of headspace volatile dissolved in methylene chloride was injected onto a Shimadzu GC17-A GC (Shimadzu Corp., Kyoto Japan) fitted with a column splitter delivering approximately half the column effluent to a flame ionization detector and half via a heated transfer line to the electroantennograph (Syntech, Hilversum, The Netherlands) with the insect preparation. Temperature programme and column specifications were conducted as described in Eigenbrode et al. (2002). Antennal responses (expressed as mV of depolarization) were standardized based on a standard sample of linalool injected through the GC/FID/EAD between each injected sample of headspace VOCs. One female was tested.

**Results**

**Olfactometer bioassay**

The results from the behavioural bioassay (Figure 1) indicate that *M. cruciger* females responded to host VOCs in the olfactometer. The weevils spent the smallest proportion of time in the *H. floribunda* quadrant of the olfactometer, approximately 20% of their time in *C. spiculifera* and the no-plant control quadrants, and the greatest amount of time (approximately 40%) in the houndstongue quadrant (One-way ANOVA; F = 4.71, P = 0.006).

**VOC analysis**

Of the 44 VOCs detected in headspace of the four species, six were shared by all and six were unique to houndstongue. *Hackelia venusta* headspace had the most compounds in common with houndstongue, followed by *C. spiculifera* and then *H. floribunda*. All
three similarity coefficients follow a similar trend, with *H. venusta* most similar to houndstongue, followed by *C. spiculifera* and then *H. floribunda* (Table 1).

### EAG and GC/FID/EAD

**EAG:** Antennal preparations exhibited depolarization (i.e., peaks in impulses indicate excitation to particular compounds) in response to puffs of extracted headspace VOCs and this response tended to be stronger for houndstongue compared to *H. floribunda* (Figure 2) (2-way ANOVA *P* values = 0.08, 0.16 and 0.28 for species, sex and interaction, respectively). GC/FID/EAD: The combined FID/EAD trace resulting from chromatography of *H. venusta* volatiles showed that an individual *M. cruciger* female antenna responded to a subset of the VOCs present.

### Discussion

Despite the recognized need to include host-choice behaviour and chemical ecology in host-range inves-

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**Table 1.** Measures of similarity for headspace volatile profiles of *Hackelia venusta, Cryptantha spiculifera* and *Hackelia floribunda*, as compared with houndstongue (*Cynoglossum officinale*) headspace volatiles.

<table>
<thead>
<tr>
<th>Species compared to <em>C. officinale</em></th>
<th>Shared components</th>
<th>Sørensen*</th>
<th>Jaccard*</th>
<th>Bray-Curtis*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. venusta</em></td>
<td>21</td>
<td>0.84</td>
<td>0.72</td>
<td>0.26</td>
</tr>
<tr>
<td><em>C. spiculifera</em></td>
<td>11</td>
<td>0.41</td>
<td>0.26</td>
<td>0.23</td>
</tr>
<tr>
<td><em>H. floribunda</em></td>
<td>9</td>
<td>0.36</td>
<td>0.22</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* The Sørensen coefficient is binary in that it is based on the presence/absence of each compound. It is calculated as: 
  \[ C_s = \frac{2a}{2a + b + c} \]
  in which *a* = the number of compounds held in common, *b* = the number of compounds unique to houndstongue and *c* = the number of compounds unique to the species compared to houndstongue. It ranges from zero for non-overlapping profiles to one for identical profiles.

* Jaccard similarity is also binary. It is calculated as: 
  \[ C_j = \frac{a}{a + b + c} \]
  and also ranges from zero to one.

* The Bray-Curtis coefficient is a quantitative index of similarity that takes into account abundance of the components. It is calculated as 
  \[ C_n = \frac{aN}{aN + bN} \], in which *N* = the sum of lesser values for those compounds shared between species and aN and bN are the sum of total values for each species. The coefficient scales from near one for equivalent profiles to zero.
Figure 2. EAG responses by male and female Mogulones cruciger to puffs of total VOC from its host, houndstongue (Cynoglossum officinale) and a non-target species, Hackelia floribunda (response standardized relative to linalool), (n= three males and seven females).

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![Diagram showing EAG responses for different plants and genders.]

C. officinale

H. floribunda

C. spiculifera

H. venusta

C. officinale

H. floribunda

Standardized EAG response

0 0.5 1 1.5 2 2.5

Investigations of candidate weed biocontrol agents (Briese, 2005; Sheppard et al., 2005), few studies have attempted this (Heard, 2000; Hopper, 2001; Schaffner, 2001). Our data provide an illustration of the type of information that can be obtained with behavioural and chemical ecological experiments.

Our olfactometer results indicate greater responsiveness of M. cruciger to odour cues from houndstongue than to the tested non-target species. We did not detect directed upwind movement by the weevils in this bioassay (data not shown), indicating that M. cruciger dispersal among potential hosts could be undirected but that host VOCs can stimulate arrested or restricted searching behaviour on or near the host. Our bioassay has some limitations for fully understanding relevant host-selection responses to target and non-target VOCs in the field. We studied walking behaviour, but flying is likely also important in host location. The three-odour choices we offered may not represent the situation encountered from isolated non-target plants in the field. Further testing is required to address these concerns.

