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Interpreting host-test results for classical biological control candidates: Can the study of native congeners improve the process?

Rhoda B. deJonge[a], Ian M. Jones[a,*], Robert S. Bourchier[b], Sandy M. Smith[a]

[a] University of Toronto, Faculty of Forestry, 33 Willcocks Street, Toronto, Ontario M5S 3B3, Canada
[b] Agriculture and Agri-Food Canada, 5403 – 1 Avenue South, Lethbridge, Alberta T1J 4B1, Canada

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ABSTRACT

When an insect candidate is identified for classical biological control, the first step is to identify its fundamental host-range. An insect's fundamental host range, however, is typically broader than its ecological host range. This discrepancy can lead to 'false positives' in host testing and hamper the development of potential agents. Here, we propose a novel tool for interpreting host-range tests and identifying 'false positives' by studying native insects closely related to the biological control candidate. We conduct a series of laboratory and field studies comparing the fundamental and ecological host ranges of *Chrysochus auratus* Fabricius (Coleoptera: Chrysomelidae), a beetle native to North America, and present throughout the range invaded by pale swallow-wort, *Vincetoxicum rossicum* (Kleopow) Barbar. (Apocynaceae). We use the results to re-evaluate the risk associated with releasing the closely-related European beetle, *Chrysochus asclepiadeus* (Pallas) (Coleoptera: Chrysomelidae), a biological control candidate for *V. rossicum* that has raised some concerns because of no-choice feeding on North American milkweed species (*Asclepias* spp.) during laboratory host-testing. Laboratory and greenhouse trials here show that native North American *C. auratus* adults can feed and complete larval development on several native *Asclepias* species (fundamental host range), however in the field where both closely-related plant genera are present, this species specialized only on plants in the genus *Apocynum*. It appears then that *Asclepias* species generate 'false positives' for *Chrysochus* beetles when only the fundamental host range is assessed in the laboratory, and there is a need to re-evaluate *C. asclepiadeus* for potential biocontrol in North America taking into account its ecological host-plant range. We advocate for the inclusion of closely-related native congeners, where appropriate species exist, to aid in interpreting host-plant testing for potential classical biological control agents.

1. Introduction

The early stages of any classical biological control program are characterized by a particular tension, that between the need to release the most effective agents, and the desire to protect non-target species. The most prominent concerns relating to biological control involve the release of agents that unexpectedly damage non-target species (Jayanth et al., 1993; Dennill et al., 1999; Withers et al., 2008). A recent meta-analysis by Hinz et al. (2019); however, revealed that such failures of host testing are increasingly rare. Of 132 specific cases of non-target attack identified by Hinz et al. (2019), occurring between 1863 and 2008, 70 (53%) were predicted by pre-release host-specificity testing, and the agents in question would not have been released if modern day values had been applied. Of the remaining 62 cases, 58 involved plants that were not included in pre-release tests (host range testing prior to 1960 did not generally include closely-related plants from the introduced range). Overall, Hinz et al. (2019) identified only four cases in which agents attacked non-target plants that had been tested prior their release. Although non-target attack is rightly seen as the primary risk factor associated with biological control, the failure to release a promising agent, perhaps through an overabundance of caution, can also have negative ecological consequences (Sheppard et al., 2003). While current host-range testing is effective at identifying insects that pose a risk to non-target species, it does little to identify 'false positives', where an agent is rejected for feeding or ovipositing on a species in the laboratory that it would rarely, or never, use in the field (Hinz et al. 2014). Identifying 'false positives' requires a clearer understanding of the relationship between the fundamental and ecological host-ranges of candidate insects, and this represents the next challenge for pre-release host-specificity testing.

* Corresponding author.
E-mail addresses: Rhoda.dejonge@utoronto.ca (R.B. deJonge), i.jones@utoronto.ca (I.M. Jones), robert.bourchier@canada.ca (R.S. Bourchier), s.smith.a@utoronto.ca (S.M. Smith).

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The main tool at the disposal of biological control practitioners for determining whether an agent is 'safe' to release is host-specificity testing carried out initially in quarantine facilities. Although host-testing protocols have been refined over recent decades (Wapshere, 1974; Briese, 2003), they are limited by an inability to replicate all conditions that occur in the invaded habitat. The common solution to this problem has been to conduct field host-specificity tests in the native range of the candidate agent (Schaffner et al., 2018). These tests have limitations of their own however, due to quarantine restrictions on the plants that need to be tested, and the costs of conducting foreign-based research (Clement and Cristofaro, 1995). Here, we propose an additional tool to compliment traditional host-specificity tests, focusing on closely-related insect species that are already present in the proposed area of release. Just as the centrifugal phylogenetic method has proven effective in elucidating the fundamental host range of candidate insects (Wapshere, 1974), we propose a phylogenetic approach that uses closely-related insects, with similar host-ranges and life-histories, to better understand their ecological host range.

In an earlier paper, we focused on the use of invasive *Vincetoxicum rossicum* (Kleopow) Barbar. (Apocynaceae) by a native beetle, *Chrysochus auratus* Fabricius (Coleoptera: Chrysomelidae) in North America. Here, we conduct a series of experiments to expand on those findings, and compare the fundamental and ecological host range of the beetle. We use this relationship to make predictions about the ecological host range of *Chrysochus asclepiadeus* (Pallas) (Coleoptera: Chrysomelidae), a potential biological control agent for *Vincetoxicum* spp. native to southern Europe.

The approach of using native insects closely related to candidate biological control agents to interpret standard host-specificity tests would be advantageous in several ways. First, plant species used by native insect congeners may require more extensive host-specificity testing than currently since related candidate agents may potentially make greater use of them than predicted (Ehrlich and Raven, 1964). Second, the ability of candidate agents to feed on certain non-target plants may be worth further investigation if those non-target plants are absent from the ecological host-range of native congeners, *i.e.,* further support may be needed to accurately predict ecological use of these particular non-targets plants. Host-range studies of native insects represent a useful tool to enhance knowledge-based decision-making during the agent development phase of biological control programs. Such a tool can help prevent the release of agents that might pose an underlying risk to non-target species or provide a second chance to identify potentially successful agents that would otherwise be eliminated from consideration.

Vincetoxicum rossicum, commonly known as pale swallow-wort or dog-strangling vine, is a perennial vine native to southwestern Ukraine (Pobedimova, 1952). The species was introduced to North America in the 1800s (Monachino, 1957) and has reached invasive status in several regions in eastern Canada and the northeastern United States (DiTommaso et al., 2005). The weed reduces native plant diversity by outcompeting neighboring plants (Christensen, 1998) with cascading effects on native arthropod assemblages (Ernst and Cappuccino, 2005). Rare alvar communities in central Canada are increasingly threatened by encroaching *V. rossicum* (Lawlor and Raynal, 2002) and the weed is a potential ovipositional sink for monarch butterflies (Casagrande and Dacy, 2007). Conventional weed control methods such as mechanical removal or herbicides are expensive at the current scale of invasion, and can damage non-target plants or be difficult to apply in remote areas. Classical biological control represents the best potential for long-term sustainable control of *V. rossicum*.

Chrysochus asclepiadeus was first identified as a classical biocontrol candidate for *V. rossicum* in 2002 (Tewksbury et al., 2002). The beetle is found throughout southern Europe (Jolivet and Verma, 2008; Schmitt, 2011) where it feeds exclusively on *Vincetoxicum* spp. (Weed, 2010). Female beetles feed on the leaves and then oviposit on the base of the plants stems where larvae feed, develop, and overwinter on the roots (Weed, 2010). *Chrysochus asclepiadeus* was determined to be the most effective herbivore on *V. rossicum* in laboratory and common garden tests conducted in Europe (Weed et al., 2011a,2011b); however, during host-range testing, the beetle demonstrated the ability to feed and develop on several native North American plants, including *Asclepias* spp. (milkweeds) (Gassmann and Louda, 2000; Gassmann et al., 2011; Sforza, 2011). As a result, screening of *C. asclepiadeus* was suspended due to the perceived risk of non-target impacts.

Chrysochus auratus is found in eastern North America and feeds exclusively on *Apocynum* spp. (Apocynaceae) (Arnett, 1968; Doussourd and Eisner, 1987; Williams, 1991; Dobler and Farrell, 1999; deJonge et al., 2017). The species provides a useful comparison to the European *Chrysochus asclepiadeus* as both species specialize on a particular genus within the Apocynaceae, and demonstrate similar life cycles with adults as foliar feeders and larvae developing on plant roots (Weiss and West, 1921; Arnett, 1968; Dobler and Farrell, 1999; Peterson et al., 2001; Jolivet and Verma, 2008). *Chrysochus auratus* also represents an ideal surrogate for *C. asclepiadeus* in this context, as it is native to the entire invasive range of *V. rossicum* (Peterson et al., 2001).

In the present study, we surveyed populations of *C. auratus* at seven sites across North America to confirm the beetle's ecological host range. Additionally, we carried out host-range testing on all life stages of *C. auratus* to determine the beetle's fundamental host range. We compared the ecological and fundamental host ranges of *C. auratus* in order to identify 'false positives', and used our observations of *C. auratus* to re-evaluate the potential risks of *C. asclepiadeus* as a biological control agent in North America.

2. Methods

2.1. Ecological host range of Chrysochus auratus

Experiment 1: Determining the ecological host range of Chrysochus auratus – Qualitative surveys were conducted to determine the ecological host range of *C. auratus* at seven sites in Washington, U.S., and British Columbia and Ontario, Canada (Table 1). The site boundaries were determined by the presence of host plants (*Apocynum* spp.). Additional sites with *Asclepias* spp. and no *Apocynum* spp. were surveyed prior to the experiment, but no *C. auratus* beetles, or signs of feeding, were found. Adult beetles were collected by hand at each study site. Hand collection was possible due to the beetles' low mobility, iridescent coloration, and aposematic behaviour. Egg masses were also collected at each study site from the underside of leaves, along stems, and on nearby vegetation. Following adult beetle collection, each site was surveyed to record vegetative cover. At sites over 100 m^2, a transect was set along the longest dimension of the site, and five evenly-spaced transects were then set perpendicular to the main transect. Four evenly-spaced, 1 m^2 quadrats were placed along each perpendicular transect to determine % coverage of the available host plants at the site. Sites less than 100 m^2 were divided into 1 m^2-quadrats and surveyed in full. All plants within each quadrat were thoroughly inspected for signs of feeding by *Chrysochus* spp.. Following site surveys, all vegetation peripheral to the site (within 50 m) was searched to record any beetles or egg masses found on plant species away from high density beetle populations.

2.2. Fundamental host range of Chrysochus auratus

Experiment 2: No-choice feeding trials with Chrysochus auratus adults – No-choice tests were conducted with adult *C. auratus* using cut leaves in the laboratory. Tests were based on a plant list from the demonstrated fundamental host range of *C. asclepiadeus* (Table 2). Adult *C. auratus* beetles were collected in the field and placed singly in 11 cm-petri dishes with moist filter paper and a single leaf from one of the test species (Table 2). Each leaf was selected from the middle stratum of the plant to maintain consistency in leaf size. Leaves were scanned prior to

Table 1
Survey locations for *C. auratus* beetles conducted in 2012 and 2013. Each site was surveyed once. The site boundaries were determined by known ecological host plant presence (*Apocynum* spp.). Sites were searched thoroughly to count adults and egg masses. Each site was surveyed to record vegetative cover.