Our focus on VOC isolates behaviour during early stages of host selection. After contact with potential hosts, appropriate tactile and gustatory cues are required for host acceptance. Further study of the so-called ‘examination’ phase of host selection is needed for a more complete understanding of factors determining the realized host ranges of candidate biological control agents. For example, M. cruciger may respond to detectable pyrrolizidine alkaloids on the plant surface. Although recent work failed to find that these alkaloids could explain oviposition preference by another specialist insect, the cinnabar moth, Tyria jacobaeae L. (Lepidoptera: Arctiidae) (Macel and Vrieleig, 2003), the response by M. cruciger to these alkaloids and other gustatory cues of target and non-target plants should be examined.

The basis of apparent M. cruciger discrimination among odours of houndstongue and non-target species remains unknown. Some insects apparently integrate information from the VOC blend of their host plants (Roseland et al., 1992), while others, including a close relative of M. cruciger, the cabbage seedpod weevil, C. obstrictus, use a few specific VOCs to orient to potential hosts (Smart and Blight, 1997). The VOC profiles of H. floribunda, H. venusta and C. spiculifera differ from houndstongue. Hackelia floribunda VOCs are the least similar to those of houndstongue. Among the three tested non-target species, the VOCs of only two, C. spiculifera and H. floribunda, were subjected to the olfactometer bioassay, where H. floribunda was once again less preferred. If specific compounds are required for host acceptance, they may be present in houndstongue but lacking in H. floribunda. Moreover, since H. floribunda appeared repellent in our tests, VOCs present in its profile, but lacking in houndstongue (i.e. benzaldehyde, undecane and dodecane) are candidate repellents. On the other hand, the weevils may integrate information from several cues during response to the VOC blends of potential hosts. For determining host-range tendencies in pre-release studies, it may not be necessary to determine these mechanisms. Our result indicates that it is feasible to include responses to cues used during host finding in assessing risks of colonization of non-targets as part of pre-release studies. Our results with M. cruciger indicate that H. floribunda may be at less risk than the other species tested here because the weevils are not attracted and possibly even
repelled by this plant, whereas \textit{H. venusta} may be at greater risk because of the similarity of its VOC pattern to that of houndstongue.

The weevil’s response to several components in the GC/FID/EAD test may help determine the basis of its host selection behaviour, as these volatiles are candidate components whose presence could attract the weevil to a non-target host that produces them. However, given that only one weevil and one plant species was used, the results presented here simply revealed that \textit{M. cruciger} can detect plant stimuli.

Our approach could help elucidate the risk \textit{M. cruciger} poses to non-targets near (e.g. Andreas, 2004) and remote from colonized houndstongue infestations. In the former setting, weevils dispersing from houndstongue into the environment will be influenced by their responsiveness to VOCs and other long-distance cues from potential hosts. If there is little or no response to non-target cues (as we may have found for two non-targets), the risk of non-target attack should be reduced. Spillover effects or central excitation/sensitization potentially alter this assessment. If weevils are sufficiently abundant and mobile, regardless of responses to VOC from potential hosts, they may encounter and colonize the non-targets (spillover). If prior contact with hosts or host odours lowers acceptability thresholds to subsequently encountered plants (central excitation/sensitization), temporary non-target attack could be facilitated. Longer-distance colonization of non-targets is also potentially mediated by VOCs. Our result suggests that, while walking, weevil colonization of the two non-targets we tested are not increased due to their released VOCs.

Our approach could help assess risks of attack of other sensitive non-targets by \textit{M. cruciger}. Three of the five endangered Boraginaceae species in the United States are among the weevil’s physiological host range. Complete development was possible on \textit{Plagiobothrys hirtus} (Greene) I.M. Johnston and \textit{Amsinckia grandiflora} (Klee. ex Gray) Kleeb. ex Greene and partial development occurred on \textit{H. venusta} (Andreas, 2004). The two remaining confamilial species that are listed as endangered in the United States, \textit{Cryptantha crassipes} I.M. Johnston and \textit{Plagiobothrys strictus} (Greene) I.M. Johnston were not tested. VOC profiles of these species could be tested to assess weevil attraction. \textit{H. venusta} VOC profiles are more similar to houndstongue than others we have tested and are potentially attractive or arrestant for \textit{M. cruciger}.

Comparisons of realized and predicted host ranges from pre-release studies (Cullen, 1990; Briese et al., 1995; Clement and Cristofaro, 1995; Briese, 1999) can evaluate the soundness of prior risk-assessment procedures (Ewel \textit{et al.}, 1999; Hopper, 2001). Selection behaviour and its phytochemical basis could, in contrast, greatly improve predictions of eventual host ranges of released agents (Marohasy, 1998; Thomas and Willis, 1998; Heard, 2000; Schaffner, 2001). By using methods similar to those presented here, behavioural and electrophysiological bioassays could form the basis of risk-assessment studies. Specifically, the release of \textit{M. cruciger} into North America provides an opportunity to compare its realized host range with host-selection predictions based on phytochemical and behavioural studies.

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