Site	Location	Patch size (m2)	Host plant (vegetative cover % ± SD)	Adults (n)	Total no. egg masses (% on ecological host plants)	Locations of egg masses not laid on ecological host plants
Kamloops, BC	50.694167, −120.380000	400	*Ap. cannabinum* (*NA*[a])	31	250 (100.00)	–
Richland, WA	46.191944, −119.355556	400	*Ap. cannabinum* (19.38 ± 25.93)	35	308 (69.48)	dead wood, fireweed: *Chamerion* sp. (Onagraceae), St John's wort: *Hypericum perforatum* L. (Clusiaceae), and sowthistle: *Sonchus arvensis* L. (Asteraceae)
Guelph, ON	43.527778, −80.322778	350	*Ap. cannabinum* (44.33 ± 20.69)	44	270 (91.85)	common milkweed: *Asclepias syriaca*, soybean: *Glycine* max (L.) Merr. (Fabaceae), grass: *Poaceae* sp., raspberry: *Rubus idaeus* L. (Rosaceae)
Copetown, ON	43.224051, −80.055077	500	*Ap. androsaemifolium* (27.47 ± 20.50)	65	13 (92.30)	Canada goldenrod: *Solidago canadensis* (Asteraceae)
Dundas, ON	43.266308, −79.941197	75	*Ap. cannabinum* (57.00 ± 25.55)	112	21 (85.71)	thistle: *Cirsium vulgare* (Savi) Ten. (Asteraceae), grapevine: *Vitis riparia* Michx. (Vitaceae)
Toronto, ON	43.648866, −79.462608	250	*Ap. androsaemifolium* (22.00 ± 22.10)	79	13 (76.92)	dead wood
Mabton, WA	46.245556, −120.110278	400	*Ap. cannabinum* (66.77 ± 20.00)	90	NA[b]	NA

[a] Vegetative surveys were not conducted at this site.
[b] No. of egg masses not determined, as masses from both *Chrysochus* species and hybrid are indistinguishable from each other in the field.

Table 2
No-choice *Chrysochus auratus* laboratory feeding trials. Results indicate the number of each plant species tested, with the percentage of beetles that fed in parentheses. Mean quantity of leaf material removed is also displayed, with standard errors in parentheses.

Leaf species	Adults that fed % (no. tested)	Mean feeding (± SE) mm^2
Apocynum androsaemifolium	88.0 (25)	244.38 (32.29)
Ap. cannabinum	85.7 (91)	237.76 (23.71)
Asclepias eriocarpa	33.3 (57)	22.46 (13.48)
As. fascicularis	0 (10)	0.00
As. incarnata	18.3 (60)	3.14 (1.55)
As. speciosa	24.6 (65)	18.10 (12.00)
As. syriaca	66.7 (6)	11.57 (6.95)
As. tuberosa	0 (6)	0.00
Vincetoxicum rossicum	26.6 (154)	1.09 (0.40)
Solidago canadensis[a]	1.4 (70)	0.53 (NA[a])

[a] Species tested include *S. canadensis* (Asteraceae) concurrent with potential Apocynaceae hosts to assure that host choice was not random.

placement using ImageJ software (version 1.47, Bethesda, MD) to determine their initial surface area (mm^2). Petri dishes with different leaf treatments were placed evenly on the bench to control for any variation in the laboratory environment; no dishes with the same treatment were placed next to one another. All dishes were kept at ambient conditions (20–22 °C) with a photoperiod of 16:8 h (L:D). Leaves were removed after 2 days and visually inspected to confirm the presence or absence of feeding. Feeding galleries were then traced, with the initial leaf scan overlaid, to record the amount of leaf surface area (mm^2) removed. Feeding trials were terminated after 2 days in order to limit changes in leaf surface associated with water loss. Plant leaves of *Apocynum* spp., *Asclepias speciosa*, *Solidago canadensis* L., and *Vincetoxicum* spp. were sourced from field sites in Washington, U.S., and British Columbia and Ontario, Canada. *Solidago canadensis* was included as a negative control to estimate the base level of adult exploratory feeding on a non-host plant. Leaves of the remaining species were sourced from plants grown in the greenhouse. *Asclepias eriocarpa*, *As. fascicularis*, and *As. tuberosa* were grown from seed, while *As. incarnata* and *As. syriaca* were purchased as small plants from an Ontario grower (Native Plants, Claremont, Pickering, Canada) specializing in wild-collected native seeds.

Experiment 3: No-choice greenhouse trials to determine survival and oviposition by Chrysochus auratus adults – In July 2011, 260 adult *C. auratus* beetles were collected from five sites in Ontario, Canada (Table 1). Mated beetle pairs (one per plant) were placed on tightly-netted test plants of the following: *Apocynum androsaemifolium* (10), *Apocynum cannabinum* (10), *As. incarnata* (20), *As. speciosa* (10), *As. syriaca* (20), *As. tuberosa* (20), *Vincetoxicum nigrum* (L.) Moench (20), and *V. rossicum* (20). Plants were checked daily to monitor beetle survival and to count egg masses. The experiment was stopped one week after the last beetle on a non-*Apocynum* spp. plant had died.

Apocynum cannabinum and *Vincetoxicum* spp. were grown in the greenhouse from roots collected at a field site near Toronto, Ontario. *Apocynum androsaemifolium* and *Asclepias* spp. were grown from small plants purchased in Ontario (Native Plants, Claremont, Pickering, Canada).

Experiment 4: No-choice greenhouse trials to determine development of Chrysochus auratus larvae – In July 2012, *C. auratus* adults were collected from a field site in Copetown, Ontario (Table 1) and held in clear, 4L plastic containers (12 cm × 12 cm × 26 cm) with foliage of plants from which they were collected. Egg masses laid on the plant material were removed every three days and placed singly in microcentrifuge tubes. Eggs were then stored in ambient laboratory conditions and monitored daily for emergence. Following emergence, 20 1st-instar larvae were placed at the base of each plant stem. A total of 45 plants were used, divided equally between *As. incarnata* (15), *Ap. androsaemifolium* (15), and *V. rossicum* (15). A screen was secured to the base of each plant pot, while netting was placed over the top of each plant

(mesh size = 0.5 mm) to prevent larval escape. The pots were dissected after 85 days (sufficient time for beetles to develop into late-instar larvae or pupae). All larvae found in the pots were counted and their head capsule widths measured at the widest point, with the mouthparts oriented downwards, using a digital microscope (Dino-Lite AM413TA) (Delbac et al., 2010). Larvae were then moved back onto roots of the same plant species to determine whether they could continue to develop to pupation and adulthood.

Experiment 5: Host-plant choice by Chrysochus auratus larvae – Adult *C. auratus* beetles were collected from Dundas, Ontario in July 2015 and stored as described above. Egg masses were removed from containers every three days and placed singly in microcentrifuge tubes. After emergence, 1st-instar larvae were placed in the center of sterilized 11 cm petri dishes (12) in groups of ten. Excised root segments of *V. rossicum*, *Ap. cannabinum*, *As. syriaca*, and *S. canadensis* were placed at the edge of the petri dishes in the four cardinal directions. *Solidago canadensis* (Asteraceae) was tested alongside potential Apocynaceae hosts to ensure that host choice made by *C. auratus* larvae was not random. Each petri dish had roots placed in a different order to control for environmental factors. Larvae were monitored after 30, 60, and 120 min to determine their location within the petri dish. Larvae were considered to have 'chosen' a root species if they were touching the root segment, curled underneath it, feeding on it or actively crawling over it. Root segments were excised from plants that had been collected as rootstock in Ontario during early spring and grown in 4-L pots in the greenhouse.

2.3. Statistical analysis

A generalized linear model with logistic distribution was conducted for Experiment 2 to compare the frequency of adult *C. auratus* feeding on leaf material from different plant genera. Non-feeding beetles were then removed from the analysis and the quantity of feeding among genera was compared using a Kruskal-Wallis test, since the data did not conform to assumptions of normality. Tukey HSD tests were used for pairwise comparisons. *Solidago canadensis* was removed from the latter analysis as only one beetle was observed to feed on this species. In Experiment 3, *Chrysochus auratus* oviposition and lifespan data did not meet assumptions of normality, therefore plant species were pooled by genera and compared using Kruskal-Wallis tests with pairwise comparisons. A Kruskal-Wallis test was used to compare the number of live larvae recovered from each plant species for Experiment 4 as data were not normally distributed. Because zero larvae were recovered from *V. rossicum* plants, an independent samples *t*-test was conducted to compare head capsule widths of larvae collected from *Ap. androsaemifolium* and *As. incarnata.* Finally, for Experiment 5, a Friedman test was conducted to determine whether larval choice changed significantly over time, as data did not conform with assumptions of normality. A Kruskal-Wallis test was subsequently used to compare larval choice among the four root species after 120 min. All analyses were conducted using SPSS version 25 (IBM, Armonk, USA).

3. Results

3.1. Ecological host range of Chrysochus auratus

Experiment 1: Determining the ecological host range of Chrysochus auratus – Field surveys demonstrated a clear preference by adults of *C. auratus* for *Apocynum* species. No evidence of feeding was observed on *Asclepias* spp. during the field surveys even though plants of this genus were present at three of the seven sites (Guelph, ON, Toronto, ON, and Kamloops, BC). Indeed, no feeding damage was observed on any plants outside of the *Apocynum* genus. Adult beetles oviposited on plants from a wide breadth of plant families as well as on non-plant substrates, but the majority of *C. auratus* egg masses found in the field (86%) were laid on *Apocynum* spp. (Table 1). All egg masses not laid on *Apocynum* plants were within quadrats that also contained *Apocynum* spp.

3.2. Determining the fundamental host range of Chrysochus auratus

Experiment 2: No-choice feeding trials with Chrysochus auratus adults – Adult *Chrysochus auratus* initiated feeding on seven separate plant species, however, the beetles only fed extensively on *Apocynum* spp. (known ecological hosts) (Table 2). The frequency with which *C. auratus* fed on cut leaves varied significantly among plant genera ($N = 548$, $\chi^2 = 118.47$, $df = 3$, $P < 0.001$). Beetles fed more frequently on *Apocynum* spp. (87.2%) than on *Asclepias* spp. (23.6%) ($N = 324$, $\chi^2 = 0.64$, $df = 1$, $P < 0.001$), *V. rossicum* (26.6%) ($N = 279$, $\chi^2 = -0.61$, $df = 1$, $P < 0.001$) or *S. canadensis* (1.4%) ($N = 195$, $\chi^2 = -0.86$, $df = 1$, $P < 0.001$). Beetles also fed more frequently on *Asclepias* spp. ($N = 269$, $\chi^2 = -0.22$, $df = 1$, $P < 0.001$) and *V. rossicum* ($N = 224$, $\chi^2 = -0.25$, $df = 1$, $P < 0.001$) than on *S. canadensis*. No difference in the frequency of feeding was observed between *Asclepias* spp. and *V. rossicum* ($N = 353$, $\chi^2 = -0.61$, $df = 1$, $P = 1.000$) (Table 2).

After non-feeding individuals were removed from the analysis, the quantity of leaf material removed by *C. auratus* beetles varied significantly among plant genera ($N = 197$, $z = 139.07$, $df = 2$, $P < 0.001$). Beetles consumed more leaf material on *Apocynum* spp. (251.63 ± 19.00 mm^2) (Mean ± SE) than on *Asclepias* spp. (12.28 ± 5.67) ($N = 156$, $z = -84.947$, $df = 1$, $P < 0.001$) or on *V. rossicum* (3.66 ± 2.60) ($N = 150$, $z = 106.98$, $df = 1$, $P < 0.001$). No difference in the area of leaf material removed was observed between *Asclepias* spp. and *V. rossicum* ($N = 88$, $z = 22.03$, $df = 1$, $P = 0.211$) (Fig. 1).

Experiment 3: No-choice greenhouse trials to determine survival and oviposition by Chrysochus auratus adults – There was a significant difference in oviposition by female *C. auratus* among the three plant genera ($N = 131$, $z = 88.75$, $df = 2$, $P < 0.001$). Beetles laid significantly more egg masses on *Apocynum* spp. (48.55 ± 6.17) (Mean ± SE) than on *Asclepias* spp. (0.06 ± 0.02) ($N = 91$, $z = 58.47$, $df = 1$, $P < 0.001$) or *Vincetoxicum* spp. (0.05 ± 0.03) ($N = 60$, $z = 58.47$, $df = 1$, $P < 0.001$). There was no significant difference between oviposition on *Asclepias* spp. and *Vincetoxicum* spp. ($N = 111$, $z = 0.36$, $df = 1$, $P = 1.000$) (Fig. 2A)

There was a significant difference in the adult lifespan of *C. auratus* on the three different plant genera ($N = 131$, $z = 44.09$, $df = 2$,

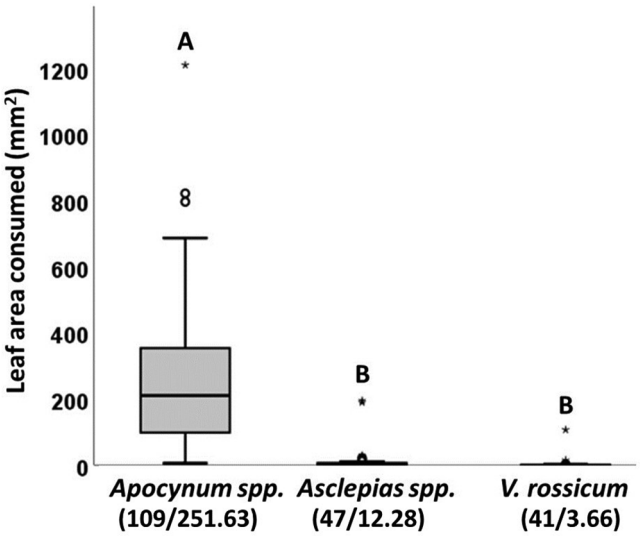

Fig. 1. Mean leaf area consumed by adult *Chrysochus auratus* during no-choice feeding trials. Plant species are grouped by genus. Box plots represent a summary of the data (minimum, first quartile, median, third quartile, and maximum). Open circles represent outliers (1.5 × interquartile range (IQR). Stars represent extreme values (3 × IQR). Letters indicate significant differences. Numbers below y axis labels indicate sample sizes and mean values.

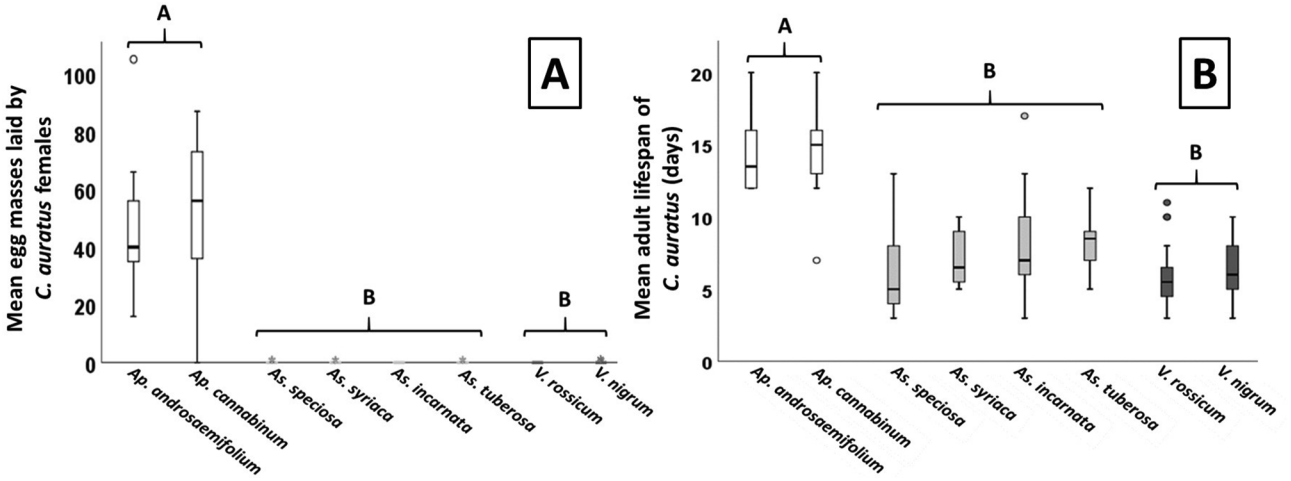

Fig. 2. Mean number of egg masses produced by female *Chrysochus auratus* (A) and mean adult lifespan of *C. auratus* (B) on potted plants. Box plots represent a summary of the data (minimum, first quartile, median, third quartile, and maximum). Open circles represent outliers (1.5 × interquartile range (IQR), while stars represent extreme values (3 × IQR). Plant species are grouped by genus for pairwise comparisons (see braces), and letters indicate significant differences between genera.

P < 0.001). Adult beetles lived longer on *Apocynum* spp. (14.8 ± 0.7 days) (Meanthan on *Asclepias* spp. (7.6 ± 0.3) (N = 91, z = 52.90, df = 1, P < 0.001) or *Vincetoxicum* spp. (6.2 ± 0.3) (N = 60, z = 71.97, df = 1, P < 0.001). Female *C. auratus* also lived longer on *Asclepias* spp. than on *Vincetoxicum* spp. (N = 111, z = 19.07, df = 1, P = 0.032) (Fig. 2B).

Experiment 4: No-choice greenhouse trials to determine development of Chrysochus auratus larvae – The number of live larvae recovered from roots after 85 days varied significantly among species (N = 45, z = 10.82, df = 2, P = 0.004). Larval survival was higher on *Ap. androsaemifolium* (4.4 ± 1.33) (Mean ± SE) (N = 30, z = 12.50, df = 1, P = 0.006) and *As. incarnata* (3.06 ± 1.1) (N = 30, z = 10.00, df = 1, P = 0.039) than on *V. rossicum* (0 ± 0), on which no larvae were recovered. No difference in larval survival was observed between *Ap. androsaemifolium* and *As. incarnata* (N = 30, z = 2.50, df = 1, P = 1.000). The head capsule width of larvae collected from *Ap. androsaemifolium* were larger (1.93 ± 0.03 mm) (Mean ± SE) than those collected from *As. incarnata* (1.80 ± 0.04 mm) (N = 104, F = 5.19, df = 1, P = 0.025) (Fig. 3). Of the 59*C. auratus* larvae recovered from *Ap. androsaemifolium*, nine individuals went on to emerge as adults. On *As. incarnata,* 45 larvae were initially recovered at 85 days, seven of which developed to adulthood.

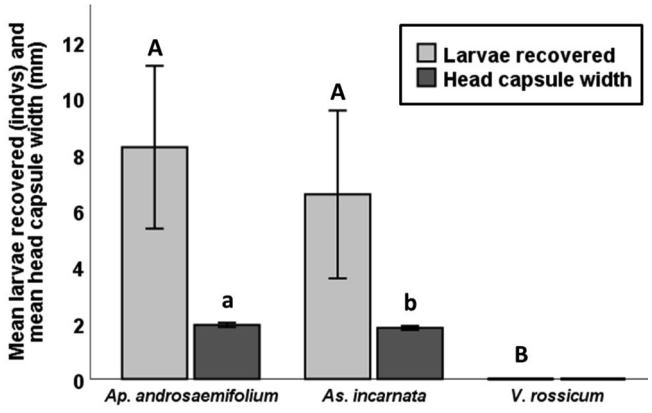

Fig. 3. Number of *Chrysochus auratus* larvae recovered from potted plants of *Apocynum androsaemifolium* (N = 15), *Asclepias incarnata* (N = 15), and *Vincetoxicum rossicum* (N = 15) after 85 days (light grey bars). Mean head capsule widths of recovered larvae (dark grey bars). Bars indicate mean values, error bars represent standard error, and letters indicate significant differences.

Experiment 5: Host-plant choice by Chrysochus auratus larvae – The Friedman test indicated that larval choice among the four root species did not change significantly over time (N = 48, χ^2 = 1.47, df = 2, P = 0.479). Consequently, a Kruskal-Wallis test was used to compare larval choice among the four test species at the 120-min observation point. Significantly more larvae were observed on *Ap. cannabinum* (1.91 ± 0.45) (Mean ± SE) than on *V. rossicum* (0.33 ± 0.18) (N = 48, z = 17.54, df = 3, P = 0.005) or *S. canadensis* (0 ± 0) (N = 48, z = 22.75, df = 3, P < 0.001). There was no difference however, between the number of larvae on *Ap. cannabinum* or on *As. syriaca* (1.75 ± 0.47) (N = 48, z = 4.71, df = 3, P = 1.000) (Fig. 4).

4. Discussion

Field and laboratory studies of adult *C. auratus* revealed a high degree of host-specificity. Exploratory feeding on non-*Apocynum* plants was observed, although it was entirely confined to the laboratory. In contrast, oviposition by adult *C. auratus* and larval feeding indicated some preference for *Asclepias* spp. over other non-host plants. Combined with knowledge of the species' biology, we use these results

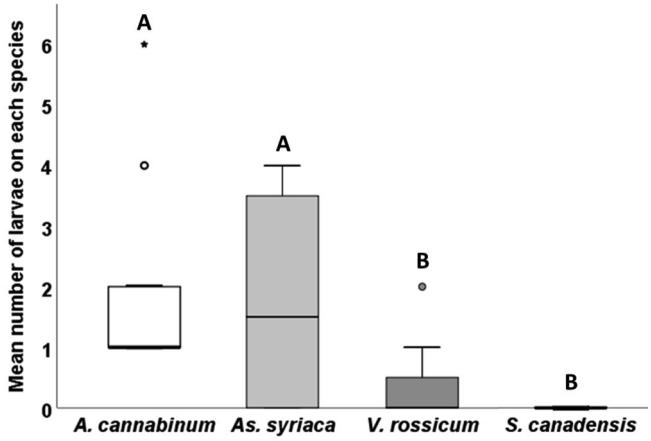

Fig. 4. Mean numbers of early instar larvae on *Apocynum cannabinum*, *Asclepias syriaca*, *Vincetoxicum rossicum*, and *Solidago canadensis* roots during choice tests (N = 12). Box plots represent a 5 number summary of the data (minimum, first quartile, median, third quartile, and maximum). Open circles represent outliers (1.5 × interquartile range (IQR). Stars represent extreme values (3 × IQR). Letters indicate significant differences.

to assess the likelihood of *C. auratus* including *Asclepias* spp. in its host-range. Furthermore, we use our observations of *C. auratus* to reevaluate the non-target risks associated with the classical biological control candidate, *C. asclepiadeus*.

Our surveys in the field corroborated several other ecological host-range studies that have characterized *C. auratus* as specific to *Apocynum* spp. (Arnett, 1968; Doussourd and Eisner, 1987; Williams, 1992; Dobler and Farrell, 1999). Weiss and West (1921) reported some use of *Asclepias* spp. by *C. auratus* in the field, but this has been refuted by all subsequent studies, including our own. We observed no feeding on *Asclepias* spp. by *C. auratus* at any of the sites surveyed, even though three of the seven sites had *Asclepias* plants intermixed with the beetle's host plants. While our field surveys confirmed a high level of host-specificity in *C. auratus*, the results of adult no-choice laboratory feeding trials were less clear. Although adult *C. auratus* only fed extensively on *Apocynum* spp., they demonstrated exploratory feeding on six of the eight non-*Apocynum* species tested, including four species within the *Asclepias* genus.

One potential factor leading to exploratory feeding on non-*Apocynum* plants in the laboratory is that growth conditions may unduly influence the palatability of plants. Laboratory-reared plants protected from competition, herbivory, and drought stress may be more vulnerable to attack by beetles than hardier plants grown in the field (Karban et al., 1997; Bernays and Graham, 1998). The use of fertilizers can also enhance plant palatability (Van Hezewijk et al., 2008). It appears that *Asclepias* spp. may be particularly susceptible to increased palatability when grown in the greenhouse. *Asclepias syriaca*, for example, exhibits fewer induced defenses when grown in full sunlight (Agrawal et al. 2012) and reduced cardenolide content when protected from drought stress (Agrawal et al., 2014). Future work should compare feeding by *C. auratus* on *Asclepias* leaves from various sources (greenhouse vs field). It is possible that adult *C. auratus* may avoid even exploratory feeding on hardier plants that have had greater exposure to stress.

Overall, we believe that adult feeding by *C. auratus* poses very little risk to *Asclepias* spp. in North America. Potted plant experiments in the greenhouse showed that adults survived significantly longer on *Apocynum* spp. than on *Asclepias* spp.. Furthermore, in choice-tests conducted by deJonge et al. (2017), *C. auratus* displayed zero or minimal feeding on non-*Apocynum* plants. In the future, two-phase field testing, in which the target plants are removed midway through the experiments (Briese et al., 2002), could be conducted to determine if there are any conditions underwhich *C. auratus* adults might utilize *Asclepias* spp. in the field.

Adult *C. auratus* females laid significantly more egg masses on potted *Apocynum* spp. than on non-host plants; however, some oviposition was observed on *Asclepias* spp. as well as on *V. rossicum*. Indeed, in common garden tests, deJonge et al. (2017) found that *C. auratus* females laid more egg masses on *Asclepias* spp. than on other non-*Apocynum* members of the Apocynaceae. These results suggest that *C. auratus* females may receive ovipositional cues from *Asclepias* spp., however, there are other possible explanations for why insects might exhibit seemingy indiscriminate oviposition during host tests. First, insects might experience central excitation due to proximity, or recent contact with a host plant (Withers and Browne, 1998). Alternatively, such indiscriminate oviposition might represent the insects normal behavior. North American *Chrysochus* beetles, in general, exhibit a rather broad ovipositional host range (deJonge et al., 2017). In the present study, *C. auratus* egg masses were found on a diversity of plants and non-plant substrates, even at field sites dominated by *Apocynum* spp.. Ovipositional behavior by *Chrysochus* beetles may be similar to that of *Pieris brassicae* L. (Lepidoptera: Pieridae) where it has been shown that females oviposit within an acceptable microhabitat, but their larvae 'update' their mother's choice by moving to the most palatable host roots within reach (Soler et al., 2012). This idea is supported by our observation that *C. auratus* egg masses, while laid in seemingly unspecialized fashion, were always found within one meter of *Apocynum* host plants. This loose placement of egg masses by *Chrysochus* females suggests that ovipositional choice may not be the most reliable indicator of non-target risk for this genus.

In potted plant experiments, *C. auratus* larvae completed their development to the adult stage on *As. incarnata*, albeit at a slower rate than they did on *Ap. androsaemifolium*. Larvae failed to develop, however, on introduced *V. rossicum*. These results might signify the ability of *C. auratus* larvae to shift onto *Asclepias* spp., however, larval feeding on *Asclepias* spp. has never been observed in the field, and our results again suggest that they may be influenced by the relatively palatable nature of laboratory-reared plants. The controlled environment of the greenhouse may also fail to reflect field conditions in other ways. For example, in the field, *As. incarnata* typically grows in wet soils (Kirk and Belt, 2011), and the water itself may act as a physical barrier against soil-dwelling enemies. While the use of *Asclepias* spp. by *C. auratus* larvae in the field is difficult to rule out entirely, their use of *As. incarnata* in the laboratory appears to represent a a 'false positive'.

In laboratory choice-tests, early-instar *C. auratus* larvae showed no preference between the roots of *Ap. cannabinum* and *As. syriaca*, but avoided the roots of an unrelated control plant, *S. canadensis*. The propensity for *C. auratus* larvae to 'choose' *As. syriaca* is cause for concern, although it should be noted that the responsibility of host-selection falls far more heavily on adult females than larvae. In the field, *C. auratus* laid very few eggs on *Asclepias* spp. (Table 1) suggesting that while larval damage to *Asclepias* spp. is possible, it will likely be extremely rare. Nevertheless, in both larval feeding and larval choice tests, *C. auratus* indicated some degree of host use on *Asclepias* spp..

There are several aspects of *C. auratus* biology that might suggest some propensity to use *Asclepias* spp. as host plants. First, *C. auratus* shares amino acid sequences with its *Asclepias*-feeding congener, *C. cobaltinus,* that are associated with the ability to digest plant toxins such as cardenolides (Labeyrie and Dobler 2004). Indeed, it appears likely that the genus *Asclepias* is an ancestral host plant for *C. auratus* (Dobler and Farrell, 1999). Here, we provide evidence of successful feeding on *Asclepias* spp. by *C. auratus* larvae, but not by adults. This is in line with previous observations demonstrating that insect larvae often hold on to ancestral hosts, even when adults do not (Janz et al., 2001). Second, *C. auratus* is known to hybridize with *C. cobaltinus* (Peterson et al., 2001), and these hybrid beetles feed on *Asclepias* spp. (deJonge et al., 2019). Such hybridization could also lead to novel host-use in *C. auratus* through gene introgression (repeated backcrossing with parental species (Rhymer and Simberloff, 1996)). In spite of these factors and the millennia of opportunity (*Asclepias* spp. are abundant throughout *C. auratus*' range), *C. auratus* has not extended its current ecological host range to include *Asclepias* spp.. The risk of adaptation to a novel host by a highly specialized insect, therefore, may be minimal in this system.

Recent reviews on non-target damage by weed biological control agents provide strong validation for the current methods for pre-release host-specificity testing, and the centrifugal phylogenetic methods by which test plant species are selected (Schaffner et al., 2018; Hinz et al., 2019). While the inadequacy or lack of post-release monitoring remains a weakness for many classical biological control programs (Havens et al., 2019), we suggest that the next frontier for pre-release biological control candidate assessment is an improved understanding of the relationship between the fundamental and ecological host-ranges. Such an understanding can save candidate agents from unwarranted rejection, and potentially pave the way for more biological control success stories. In South Africa, the chrysomelid beetle, *Leptinotarsa texana* Schaeffer, was released to control *Solanum elaeagnifolium* Cav., despite being observed to develop on several native South African *Solanum* species as well as cultivated eggplant during laboratory testing (Hill and Hulley, 1995; Olckers et al., 1995). The decision to release was made based on the fact that *L. taxana* had never been observed to feed on any of these non-target species in its native range. Since its release, *L. taxana* has exerted widespread impact on its target weed (Winston et al., 2014) and

only a single incidence of non-target feeding has been reported (Hinz et al., 2019). Examples like this demonstrate the importance of understanding how the fundamental host range of a candidate agent will translate to its behavior in the field.

Our study of closely-related native insects can be extended to other biological control systems where native or adventive congeners are available. For example, a European weevil, *Ceutorhynchus scrobicollis* Nerensheimer & Wagner (Coleoptera: Curculionidae), has recently been approved for release against garlic mustard (*Alliaria petiolata*) in North America, despite demonstrating limited ability to feed on a non-target plant (*Rorippa sinuata* (Nutt.) Hitchc. (Brassicaceae)) in the laboratory (Blossey et al., 2002). There are a number of native and adventive North American *Ceutorhynchus* spp. specializing on plants in the Brassicaceae that could have been investigated for better interpretation of *C. scrobicollis'* host tests. While the risk to *Rorippa sinuata* appears very low, this system serves to highlight the potential for native congeners to enhance the risk-assessment process for classical biological control programs.

Although closely related congeneric insects often express similar feeding patterns and niche requirements (Kirmse and Ratcliffe, 2019), such similarity cannot be taken for granted. Many closely related insect species exhibit markedly different host preferences (Frey et al., 1992), indeed, the degree of host specificity can even vary among individuals of the same species (Haines et al., 2013). As a result, care must be taken to select appropriate species for comparison, and results must be treated with caution. Nevertheless, the study of congeneric insects, present in the proposed area of introduction, can be a valuable tool for understanding the relationship between an insects fundamental and ecological host-range.

4.1. Recommendations for the further study of C. Asclepiadeus

In the first two experiments of our study, *C. auratus* adults demonstrated a high degree of specialization on *Apocynum* spp.. Any exploratory use of *Asclepias* spp. by adults in the laboratory appears highly unlikely to translate to the field. Our results invite scrutiny of the original decision to terminate host testing of European *C. asclepiadeus* for biological control in North America due to non-target feeding concerns on *Asclepias* spp (Table 3). Further exploration of the relationship between the fundamental and ecological host ranges of this species should be conducted by carrying out field host-specificity trials with *Asclepias* spp. in the beetles' home range. Such trials would be facilitated by the fact that *As. syriaca* has become naturalized in many areas of Europe and is now considered invasive (Pauková et al., 2014; DAISIE European Invasive Alien Species Gateway, 2017). Should *C. asclepiadeus* feed on *As. syriaca* during such trials, its candidacy as a classical biological agent for *V. rossicum* in North America should be terminated, although in this case, the beetle might then be considered as a native biological agent for *As. syriaca* in Europe.

During initial host-testing of *C. asclepiadeus*, only one *Apocynum* species was included (*Ap. cannabinum*). Given its close relation to *C. auratus* (an *Apocynum* specialist), and the potential for hybridization, more extensive testing should be conducted on *Apocynum* spp. before *C. asclepiadeus* is reconsidered for release in North America. Our results from experiments four, five, and six provide evidence that *Asclepias* spp. are prone to generating false positives in *Chrysochus* beetles, and this tendency should be considered when interpreting the results of *C. asclepiadeus* host-specificity tests. Insights such as this highlight the value of studying native congeners to support the host-range testing of classical biological control candidates.

5. Conclusions

Identifying 'false positives' in host-range testing represents an important part of biological control agent development. An excess of caution may result in the rejection of otherwise promising insects (Sheppard et al., 2003), while the costs associated with insufficient caution are well documented (Simberloff and Stiling, 1996a,b). Our work shows that the study of native congeners can inform this risk assessment process in a number of key ways. The lack of logistical constraints allows for more comprehensive ecological studies through which the relationship between the fundamental and ecological host ranges may become clearer. In the present study, we identified a plant genus (*Asclepias*) that was more likely to generate 'false positives' in *Chrysochus* beetles. Conversely, we also identified negative traits, such as unspecialized oviposition behavior and the ability to hybridize with broader feeding species, that might increase the risks associated with this genus. Insights like these can substantially aid in the interpretation of host-testing and, ultimately, the decision to release novel natural enemy species as part of classical biological control programs.

Table 3
Summary of the fundamental and ecological host ranges of *Chrysochus asclepiadeus* and *C. auratus,* compiled from both no-choice and choice tests on leaves and plants, as well as available survey data (deJonge et al. 2017; Gassman et al. 2010a,b, and Sforza, 2011). Dashes (–) refer to lack of use by beetle at this life stage. Blank spaces refer to no testing done. Scores in bold are plant species only in beetles' fundamental host range. Scores in grey are plant species in beetles' ecological host range.

	C. asclepiadeus			*C. auratus*		
	Adult[a]	Larvae[b]	Ovpstn[c]	Adult	Larvae	Ovpstn
Apocynum androsaemifolium				+ + +	+ + +	+ + +
Ap. cannabinum	–	+ + +	–	+ + +	+ + +	+ + +
Asclepias eriocarpa				+ +	+ +	
A. fascicularis	+ + +	+ + +	–	+		
A. incarnata	+ +	+ + +	+	+	+ + +	+ +
A. speciosa	+ +	+ + +	+	+ +		+
A. syriaca	+ +	+ + +	+	+ +	+ +	+
A. tuberosa	+ +	+ + +	+	–		+
V. nigrum	+ + +	+ + +	+ + +	–	–	+
Vincetoxicum rossicum	+ + +	+ + +	+ + +	+	+	+
Solidago canadensis				–	–	+

[a] Adult feeding: + nibbling, + + moderate feeding, + + + extensive defoliation.
[b] Larval development: + one molt, + + development beyond 2nd instar, + + + complete development.
[c] Oviposition: + very few masses observed on plant in lab tests, + + moderate oviposition + + + extensive oviposition observed in lab and field.

CRediT authorship contribution statement

Rhoda B. deJonge: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **Ian M. Jones:** Data curation, Formal analysis, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Robert S. Bourchier:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Sandy M. Smith:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing - review & editing.

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